University of Novi Sad
INSTITUTE OF FOOD TECHNOLOGY IN NOVI SAD

FINS

food congress
28-30. 10. 2014.
Novi Sad, Serbia

II International Congress
Food Technology, Quality and Safety

ISEKI - Food Association

EFFoST
European Federation of Food Science and Technology

PROCEEDINGS

Organization of Symposium:

- INSTITUTE OF FOOD TECHNOLOGY, University of Novi Sad, Serbia

Symposium is supported by:

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- Provincial Secretariat for Science and Technological Development - Novi Sad
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CONTENT

INFLUENCE OF GOJI BERRY, PUMPKIN POWDER AND ROSE PETAL EXTRACT ON THE QUALITY OF COOKED SAUSAGES WITH REDUCED NITRITE CONTENT

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ABSTRACT

The influence of dried fruit of goji berry (GB) (*Lycium chinense*), butternut pumpkin powder (PP) *(Cucurbita moschata)* and rose petal extract (RPE) (Rosa damascena *Mill*) on lipid and protein oxidation in sausages with 1/2 reduced nitrite content was studied. The sausages were produced with 0.05 g/kg sodium nitrite, and 5 or 10 g/kg dried goji berry fruits resp. pumpkin powder and 0.05 or 0.1 % rose petal extract. The controls with 0.05 or 0.10 g/kg sodium nitrite only were used. In comparison with control samples C, on 6th d of storage statistically significant (p ≤ 0.05) reduction of total protein carbonyls in samples 0.05% RPE, 0.1% RPE, 0.5 % and 1% GB (50,58%, 50,00%, 44,00% и 46,15% resp.) was detected. The addition of 0.5% and 1% pumpkin powder showed weak antioxidant activity, but the addition of 0.05% to 0.1% rose petal extract or 0.5 to 1.0% goji berry effectively inhibit the protein hydrolysis and oxidation, lipolysis, and lipid oxidation (p ≤ 0.05) and can be used successfully as additives for development new functional sausages with halfway reduced nitrites content.
Keywords: functional meat products, rose petal extract, goji berry, lipid and protein oxidation, reduced nitrite content

INTRODUCTION

In the last years much attention has been paid to develop new meat products which may prevent the risk of diseases (Zhang et al. 2010). It is known that foods with tertiary function has anticarcinogenic, antimutagenic, antioxidative activity and antiaging activity (Dentali, 2002).
Numerous studies have tried to demonstrate the possibility of changing the image of meat products from traditionally accepted to one of healthy living thanks by the addition of vegetables, extracts, and fibers, elimination of fats, and reduction of additives (Fernández-Ginés et al. 2005). Recent innovations in the meat industry are based on either reducing the content of unhealthy substances i.e. less added nitrate and nitrite (Toldrá, 2011).
A pumpkin is a well-known traditional food, which antioxidant, antidiabetic, anticarcinogenic, antiinflammatory (Yadav et al., 2010), and antihypercholesterolemia properties (Adaramoye et al., 2007). Pumpkin contents alkaloids, flavonoids, palmitic, oleic and linoleic acids (Yadav et al., 2010), carotenoids of which more than 80% β-carotene, and less amounts of lutein, lycopene, α-carotene and cis-β-carotene content (Seo et al., 2005).
Antioxidant properties of goji berry fruits improves neurological/psychological performance and gastrointestinal functions (Seeram, 2008). It shows hepatoprotective, antdiabetic and anticancer effect (Amagase and Farnsworth, 2011).
Rose petal extract, as by-product of rose oil (Rosa damascena *Mill)* processing is disposal problem for the food industry, but on the other hand is promising source of polyphenols (Kammerer et al., 2005) with antioxidant (Wang et al., 2006) and antibacterial activity (Özkan et al., 2004). Moreover, strategies for the exploatation of polyphenol-rich extracts as

functional (Larrosa et al., 2002) or technological (Ivanov et al., 2009) food additives have been proposed.

In the available literature there are no reports of application of butternut pumpkin powder *(Cucurbita moschata*) (PP), dried fruits goji berry (*Lycium chinense)* or rose petal extract (Rosa damascena *Mill*) (RPE) during sausages production.

Therefore the purpose of this study was to identify the technological capabilities for developing of new functional cooked sausages with half reduced nitrite content, by enrichment with butternut pumpkin polder, dried fruits goji berry or rose petal extracts.

MATERIAL AND METHODS

Meat raw materials
The chilled to 0 – 4ºC beef (pH 6.60) and pork (pH 6.55) were used in this experiment. The beef and pork meat were supplied by the company Kartevi brother's Ltd, village Benkovski, district Plovdiv, Bulgaria.

Other ingredients and additives
The sodium chloride (salt), sodium tripolyphosphates (STPP, E451$_{(i)}$), sodium nitrite (E250), fresh goji berry (*Lycium chinense)* fruits and butternut pumpkin *(Cucurbita moschata)* were bought from the local market.

The goji berry fruits were dried in the Department of Processes and Apparatus from University of Food Technologies, Plovdiv, Bulgaria. The dry fruits were grinded before use.

The butternut pumpkin was cleaned of peel and seeds, shredded into small particles and dried in a spray dryer in the Department of Heat Engineering from University of Food Technologies, Plovdiv, Bulgaria.

The rose petal extract (Rosa damascena *Mill*) was produced in the Department of Food Preservation and Refrigeration Technology, Technological Faculty, University of Food Technology, Plovdiv, Bulgaria.

Sample preparation
Sausages were manufactured by traditional technological process following the requirements of Bulgarian Government Standard (BGS 127-83) (Table 1). The meat pieces were mixed with salt and phosphates on blender. The filling mass was prepared by cutter adding the flake ice and, NaNO$_2$. The prepared filling mass was separated of eight equal portions and was filled in moisture and gases no-permeable five-layer polymer casings. The sausages were cooked to an internal temperature of 72°C and chilled in cold water. The experiments were produced with 6 samples - GB1 (containing 5 g/kg dried fruits of goji bery), GB2 (containing 10 g/kg dried fruits of goji bery), P1 (containing 5 g/kg pumpkin powder), P2 (containing 10 g/kg pumpkin powder), RPE1 (containing 0,05% rose petal extract), RPE2 (containing 0.1% rose petal extract). The controls with 0,1 or 0,05 g/kg sodium nitrite only were used. First half of the samples was immediately studded (1d), and the second half was stored 6 day at 0 - 4ºC (6d). The samples were obtained according ISO 3100-1:1991.

Determination of the degree of lipolysis by acid value
As a standard of the rate of lipolysis, the acid value (AV) of the extracted lipids was measured following EN ISO 660:2001 procedure (Kardash and Tur'yan 2005).

Determination of degree of lipid oxidation by 2-thiobarbituric acid reactive substances
TBARS were determined by the method described by Botsoglou et al. (1994). The duble beam UV-VIS spectrophotometer Camspec model M550 (Camspec Ltd, Kembridge, UK) was used.

Determination of free amino nitrogen
The FAN in samples was determined by method described Lorenzo et al. (2008).

Table 1. Formulation of different samples functional cooked sausages

Ingredients	Samples							
	C	C1/2	GB1	GB2	P1	P2	RPE1	RPE2
Beef topside, g/kg	500	500	500	500	500	500	500	500
Pork chest, g/kg	500	200	200	200	200	200	200	200
Sodium chloride, g/kg	20	20	20	20	20	20	20	20
Sodium nitrite, g/kg	0,1	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Dried fruits of goji berry, g/kg	-	-	5	10	-	-	-	-
Pumpkin powder, g/kg	-	-	-	-	5	10	-	-
Rose petal extract (%)	-	-	-	-	-	-	0.05	0.1
Sodium polyphosphates, g/kg	2	2	2	2	2	2	2	2
Flake ice, g/kg	200	200	200	200	200	200	200	200

Determination of total protein carbonyls
A protein oxidation was measured by estimation of carbonyl groups formed with some modifications. Protein concentration was calculated at 280 nm in the HCl control using BSA in 6 M guanidine as standard. Carbonyl concentration was measured on the treated sample by measuring DNPH incorporated on the basis of an absorption of 21.0 mM-1cm-1 at 370 nm for protein hydrazones. The results were expressed as nanomoles of DNPH fixed per milligram of protein (Merciera et al. 2004)

Statistical analysis
The data of different samples were analyzed independently by SAS software (SAS Institute, Inc. 1990). The Student-Newman-Keuls multiple range test was used to compare differences among means. Mean values and standard errors of the mean were reported. Significance of differences was defined at $p \leq 0.05$.

RESULTS AND DISCUSSION

Hydrolytic changes in total lipids set by the indicator acid value (AV)
During the 6d of storage at 0 - 4 º C statistical significant increases ($p < 0.05$) of AV at control samples C and C1/2 and samples P1 and P2 was established (Fig.1).
On the other hand, a minimal decrease ($p>0.05$) in AV was established in samples enriched with rose petal extract and dried fruits of goji berry. In this samples (RPE1, RPE2, GB1 and GB2) on the first and six day of the experiment the changes in AV were no statistically significant ($p > 0.05$) different. On the 6 day TBARS in RPE1 and RPE2 was approx. 2 times lower compared to control sample C (Fig.1).
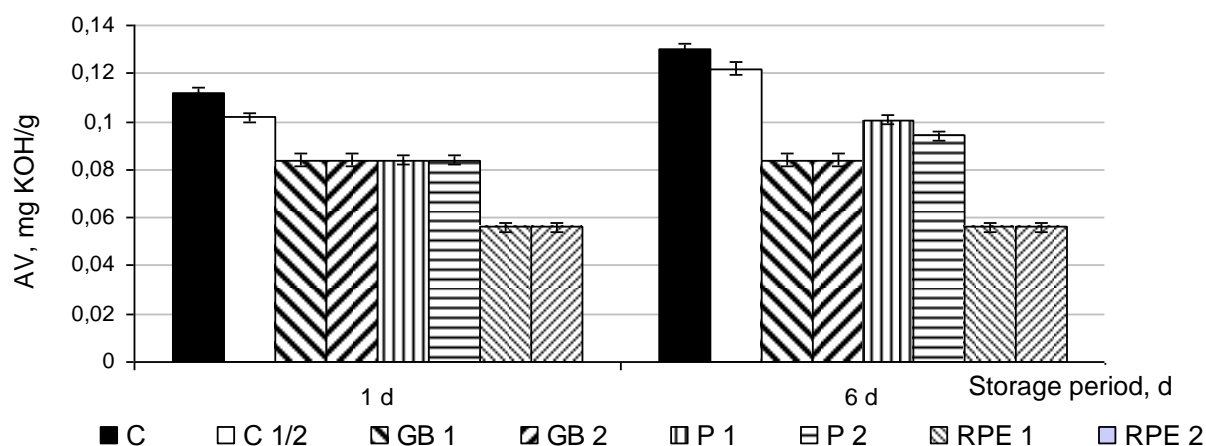
Figure 1. Changes in acid value of studied sausages

Changes to the content of the secondary products of lipid oxidation in examined samples set by the indicator TBARS

During the 6d of storage at 0 - 4 º C the increase of TBARS at control samples C and C1/2 and samples P1 (Fig.2) with 11.7%, 9.0% and 4% resp. was established. After addition of 1% dried goji berry no statistical difference (p>0.05) in TBARS at first and six day was found. In contrast, in the samples with rose petal extract (RPE1 and RPE2) the opposite changes occur and during the storage period TBARS decreased with 11.9%.
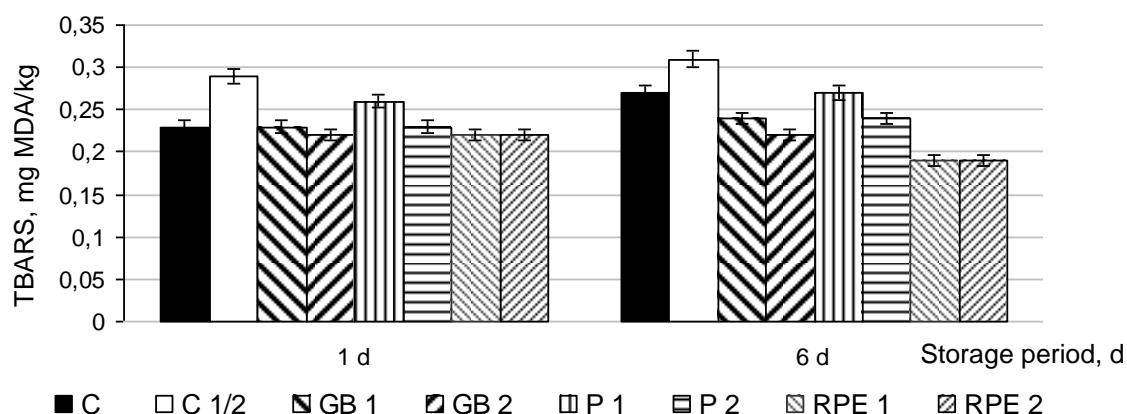
Figure 2. Changes in TBARS of studied sausages

Probably due to higher antioxidant activity the addition of 0.5% or 1.0% goji berry (*Lycium barbarum*) and 0.05% or 0.1% rose petal extract (Rosa damascene *Mill*) has been proved to be more effective for lipid oxidation inhibition in comparison with the pumpkin powder.
The addition of butternut pumpkin powder (*Cucurbita moschata*) was not appropriate for inhibition of hydrolytic and oxidative changes in total lipids of studied cooked sausages with half nitrite content.

Changes in protein oxidation

In comparison with control sample C, a statistically significant (p < 0.05) reduction of total protein carbonyls on 6 d of storage at both 0.5 and 1 g/kg goji berry and 0.05% or 0.1% rose petal extract addition was determined (Fig. 3). The reduction of total protein carbonyls in samples RPE1, RPE2, GB1 and GB2 was with 50,58%, 50,00%, 44,00% и 46,15% resp.
The conclusion was made that addition of 0.05% to 0.1% RPE or 0.5% to 1.0% goji berry can be used for effective inhibition of protein oxidation in the studied cooked sausages. The

enrichment of cooked sausages with halfway reduced amounts of nitrites with butternut pumpkin powder *(Cucurbita moschata)* was not appropriate for inhibition of protein oxidation.
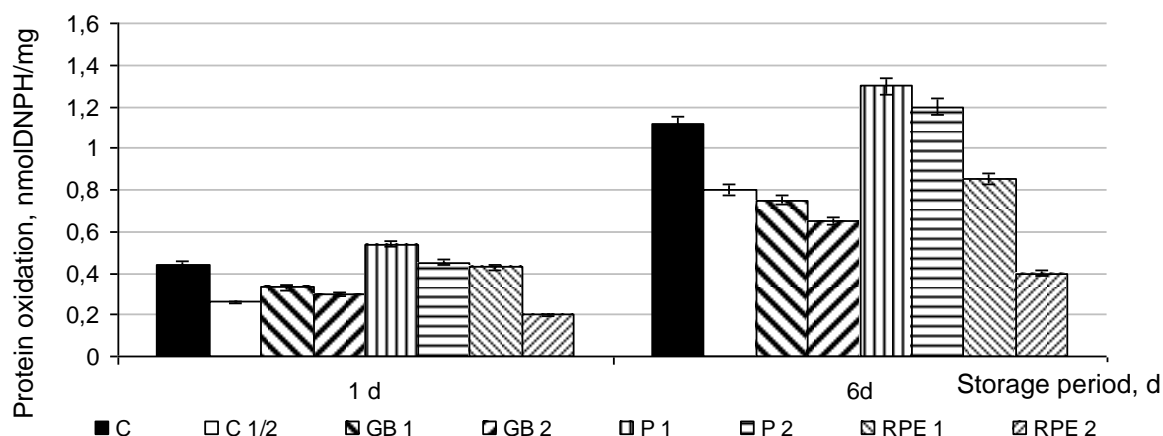
Figure 3. Protein oxidation of studied sausages

Degree of proteolysis

On the 1d of the experiment the highest free amino groups (FAN) contents (used as indicator of degree of proteolysis) was found at samples P1, C1/2 and C, and the lowest in samples with 0.5% and 1% dry fruits goji berry (Fig. 4). At the 6 d of storage at 0 - 4ºC no statistically significant ($p > 0.05$) difference of FAN content at all examined samples was established (Fig. 4). Pumpkin polder, dried fruits goji berry and rose petal extracts can be used for inhibition of proteolitical changes during production of cooked sausages with half nitrite content.
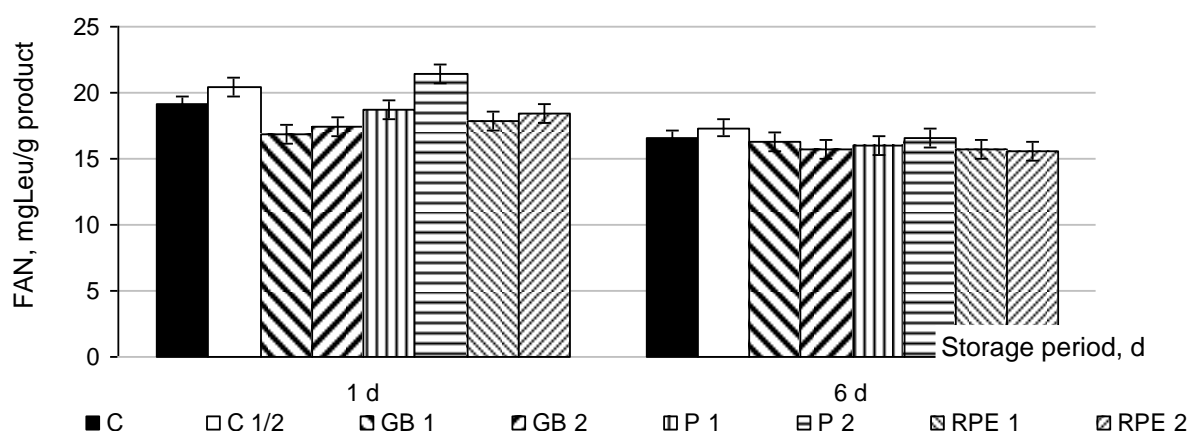
Figure 4. Free amino groups (FAN) contents of studied sausages

CONCLUSIONS

The addition of 0.5% and 1% pumpkin powder showed weak antioxidant activity, but the addition of 0.05% to 0.1% rose petal extract and 0.5 to 1.0% goji berry effectively inhibit the protein hydrolysis and oxidation, lipolysis, and lipid oxidation ($p \leq 0.05$) and can be used as additives for development new functional cooked sausages with halfway reduced nitrites content. Future experiments will be need for future optimization of the formulation new functional cooked sausages.

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DETECTION OF HEPATITIS A VIRUS RNA IN SLICED HAM

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ABSTRACT

Sliced dry meat products can be contaminated with hepatitis A virus (HAV) during slicing. This is due to deficient hygienic practices (dirty hands and fingernails and/or the inability to wash hands appropriately) since HAV infection is acquired primarily by the fecal-oral route by either person-to-person contact or ingestion of contaminated food or water. HAV infection dose in humans is 10-100 viral copies.

In this paper we evaluated HAV detection in artificially contaminated sliced ham using molecular approach. Sliced ham samples were gamma irradiated with 25 kGy Cobalt-60. Afterwards, these were contaminated with ten-fold dilution of respective aqueous solution of HAV (certified reference material at $4,8 \times 10^4$ viral copies per mL) and let stand until full absorption. Virus elution has been performed by PEG/NaCl precipitation whereas extraction of viral RNA was completed using Trizol/Chloroform method. Reverse transcription and one-step qPCR (RT-qPCR) were used to detect cDNA of HAV's RNA.

Repeatability studies demonstrated that average HAV recovery rate from contaminated ham was 46%. Furthermore, RT-qPCR despite being able to detect 100 copies of viral RNA "in vitro" was found to have a LOD50% at 211 viral copies per gram while LOD95% 554 viral copies per gram.

The results of this study show that the detection of HAV in meat products is challenging, and more efforts to improve elution methods are still needed to detect HAVs in meat products containing viruses in low copies.

Keywords: hepatitis A, detection, ham

INTRODUCTION

Hepatitis A virus (HAV), a single stranded RNA virus, is one of the main causes of acute hepatitis. HAV is primarily propagated by the fecal–oral route, through person-to-person contact, or by ingestion of contaminated food or water. In approximately 50% of hepatitis A cases, the infectious source remains unidentified (Fiore *et al.*, 2006). Up to 3% of reported cases are identified as a part of recognized foodborne outbreaks, though a significant percentage of sporadic cases might be foodborne (Fiore, 2004).

Contrasting to most food-borne bacteria, viruses cannot grow in the environment since they need specific host cells to replicate (Koopmans and Duizer, 2004). HAV virus isunenveloped, as such it is highly resistant to environmental stress factors like heat, pH, drying, UV exposure (Baert *et al.*, 2009 and Vasickova *et al.*, 2010),makingit infective in foods for periods from 2 days up to 1 month (Bidawid *et al.*, 2001, Hewitt and Greening, 2004 Butot *et al.*, 2008, and McCaustland *et al.*, 1982).

The majority of foodborne HAV outbreaks occur at retail when food is contaminated due to poor hygienic practices at infected food handler (dirty hands and fingernails and/or the inability to wash hands appropriately) but food contamination prior to retail distribution have also been described.The fatality rate in HAV infections is lower than 0,1%, although recent HAV outbreaks tend to be more severe (Kanda *et al.*, 2002), e.g. the recent outbreak associated with the ingestion of contaminated green onions in Pennsylvania which caused mortality in three among 601 cases (Wheeler *et al.*, 2005).

Furthermore, HAV has very low infectious doses of 10–100 infectious viral particles (Teunis *et al.*, 2008). Therefore sensitive methods are needed forscreening of food-borne viruses'

presence in food products.Detection of this virus in foodwas currently performed usingmolecular methods.

Aim of this study was to evaluate HAV detection in artificially contaminated sliced ham using molecular approach.

MATERIAL AND METHODS

Virus - Certified reference material (PHE, UK) of HAV ($4,8 \times 10^4$ viral copies per lenticule) was reconstituted according to the manufacturers instruction.

Samples - Sliced pork ham samples were gamma irradiated with 25 kGy Cobalt-60 in order to sterilize samples and generate controlled model.Afterwards, each of 25 g samplewas contaminated with 1 mL of ten-fold dilution of respective aqueous solution of HAV and leftat room temperature for 20 min until totally dried.

Virus detection - Virus elution has been performed by alkaline/PEG/NaCl precipitation whereas extraction of viral RNA was completed using Trizol/Chloroform methoddescribed by Chomczynski and Sacchi (1987) (Life Technologies, Rockville, USA) followed by RNA purification using RNeasy Mini Kit (Qiagen, Germany). Reverse transcription was carried out at 42°C for 1 h with random primer (Life Technologies, USA) and using Moloney murine leukemia virus reverse transcriptase (Life Technologies, USA).Detection of HAV'scDNA was achieved using Brilliant III UltraFast SYBR Green Master Mix (Agilent, USA). The primers were identified using the Primer Express program, forward primer 5′-TCACCGCCGTTTGCCTAG -3′ and reverse primer 5′-GGAGAGCCCTGGAAGAAAG -3′. All PCR reactions were carried out using the Stratagene Mx3005P real-time PCR system (Agilent, USA). The thermal cycling conditions comprised an initial step at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min.For each PCR run, a master mix was prepared on ice as instructed and 5 µL of respective cDNA was added.

RESULTS AND DISCUSSION

Results of testing qPCR assay efficiency are shown in Figure 1 and demonstrate that it would be possible to detect HAV at levels < 10 copies per mL at 92% efficiency. However, repeatability studies demonstrated that average HAV recovery rate from contaminated ham was 46%. Furthermore, RT-qPCR despite being able to detect less than 10 copies of viral RNA "in vitro" was found to have a LOD50% at 211 viral copies per gram while LOD95% 554 viral copies per gram in case of artificially contaminated sliced ham samples (figures not shown). Furthermore,it is likely that viral exposure to proteolytic enzymes from meat (cathepsins – B, D, H & L, and calpains) results inrecovery losses which should be taken into account when assessing health risk in cases of low viral copies.
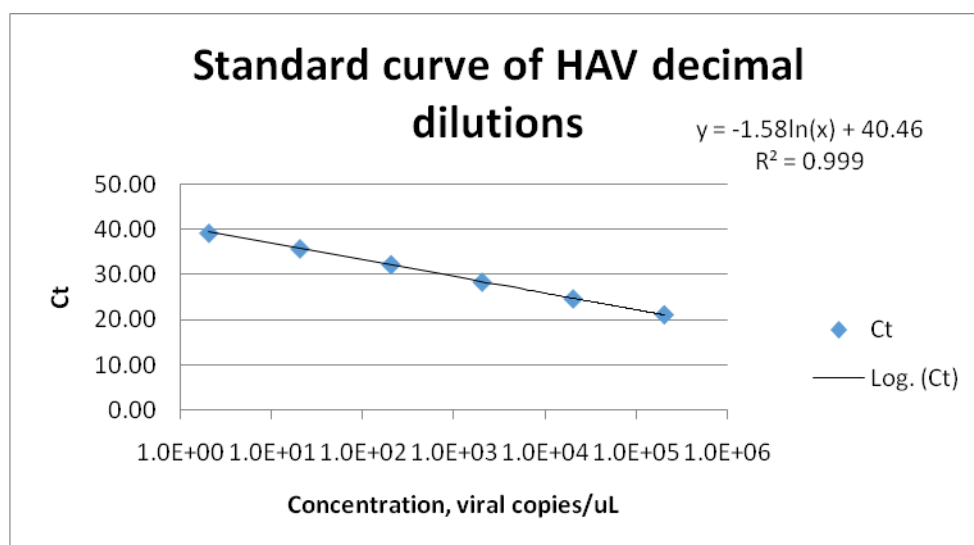
Figure 1.Standard curve of HAV decimal dilution

CONCLUSIONS

Although theresults of this study indicatethat HAV detection in meat products is very challenging more efforts are still needed to improve elution and extraction strategy to detect HAV in meat products, especially when it comes in low copy numbers. Also, HAV detection in food is more difficult than other viral agents due to long incubation period of HAV infection, which obstructs the analysis of the implicated food, unless control portion of this food is stored to enable later assay.

ACKNOWLEDGEMENTS

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IMPROVEMENT OF THE FREEZING PROCESS OF SPINACH BY USING CRYOPROTECTANTS

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ABSTRACT

Industrial freezing of spinach is made in blast freezers, using rapid freezing, in forms of briquettes. Besides formation of small crystals, rapid freezing, can still deteriorate the quality of spinach and decrease its commercial value due to texture loss caused by re-crystallization and agglomeration of crystals during the thawing phase.
Cryoprotective treatment maintains the natural quality of food by inhibiting the growth of small crystals. In this study five cryoprotectants: glucose, trehalose, glycerol, mannitol and sodium tripolyphosphate were tested. They were introduced into the spinach tissue by means of vacuum infusion and ultrasound. Afterwards, samples were frozen in liquid nitrogen to simulate rapid freezing and stored at -18°C for a period of 30 days. The quality of the spinach leaves was evaluated in terms of drip loss, color and texture changes.
Cryocomponents reduced the drip loss during storage from 6 to 40% in all treated samples. In general, the combination of ultrasound and vacuum infusion ensured better cryoprotection for the spinach than using just vacuum infusion for incorporation of the cryoprotective substances in the tissue. Spinach leaves treated with cryoprotectants preserved their turgidity after freezing/thawing in comparison with the enervated control sample shown by the reduced burst strength. There was a significant loss of elasticity of the samples shown by the decreased distance to burst. The results from the evaluation of the color of stored samples (30 days at -18°C) after thawing demonstrated retention of the color in all samples treated with cryoprotectants compared to the 53% loss of the green color of the control sample.
Keywords: spinach, vacuum infusion, ultrasound, cryopreservation

INTRODUCTION

Freezing is a widely used preservation process for fruits and vegetables focused on maintaing the quality of the food. However, loss of the fresh-like characteristics of the products due to freeze-induced damage of cell membranes, turgor loss and the consequent loss of cell viability, could not be avoided (Velickova *et al.*, 2013). The freezing of leaf vegetables, such as spinach, can cause serious quality losses in the flavor and texture and therefore decrease its placement on the market. Industrial blast freezers are broadly used to preserve the quality of the spinach. The rapid freezing methods, such as the cryogenic freezing, are supposed to preserve the stuctural integrety of the plant by formation of small ice crystals thus causing less cell disruption and maintaining the desireble texture. However, recrystallization, which is inevitable during thawing, will still damage the cell membrane, increase the drip loss and the loss of nutrients. Furthermore, the plant tissue can disintegrate during thawing resulting in loss of texture due to loss of cells water capacity reversing the advantages of rapid freezing. The quality of the food should be preserved not only during freezing, but also during frozen storage and during thawing since all the frozen food is consumed after thawing (Van Buggenhout *et al.*,2006).
Recently, it became clear that using cryoprotective substances (amino acids, quaternary ammonium compounds, numerous sugars, alcohols and sodium salts) which reduce the freezable water by osmotic dehydration could protect plant tissue from disruption during freezing and thawing. Phoon *et al.,* (2008) significantly improved the freezing tolerance of spinach leaves by using trehalose. Trehalose was distributed in intra- and extracellular

spaces by means of vacuum infusion and pulsed electric fields. This strategy successfully maintained cell vitality, turgidity and fresh-like characteristics of the leaves after thawing. Van Buggenhout *et al.* (2006) used calcium and pectinmethylesterase (PME) impregnation by vacuum infusion to maintain the texture after freezing/thawing.

The present study reports on the use of vacuum infusion combined with ultrasound forincorporation of cryoprotectants (glucose, trehalose, glycerol, mannitol and sodium triplyphosphate) inside spinach leaves before freezing. The effect of the cryoprotectants on the freezing tolerance of spinach was evaluated by measuring the drip loss, color and textural changes, and sodium chloride content.

MATERIAL AND METHODS

Raw Material
Fresh spinach (*Spinacia oleracea)* flat leaves were purchased from the local green market on daily basis from the same supplier.

Cryoprotective solutions
The following cryoprotectants were used to prepare aqueous solutions: glucose (Merck-Alcaloid, Skopje, Macedonia), trehalose (Cargill*Ascend 16400, Copenhagen, Denmark), glycerol, mannitol, and sodium tripolyphosphate, Na-TPP, (Sigma-Aldrich, Munich Germany). Five different solutions were prepared: 15% (w/w) glucose solution, 15% (w/w) trehalose solution, 15 % (w/w) glycerol solution, 15 % (w/w) mannitol solution and a 1,5% (w/w) TPP solution.

Vacuum impregnation and ultrasound treatment
The spinach leaves were vacuum infused separately with 5 different cryoprotective solutions. 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. For the second experiment the vacuum infusion was coupled with ultrasound treatment. The spinach leaves immersed in the cryoprotective solutions were placed in an ultrasonic bath filled with distilled water and previously degassed and treated for 10 min prior the vacuum infusion step.

Freezing process
Freezing was completed by immersing the leaves in liquid nitrogen for 10 s. The frozen spinach was placed in polyethylene bags and stored in a conventional freezer at a temperature of -18°C for a period of 30 days. Control samples without any treatments were also frozen and stored for the same period of time.

Solute uptake
The solute uptake of the treated spinach leaves was calculated from the following equation, in percent:

$$\% \text{ solute uptake} = \frac{m_1 - m_2}{m_1} \cdot 100 \quad (1)$$

Where m_1 is the initial mass of the fresh spinach leaves and m_2 is the mass of the infused spinach leaves.

Drip loss
Drip loss was calculated on the base of the weight of spinach leaves after freezing and thawing. The following equation was applied:

$$\% \text{ drip loss} = \frac{m_1 - m_2}{m_1} \cdot 100 \quad (2)$$

Where, m_1 is the mass of the spinach sample after freezing, and m_2 is its mass after thawing. All the samples were measured on analytical scale in triple.

Measurement of color

To measure the color of fresh spinach as well as the color of the treated spinach samples, Dr. Lange spectro-color colorimeter was used. The instrument was calibrated against the black and white tile before use. Samples were placed above the light source and the four parameters, that is L* (lightness), a* (greenness), b* (yellowness) and C (hroma) were measured. Data were collected from 15 measurements on each sample. Total color difference ΔE was calculated using the following equation:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (3)$$

Texture

The texture of the spinach leaves was evaluated by using 2 different tests: the measurement of the burst strength of spinach leaves and the wilting test using texture analyzer (TA-XT2i of Stable Micro Systems, Godalming, England). For the measurement of the burst strength five square samples (20 x 20 mm) were cut out from the spinach leaves. The puncture was carried out with 2 mm probe. The initial distance was set to be 5 mm. The leaves were punctured in the center with cross-head speed of 1 mm/s and the force and distance necessary to puncture them were recorded. The peak force is the burst strength and the displacement is the distance to burst, which is an indication of the flexibility of the spinach leaf.The turgidity of the treated spinach samples with the wilting test was evaluated by simply holding the centre of the sample with a small pincer and observing whether the leaves would bend or not.

RESULTS AND DISCUSSION

The solute uptake and the drip loss of the control and treated spinach leaves are presented in Table 1. The results proved that the amount of absorbed cryoprotectant depended on the infusing method. When applying vacuum infusion, during the increase of the vacuum and holding time, the cellular content leaked from the cell and mixed with the cryoprotective solution. Releasing the vacuum on the other hand supports the uptake of the mixture of cytosol and cryoprotectant inside the cell (Phoon *et al.,* 2008). The mass gain with the vacuum infusion was from 9.6±0.85 to 13.13±1.18% for all cryoprotectants and was not affected by the type of cryoprotectant. The combined treatment, that consisted of ultrasound assisted vacuum infusion, significantly increased the solid gain from 34.7±3.13 to 47.2±3.05% (Table 1). The ultrasound enhances the permeability of cell membrane by cavitation shear forces and facilitates the transfer from the cell to the solvent and reverse (Jovanovic-Malinovska *et al.*, 2014). The only exception was noticed for the absorption of Na-TPP, where the low initial concentration of the cryoprotective solution (1.5% w/w) kept the solute uptake at 15%.

The major concern of the freezing process is the agglomeration and re-crystallization of the small crystals during thawing which causes mechanical ruptures of the cell and cell leakage resulting in high drip loss. The reduction of the free water content can be achieved by introducing solutes inside the cell and reducing the re-crystallization of the ice crystals (Velickova *et al.*, 2013). The drip loss of the treated spinach samples was decreased from 5.7% to 40.23% compared to the control sample. The best cryoprotection was obtained in the samples impregnated with mannitol when combined infusing methods were used. This might be due to the fact that mannitol is a sugar alcohol that forms strong bonds between its hydroxyl groups and the water molecules. There was no correlation between the amount of solute uptake and the drip loss.

Table 1. Solute uptake and drip loss of spinach samples stored at -18°C for 30 days after freezing/thawing

Spinach	Solute uptake (%)	Drip loss (%)
control	/	62.43±2.33
vacuum infusion		
glucose	13.13±1.18 [a]	44.01±2.89
trehalose	10.8±0.96 [a]	53.02±3.54
mannitol	12.4±1.79 [a]	55.46±2.06
glycerol	9.6±0.85 [a]	54.45±1.98
Na-TPP	11.5±1.28 [a]	58.32±3.33
ultrasound & vacuum infusion		
glucose	41.7±1.86 [a]	53.64±4.87
trehalose	42.7±2.09 [a]	58.83±4.55
mannitol	34.7±3.13 [b]	37.31±1.76
glycerol	47.2±3.05 [c]	53.76±2.67
Na-TPP	15.8±1.05 [d]	56.10±1.83

*Reported are average values and standard deviations.
Values with different letter in a given column are significantly different (p < 0.05)

Color is important parameter for the appearance of the product and has high influence on the consumer choice. The color parameters of the control and treated spinach leaves are given in Table 2. The most important color parameter for the spinach is the a* value since it represents the green color. The results proved that there were slight differences between the fresh and treated samples in regard to this parameter, indicating that cryoprotectants retained the typical green color from 30 to 60 %. Only the control, untreated sample, exhibited significant loss, 52%, of the green color due to the chlorophyll's pheophytilisation that occurred during freezing/thawing cycle (Martins *et al.* 2002). The b*-values demonstrated greater degradation rate decreasing from initial value of 21.6 for the fresh sample to 6.1 for the control sample. Still, samples treated with cryoprotectants , all the differences above 3.5 are perceptible by the human eye (Barrett *et al.,* 2010).

Table 2. Color of the spinach leaves stored at -18°C for 30 days after freezing/thawing

Spinach	L	a	b	c	ΔE
fresh	32.7±3.89	-10.8±0.60	21.6±4.59	24.1±4.33	/
control	35.2±0.12	-5.1±0.04	6.1±0.38	7.9±0.30	15.0±1.20
vacuum infusion					
glucose	22.1±2.22	-8.8±0.90	8.6±2.05	12.4±1.93	12.9±0.88
trehalose	20.4±3.91	-10.3±0.88	11.3±2.77	14.7±2.72	8.5±0.65
mannitol	24.4±4.28	-9.8±1.08	15.2±3.22	17.5±3.25	13.5±1.00
glycerol	20.3±3.42	-9.9±0.89	8.6±1.67	13.3±1.98	7.9±0.54
Na-TPP	27.7±3.18	-10.0±1.36	17.8±3.60	20.5±3.53	4.2±0.38
ultrasound & vacuum infusion					
glucose	23.5±3.99	-10.3±0.80	14.8±3.36	15.9±3.86	8.9±1.02
trehalose	19.7±2.88	-9.7±0.94	9.5±2.18	13.1±2.18	14.6±1.33
mannitol	32.7±6.61	-7.5±1.55	10.87±2.66	13.1±2.74	5.9±0.76
glycerol	21.7±1.99	-11.2±0.87	12.0±1.36	16.1±1.98	8.2±0.64
Na-TPP	27.2±2.21	-10.9±0.92	14.1±2.75	18.0±3.60	5.6±0.82

The textural changes of the spinach after being frozen for 30 days and thawed are given in Figures 1 and 2. The pre-treatments, vacuum infusion and ultrasound assisted vacuum infusion, performed prior to freezing did not affect the texture of the spinach leaves (data not shown). After storage it could be seen that the treated leaves maintained their hardness, unlike the control sample that was completely enervated. Despite the use of the cryoprotectants, the flexibility of the leaves could not be preserved.
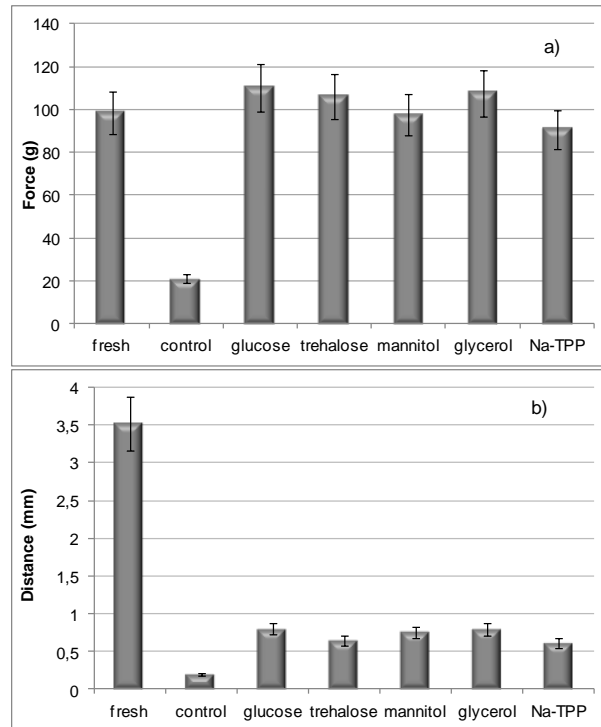
Figure 1.Hardness,a) and flexibility, b) of vacuum infused spinach leaves stored for 30 days at -18°C
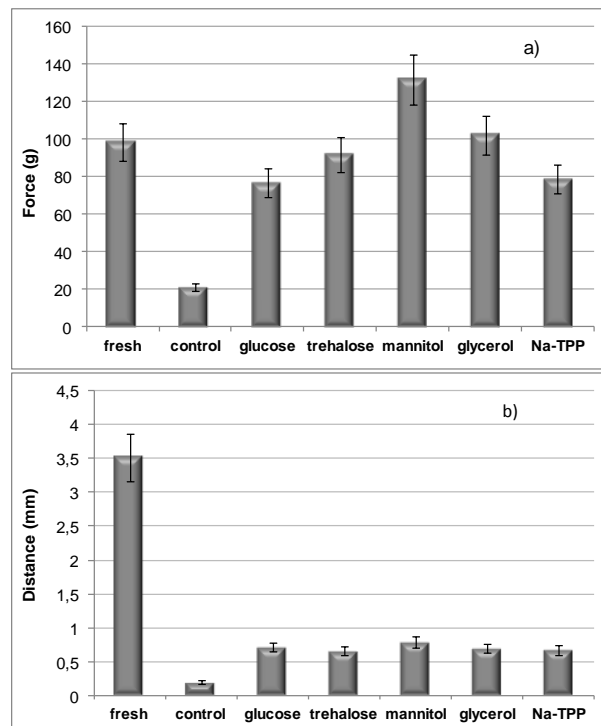
Figure 2.Hardness,a) and flexibility, b) of ultrasonicated and vacuum infused spinach leaves stored for 30 days at -18°C;

The turgidity of the treated spinach leaves stored for 30 days at -18°C is given in Figures 3 and 4. The photographs present only slight wilting of the spinach leaves.
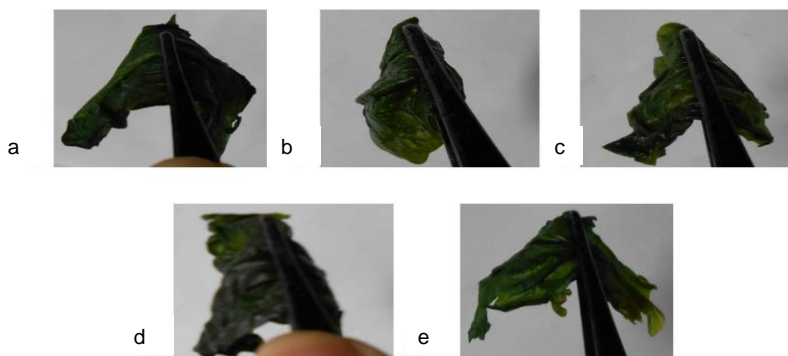
*Figure 3. Turgidity of vacuum infused spinach leaves stored for 30 days at -18°C;
a) glucose, b) trehalose, c) mannitol, d) glycerol, e) Na-TPP*

The results suggested that the infusion methods used allowed the uptake of cryoprotectants by the tissue, thus increasing the freezing resistance of the samples. In general, the addition of the ultrasound step to the infusing treatment improved the freezing tolerance of the samples, probably due to cellular restructuring under the ultrasonication.
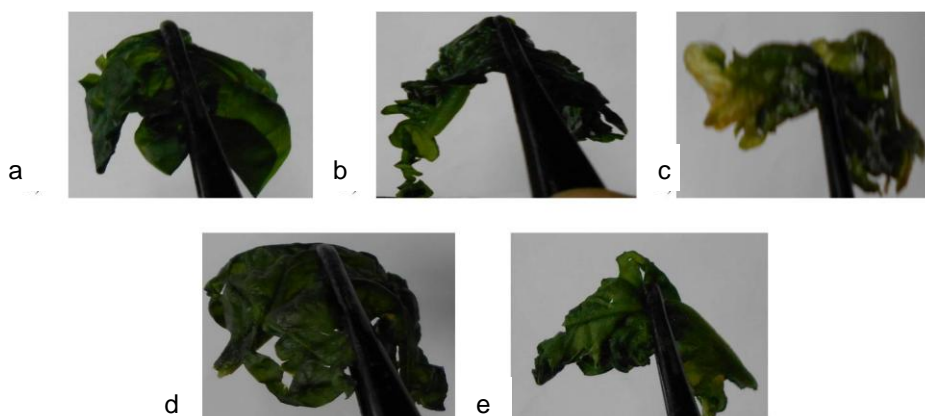
*Figure 4. Turgidity of ultrasonicated and vacuum infused spinach leaves stored for 30 days at -18°C;
a) glucose, b) trehalose, c) mannitol, d) glycerol, e) Na-TPP*

CONCLUSIONS

The impregnated spinach leaves with the cryoprotectants glucose, trehalose, glycerol, mannitol and sodium tripolyphosphateexhibited improved freezing tolerance compared to control, untreated samples. Higher freezing resistance of spinach leaves was achieved when the vacuum infusion process was used in combination with ultrasound.The combined methods probably allowed presence of higher concentrations of cryoprotectants and therefore enable better preservation of the spinach tissue during freezing and thawing process.

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THE INFLUENCE OF VACUUM PACKAGING AND STORAGE ON LIPID OXIDATION IN TRADITIONAL *PETROVSKÁ KLOBÁSA* SAUSAGE

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ABSTRACT

The effect of vacuum packaging on lipid oxidation of dry fermented sausage (*Petrovská klobása)* was investigated. Fatty acid profile, TBARS value and sensory analysis of odor and taste were determined after drying process and during seven months of storage. Sausages were produced in industrial conditions and subjected to smoking, drying and ripening processes during 60 days. The produced sausages were stored in unpacked and vacuum packed form. During the storage period, vacuum packed sausages showed better oxidative stability. ∑PUFA and PUFA/SFA in vacuum packed sausages were significantly higher than in unpacked sausages. 2-thiobarbituric acid reactive substances (TBARS) value in vacuum packed sausages was 0.25 mg MDA/kg at the end of investigated storage period, and this content was significantly lower ($P<0.05$) compared to unpacked sausages (0.73 mg MDA/kg). After 2 and 7 months of storage, vacuum packed sausages are characterized by higher sensory scores for odor and taste. Results suggest that vacuum packaging can be successfully applied to protect dry fermented sausages from lipid oxidation during investigated storage period.
Keywords: vacuum packaging, lipid oxidation, traditional sausage

INTRODUCTION

Petrovská klobása is a traditional dry fermented sausage produced in Backi Petrovac (The Province of Vojvodina, Serbia) exclusively from pork meat and fat with the addition of red hot paprika powder, salt, garlic, caraway and sugar (Ikonić *et al.,* 2011). Fermented sausages are products that contain a high percentage of fat. Fat is responsible for numerous properties of the fermented sausages. From a physiological aspect, fat is an important source of energy as well as of essential fatty acids and liposoluble vitamins. Products formed during lipolysis and lipid oxidation have an important role in the formation of odor, taste and texture of the final product. However, fermented sausages also show some negative properties as a consequence of high content of animal fat. Lipolysis is the first step in the process of auto-oxidation of free fatty acids. Oxidation can also affect the nutritional value of food by decomposition of vitamins and unsaturated essential fatty acids (Olivares *et al.,* 2009; Šojić *et al.,* 2014).

In the modern food chain system, it is hardly conceivable to distribute foodstuff without packaging. Food packaging preserves and protects 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 (Šuput *et al.,* 2013). Vacuum packaging has also been introduced as a commercial way for the retail selling of meat products (Samelis *et al.,* 2000). Numerous authors have investigated effect of vacuum packaging on oxidative stability and shelf-life of fermented sausages, but reported results differ among papers. Ansorena and Astiasarán (2004) reported that sausages packed in vacuum showed better oxidative stability compared to the unpacked sausages, while Summo *et al.* (2006) did not determine significant effect of vacuum packaging on lipid oxidative stability in fermented sausages. Valencia *et al.* (2006) and Rubio *et al.* (2008) did not observe significant difference between the effects of vacuum packaging on oxidative stability of fermented sausages during long storage period.

Petrovská klobása is a traditional product that is currently being transferred from a small-scale production to industrial. At this point, preservation of product during distribution and storage is very important. The aim of this research was to investigate the effects of vacuum packaging of *Petrovská klobása* sausage on lipid oxidation intensity of oxidative changes, as well as on sensory properties during long storage period.

MATERIAL AND METHODS

The *Petrovská klobása* sausage was subjected to the traditional conditions of smoking (5 °C–10 °C and RH = 75%–85%), and drying (8 °C–10 °C, RH = 90%–75%), during 60 days. After that time, sausages were divided into two groups. The first group consisted of unpacked sausages, while the sausages from the second group were packed under vacuum. After packing, sausages were stored in a chamber with controlled temperature (15 °C) and relative humidity (75%) for seven months. Fatty acids composition, TBARS value , as well as sensory evaluation of odor and taste were determined after drying process and after two and seven months of storage.

Fatty acid profile determination
The method of Folch *et al.* (1957) was used for the extraction of lipids from sausages. The fatty acid composition was determined by gas chromatography. A Perkin–Elmer Varian, series 1400 gas chromatograph fitted with a packed column (3 m x 3.0 mm, a stationary phase GP 10% SPTM-2330 on inert carrier 100/120 Chromosorb WAW) and flame ionization detection was used (Perkin-Elmer, Waltham, Massachusetts, USA). Fatty acids methyl esters were quantified as percentage of total methyl esters.

TBARS determination
TBARS (2-thiobarbituric acid reactive substances) test was performed using the method of Bostoglou *et al.* (1994), with modifications. TCA solution (10%) was added to the sample and extraction was performed in ultrasonic bath (XUB 12, Grant Instruments, Cambridge, UK) (Mandić *et al.,* 2007). The absorbance was measured at 532 nm using a spectrophotometer (Jenway 6300, Felsted, United Kingdom). TBARS values were expressed as milligrams of malondialdehyde per kilogram of sample.

Sensory evaluation of odor and taste
A panel consisting of seven trained panelists of different ages performed sensory evaluation of odor and taste. Casing was removed; sausages were cut into slices of approximately 4 mm thickness and served at room temperature on white plastic dishes. Three slices were served from each batch. Water and unsalted toasts were provided to cleanse the palate between samples. Evaluation was performed according to quantitative descriptive analysis (QDA), using a scale from 0 to 5, with a sensitivity threshold of 0.25 points (Stone and Sidel, 2004). Each mark means distinctive quality level, described as follows: 5 – extraordinary, typical, optimal quality; 4 – observable deviations or insignificant quality defects; 3 – drawbacks and defects of quality; 2 – distinct to very distinct drawbacks and defects of quality; 1 – fully changed, atypical properties, product unacceptable; 0 – visible mechanical or microbiological contamination, atypical product.

Statistical analysis
Statistical analysis was carried out using STATISTICA 8.0 (2008). All data were presented as mean value with their standard deviation indicated (mean ± SD). Variance analysis (ANOVA) was performed, with a confidence interval of 95% ($p < 0.05$). Means were compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

Table 1 shows the fatty acid composition of the *Petrovská klobása* sausages at the end of drying process and after 2 and 7 months of storage. There were no significant differences (P>0.05) in ∑SFA and ∑UFA between vacuum packed and unpacked sausages after two and seven monts of storage. Similar results were obtained in our previous study (Krkić *et al.,* 2013), when the antioxidative effect of chitosan-oregano coating on lipids of Petrovskà klobàsa sausages was examined. However, after 2 and 7 months of storage, ∑PUFA and PUFA/SFA were significantly higher (P<0.05) in vacuum packed compared to unpacked sausages. Obtained results correspond to literature data (Valencia *et al.,* 2006).

Table 1. The influence of vacuum packaging (VP) and storage time on fatty acid profile in Petrovská klobása sausage

Fatty acid	At the end of drying	Storage time (months)			
		2		7	
		Unpacked	VP	Unpacked	VP
∑SFA	34.07 ±0.39	34.56 ±0.63	33.96 ±0.60	34.35 ±0.75	33.54 ±0.53
∑UFA	65.87 ±0.49	65.44 ±0.56	66.03 ±0.54	65.60 ±0.76	66.43 ±0.48
∑PUFA	16.83[a] ±0.09	15.68[c] ±0.19	16.81[a] ±0.10	16.30[b] ±0.42	16.92[a] ±0.33
UFA/SFA	1.93[abc] ±0.04	1.89[c] ±0.03	1.94[ab] ±0.02	1.91[bc] ±0.04	1.98[a] ±0.04
PUFA/SFA	0.49[a] ±0.01	0.45[b] ±0.00	0.49[a] ±0.00	0.47[b] ±0.00	0.50[a] ±0.01

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

At the end of drying process of *Petrovská klobása* sausage, TBARS value was 1.19 mg MDA/kg. After two months of storage, TBARS value decreased significantly (P < 0.05) for both tested sausages. Nassu *et al.* (2003) and Ansorena and Astiasarán (2004) obtained similar results. The decrease of malondialdehyde content is probably due to reactions between malondialdehyde and carbohydrates, nitrites and amino acids, produced during ripening process (Janero, 1990). At the end of storage period, TBARS value in the vacuum packed sausages was 0.25 mg MDA/kg and this content was significantly lower (P<0.05) compared to the unpacked sausages (0.73 mg MDA/kg). Examining vacuum packaging effect, Ansorena and Astiasarán (2004) obtained similar results.
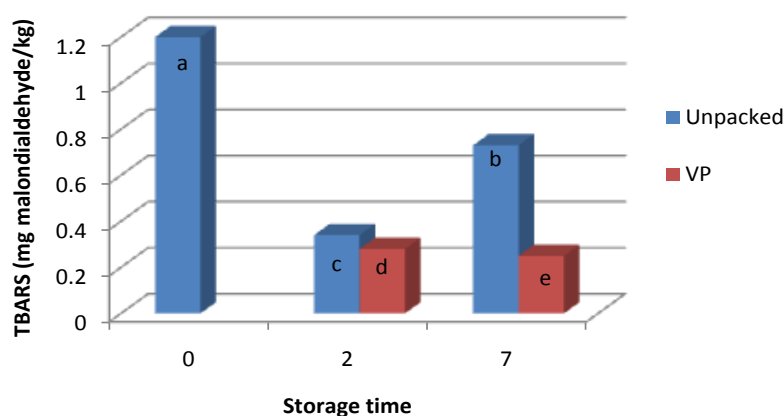
Figure 1. The influence of vacuum packaging (VP) and storage time on TBARS value in Petrovská klobása sausage

Sensory score of odor and taste (Figure 1) was the highest for VP sausages after 2 months of storage (4.54). During the whole storage period, VP sausages had significantly higher ($P<0.05$) scores for odor and taste, compared to unpacked sausages. This result is in negative correlation with the TBARS values in VP and unpacked sausage after two and seven months of storage. Similar results were reported by Krkić et al. (2012).
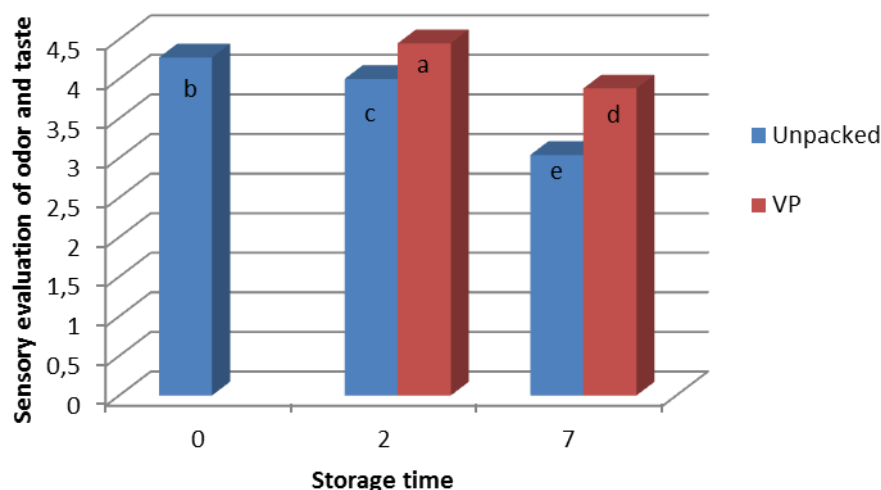
Figure 2. The influence of vacuum packaging (VP) and storage time on sensory evaluation of odor and taste of Petrovská klobása sausage

CONCLUSIONS

Vacuum packaging of *Petrovská klobása* sausage resulted in lower degradation of polyunsaturated fatty acids compared to unpacked sausage during the investigated storage period (7 months). TBARS value was also lower for vacuum packed sausage. Sensory score for odor and taste of vacuum packed sausage was better than for the unpacked sausage. It can be concluded that vacuum packaging can prolong oxidative changes of traditional *Petrovská klobása* sausage and ensure its better sensory score.

ACKNOWLEDGEMENTS

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HYGIENIC EQUIPMENT DESIGN AND MEAT PROCESSING OPERATIONS

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ABSTRACT

Hygienic design refers to those building design features that may be unique to a meat processing plant and are intended to reduce the risk of contamination by biological, physical and chemical hazards from meat processing operations. The task of hygienic design is to minimize risks of contamination and to make easier the challenges of cleaning and maintaining the plant and equipment. Poorly designed equipment might increase the risk of contamination of meat and meat products with micro-organisms and different stages of processing and manufacturing could demand different levels of hygienic design. The fundamental principle, however, is that the design of any piece of equipment must not allow any increase in the concentration of relevant contaminants.

The European Directive 2006/42/EC for machinery already sets up requirements for food processing machinery, which have to be obtained in all meat processing machines. Chapter 2.1. states that all machines have to be cleanable, drainable and all surfaces have to be smooth. These general statements are detailed in the following standards: The EN ISO 14159 "Safety of machinery - Hygiene requirements for the design of machinery" and the EN 1672-2 "Food processing machinery - Basic concepts - Part 2: Hygiene requirements". They also give the presumption of conformity for these machines build according to these standards, as they are listed under the machinery directive in the European journal, as harmonized standards.

Within Europe and the USA, a number of organisations exist to foster consensus in hygienic design and the use of these organisations' guidelines can have a quasi legal status. In 1989 an independent group, the European Hygienic Equipment Design Group (EHEDG) was formed; it consists of approximately 100 members who are equipment manufacturers, food processors, research organisations, or government representatives from many countries including Serbia.

Keywords: *hygienic design, meat, processing, equipment*

INTRODUCTION

Hygiene problems in equipment are caused when microorganisms become attached to the surfaces and survive on them and later become detached from them contaminating the product. This can be due to bad hygienic design in cases where the machines cannot be cleaned properly (Wirtanen, 1995). Food-processing equipment has been shown to be a source of contamination, e.g. *Listeria monocytogenes*, in many studies (Arnisalo *et al.,* 2005).

Raw meat, if red or white (e.g. chicken), is one of the most critical products within food processing. The meat itself can be a source of micro-organism, which can spoil the food, reduce shelf live and could be harmful for the consumer. In most cases minor contamination with less harmful micro-organism of the raw meat is not a problem, as long as the facility, in which the product is prepared, is readily cleanable and is not harbouring product and micro-organism itself. During meat production and processing, contamination may additionally occur from micro-organisms present in the factory and so the operating environment and the behaviour of the employees also becomes an important factor.

Food processing equipment has traditionally been designed and built to be suitable for purpose. For example, a mixer for raw meat has rarely been designed to the same hygienic level as a slicer of cooked meats. Similarly, aseptic fillers have usually been designed to a much higher hygiene standard than can filling machines. The reason for this has been

related to the risk of a hazard being transferred from the equipment to the product produced and thus the consumer.

GENERAL PRINCIPLES OF SANITARY DESIGN

Poorly designed equipment might increase the risk of contamination of meat and meat products with micro-organisms and different stages of processing and manufacturing could demand different levels of hygienic design. The fundamental principle, however, is that the design of any piece of equipment must not allow any increase in the concentration of relevant contaminants.

The American Meat Institute (AMI) has developed a checklist, based on NSF International (formerly "National Sanitation Foundation"-USA) and AMI Guidelines and splits the basic requirements into 10 principles (Seward*, 2007*) which are:

1) Cleanable to a microbiological level
2) Made of compatible materials
3) Accessible for inspection, maintenance, cleaning & sanitation
4) No (product or) liquid collection
5) Equipment is designed to eliminate or minimize hollow areas
6) No niches
7) Sanitary operational performance
8) Hygienic design of maintenance enclosures (and human machine interfaces)
9) Hygienic compatibility with other systems
10) Validated cleaning and sanitizing protocols

Each principle of this AMI document provides an explanation with a few sentences and questions are asked, checking, if these principles are covered. Depending on the level of fulfillment the tested equipment gets proportionate points of the by AMI proposed available points, which are given. At the end the sum of all reached points indicates, how good the equipment fulfills the Hygienic Design requirements.

Guidelines for the hygienic construction of commercial or custom-built equipment are based on various international standards:

1. Hygiene Requirements for the Design of Meat and Poultry Processing Equipment (ANSI, 1999, 2010) (www.ansi.org);
2. 3-A sanitary standards (www.3-a.org);
3. National Sanitation Foundation International Standards (NSF International; www.nsf.org);
4. European Norms for Food Processing Machinery (CEN/TC 153 - Machinery intended for use with foodstuffs and feed, www.cen.eu);
5. International Organization for Standardization (ISO, www.iso.org);

EUROPEAN STANDARDS AND REGULATIONS CONCERNING HYGIENIC DESIGN

In the EC, the Council Directive on the approximation of the laws of Member States relating to machinery (98/37/EC) was published. The Directive includes a short section dealing with hygiene and design requirements which states that machinery intended for the preparation and processing of foods must be designed and constructed so as to avoid health risks and consists of seven hygiene rules that must be observed. These rules are concerned with the suitability and cleanability of materials in contact with food; surface finish and design features such as joints, absence of ridges and crevices; avoidance of the use of fasteners, e.g. screws and rivets; the design of internal angles and corners; drainage of residues from equipment surfaces; dead spaces and voids, and lastly bearings and shaft seals. The Directive requires that all machinery sold within the EC after January 1995 shall meet these basic standards and be marked accordingly to show compliance (the 'CE' mark).

The revised Machinery Directive 2006/42/EC did not introduce any radical changes compared with the old Machinery Directive 98/37/EC but aims to consolidate the achievements of the Machinery Directive in terms of free circulation and safety of machinery

while improving its application. Chapter 2.1. states very clearly that all machines have to be cleanable, drainable and all surfaces have to be smooth. In addition to these hygiene rules, this section also contains a requirement that machinery manufacturers must indicate the recommended products (chemicals) and methods for cleaning, disinfecting and rinsing both open equipment (e.g. conveyors) and closed equipment (e.g. pipelines, valves and pumps) where clean-in-place (CIP) procedures need to be used.

A basic standard about hygiene requirements for the design of machinery is the ISO 14159:2004, Safety of Machinery–Hygienic Requirements for the Design of Machinery. Anoter important basic standard is the Standard EN 1672-2:2005, Food processing machinery - Basic concepts-Part 2: Hygiene requirements. These two standards give a good overview and are describing, what is needed in order to fulfill the requirements of the Directive 2006/42/EC for machinery (short: Machinery Directive).

It is impossible to provide hygienic design guidelines for all individual food processing equipment. The European committee for standardization (CEN) issues standards for equipment manufacturers to be able to full the requirements of the directive. The approach of the CEN/TC 153 was, therefore, to define the basic hygienic design requirements and they consists of two headings: covering food contact (broken down into eleven subsections) and non-food contact areas. Most of these standards cover this topic by an annex for hygienic requirements. Most of the requirements are not very specific, but the defined surface roughness, depending on the hygiene zone, could help to know, what's acceptable.

However, there are also guidelines and methods published e.g. by the European Hygienic Engineering and Design Group (EHEDG, http://www.ehedg.org) available for helping in the design of new hygienic equipment.

There are existing standards issued by CEN/TC 153 with relevance to meat processing equipment (EN, 1974; EN, 2003a, 2003b, 2003c, 2003d, 2004, 2005a, 2005b,2006, 2008a, 2008b ; CEN, 2008; EN, 2008, 2012) and EHEDG guidelines (EHEDG, 2004a,2004b).

Nowadays, there is intention, over EHEDG established working subgroups to make specific guidelines for other meat processing equipment which are not covered with international standards including deboning and trimming-conveyor belts, freezers, marinating, tumbling and Injection equipment, and also equipment for forming, wraping, packaging and weighing. The EHEDG organisationa is a consortium of equipment manufacturers, food industries, research institutes as well as public health authorities and was founded in 1989 with the aim to promote hygiene during the processing and packing of food products.

The principal goal of EHEDG is the promotion of safe food by improving hygienic engineering and design in all aspects of food manufacture.

EHEDG actively supports European legislation, which requires that handling, preparation processing and packaging of food is done hygienically using hygienic machinery and in hygienic premises (EC 2006/42, EN 1672-2 and EN ISO 14159).

EHEDG is aiming to provide practical guidance on hygienic engineering and design in all aspects of food manufacture and is offering a platform to discuss technical developments and best practices under hygienic design aspects.

A major task of EHEDG is to provide guidelines and recommendations on essential hygienic design standards and practices, based on science and technology and to periodically review them. These documents provide guidance to equipment manufacturers and users on compliance with national and international legislation. However, the EHEDG guidelines are merely non-binding documents and the industry is free to follow or not.

CONCLUSIONS

Food processing equipment that is designed hygienically has three key advantages:

Food quality-Good hygienic design maintains product in the main product flow. This ensures that product is not 'held-up' within the equipment where it could deteriorate and affect product quality on rejoining the main product flow. Or, for example in flavourings

manufacture, one batch could not cross-contaminate a subsequent batch to give sensory problems.

Food safety-Good hygienic design prevents the contamination of the product with substances that would adversely affect the health of the consumer. Such contamination could be microbiological (e.g. pathogens), chemical (e.g. lubricating fluids, cleaning chemicals) and physical (e.g. glass).

Cost reduction- Good hygienic design reduces the time required for an item of equipment to be cleaned. This reduction of cleaning time is significant over the lifetime of the equipment such that hygienically designed equipment which may initially be more expensive (compared to similarly performing poorly designed equipment), will be more cost effective in the long term. In addition, reduced down time for cleaning may lead to the opportunity for increased production.

Of these three factors, safety is the most important to the food consumer. There have been many examples of product recalls, lost production, and indeed site closure, due to contamination arising from poorly designed equipment. Physical foreign body contaminants, such as pieces of plastic, affect the wholesomeness of food but rarely receive media attention. Physical contaminants of a more serious nature e.g. glass fragments or caustic CIP fluids, however, are much more serious. Perhaps of the most concern are pathogenic microorganisms such as *Listeria* or *Escherichia coli* O157:H7, which may be harboured in equipment and then subsequently grow during production and contaminate the product.

The importance of hygienic design cannot be overstated. Regardless of the quality of the general design (fit for purpose) and execution of the sanitation programme, contamination will never be controlled unless the areas in which soils can accumulate are either avoided by good hygienic design or can be readily exposed for cleaning.

Both equipment designers and persons responsible for the selection of equipment, hopefully after a dialogue with engineers, microbiologists and production, must be familiar with the principles of hygienic design to ensure that suitable equipment is purchased. Further to this, persons responsible for the management of sanitation programmes, especially in 'high risk' food manufacturing areas, must also be familiar with basic hygienic design concepts to be sure that equipment is dismantled sufficiently so that surfaces are safe for food production. Similarly, anyone involved in the inspection of hygienic design from a regulatory viewpoint must be specifically trained in this field to ensure that suitable equipment is being used. Hygienic design training should, therefore, be targeted at engineers during their higher education and at key food processing staff.

ACKNOWLEDGEMENTS

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MERCURY IN FISH AND FISH PRODUCTS

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ABSTRACT

Mercury is a contaminant that belongs to the group of heavy metals. There is evidence that mercury toxic effect on the human body. The man living organism coming through air and water. However, the main way to enter the living organism is food that is contaminated with mercury. One of the largest sources of mercury is fish meat, which is why the consumption of fish and fish products may pose a risk to human health. These findings have led to improved control of mercury content in these products. In order to protect consumers adopted the regulations on the maximum allowable amount of mercury in food. In order to determine the mercury content in fish and fish products placed on the market and the Serbian Republic of Bosnia and Herzegovina, conducted extensive research. Part of this research is presented in this paper.

Mercury was determined using spectrometric method by Advanced Mercury Analyser AMA - 254, and the measured concentrations are shown in mg/kg of fish. The study included 48 samples of fish and fish products. The largest amount of mercury was found in canned tuna from pieces in vegetable oil (0,256 mg/kg) and a piece of tuna in cans (0.229 mg/ kg), while the lowest content was measured in the meat fresh trout and frozen pangasius (0,005 mg/kg) and slightly higher results than the minimum specified amounts, were found in fresh carp (0,006 mg/kg) and picarel (0,008 mg/kg). When comparing the results obtained with the maximum level of this element can be concluded that the consumption of fish and fish products in a balanced diet, do not pose a risk to human health.

Keywords *:* mercury, heavy metals, residues, fish, fish products

INTRODUCTION

Nutritional and health significance that is achieved by using fish and fish products in the diet is one of the reasons for the continuous growth in demand for these products on the market (Pavlićević *et al.*, 2014, Burger and Gochfeld, 2009; Sveinsdóttir *et al.*, 2009; Baltić and Teodorović, 1997).

What fish, as food, makes it particularly attractive for the consumer is, in addition to its high content of protein, minerals and vitamins, low levels of saturated fat and that is a very rich source of fatty acids, of which some are considered essential, such as omega -3 polyunsaturated fatty acids. These acids cannot be synthesized in the body and play a role in the prevention of many diseases such as coronary disease, especially myocardial infarction, arteriosclerosis, hypertension and other cardiovascular disorders (Cirkovic, 2002; Kilibarda, 2006; Connor, 2000; Zatsick and Mayket, 2007, Hunter and Roberts, 2000; Mozaffarian *et al.*, 2005, Anon, 2003; Baltić and Tadić, 2001).

Although the role of fish and fishery products in human nutrition is very important, there is growing concern regarding proportional presence of fish in the human diet.

In fact, if people consume large quantities of fish, it may pose a risk due to the presence of contaminants which have undesirable consequences for human health (Hites *et al.*, 2004, Mazloomi *et al.*, 2008; Rom 2007; Storelli, 2008; EFSA, 2005).

Contaminants, such as pesticides, heavy metals, polychlorinated biphenyls, dioxins, furans, polycyclic aromatic hydrocarbons and radioactive elements are all environmental pollutants. However, although their concentration in the environment is usually quite low, they are readily taken up by water-dwelling organisms. Moreover, as a result of bioaccumulation and bioconcentration processes, levels of these compounds are progressively increased along the food chain, particularly in organisms with longer life span and predatory fish.

Based on the literature, fish consumption can be considered as one of the main sources of human exposure to the above-mentioned environmental pollutants (EFSA, 2005; Storelli, 2008).

Mercury (Hg) is a chemical element belonging to the heavy metals and is the only metal that is at room temperature is in the liquid state. Mercury exists as elementary, organic and inorganic form in the environment.

It is one of the most dangerous pollutants and can be found in all four sphere of earth (Table 1), because this element comes to environment from natural and anthropogenic sources (Pacyna, 2010, EFSA, 2005; Storelli, 2008; Gerbersmann *et al.*, 1997; Cai, 2000; Queuvallier *et al.*, 2000).

Due to its persistence in the environment, its atmospheric transport, bioaccumulation, biotransformation and biomagnification in food chain, mercury is recognized as a global problem (WHO 1990).

Certain microorganisms are capable of converting all of inorganic mercury in organic methyl mercury, which is then released into the water and may accumulate in the marine organisms (Bose O`Relly *et al.*, 2010). All forms of mercury and its compounds are potentially toxic and their toxicity depends on a concentration in individual organisms (Srebočan *et al.*, 2007). In the human body can be entered by inhalation or ingestion. As of the total mercury intake of food, 60 to 90% is represented by fish, fishery products, bivalve molluscs and cephalopods, They are a representation of its main sources (Berglund *et al.*, 2005; Elhamri *et al.*, 2007). Contamination with mercury is very high in some fish causing harmful effects to human health in people who consume large amounts of fish (Hites *et al.*, 2004).

Table 1. Mercury in the environment

Environment	Quantity
Earth's crust	0.08 mg/kg
Ground	0.1-0.5 µg/kg, contaminated soil to 0.2 mg/kg
Atmosphere	40 ng/kg
Seawater	2-10 ng/m³, deposits of ore and 1.500 ng/m³, in rainwater 2-5 ng/kg

These findings have led to improved control of these products in the world why in the BH set Regulation on maximum permitted levels for certain contaminants in foodstuffs ("Official Journal of Bosnia and Herzegovina", no. 37/09 and 39/12).

As the aim of this study was determined to establish the content of total mercury in fish and fish products to assess the contamination with these toxic metals, determine compliance with valid regulations, contributing to the considerations in the risk from consuming fish and fish products marketed Republic of Srpska.

MATERIALS AND METHODS

The total mercury concentration was measured in samples of fish and fish products that are given to testing upon the request of veterinary inspection with the Republic of Srpska. The study included 48 samples of fish and fish products: fresh fish, 33 samples of frozen fish samples 9 and 6 samples of canned fish (tuna four samples, one sardine and mackerel one). These data were collected at the end of 2013th and during the 2014th year. Fish, which is delivered in the fresh or frozen, stored at -20 °C until the time of analysis, and the temperature for canned refrigerator. Before the analysis, edible parts of fresh and frozen fish are chopped into pieces 2-3 cm thick and homogenized, while the cans after tempering at room temperature, homogenized immediately after opening. Quantification of mercury was 0,005 mg/kg.

The amount of total mercury was determined by mercury analyzer Advanced Mercury Analyser AMA-254 (Altec, Prague, Czech Republic). In the combustion furnace, the homogenised sample (100 mg (µL)) is first dried at 120 °C for 60 s, and then under a stream

of oxygen at 850 ^{0}C undergoing phase decomposition during 150 s. Decomposition Products, a stream of oxygen, passing through a catalytic furnace at 700 ^{0}C at which retain nitrogen and sulfur oxides. Mercury is catching on Au-amalgamator. Selectively releasing trapped alive with amalgamat short warming and mercury vapor is transported into the cell for measurement in which the mercury finally quantify (measurement cycle, 45 s) at the appropriate wavelength (253.65 nm).

For the preparation of standard solution used was a standard mercury concentration of 1000 mg/l. In order to improve the stability of the diluted primary standards, the following chemicals were used: 10% (m/v) solution of $K_2Cr_2O_7$, conc. HCl and conc. HNO_3 and was used as a solvent with ultra-pure water.

RESULTS AND DISCUSSION

Mercury has been detected in all samples of fish and fish products (48 samples). Results of the analyses of different types of fish and fish product are shown in Table 2. Based on these results, it can be concluded that there are differences in the content of mercury in fish and fish products. The concentration of mercury in fresh fish ranged from 0,006 to 0,136 mg/kg (average 0,049 mg/kg), frozen fish from 0,005 to 0,026 mg/kg (average 0,017 mg/kg), canned mackerel and sardines from 0,045 to 0,060 mg/kg and canned tuna from 0,111 to 0,256 mg/kg (average 0,178 mg/kg). The greatest mercury content variations were found in canned tuna in oil (Figure 1*).*

According to the Regulation on the maximum residue limits for certain contaminants in foodstuffs ("Official Journal of BiH", no. 37/09 and 39/12) the Maximum Residue Limit (MRL) for mercury in tuna is 1 mg/kg, while for all other species, which were the subject to testing, are 0,5 mg/kg. In all tested samples, the mercury content is below the Maximum Residue Limits (MRL).

Based on the test results, we found that mercury concentrations were significantly higher in samples of canned tuna compared to mercury concentrations determined in fresh and frozen fish. The largest amount of mercury was found in canned tuna meat pieces in vegetable oil (0,256 mg/kg) and tuna meat pieces in olive oil (0,229 mg/kg), while the lowest content was measured in the fresh trout meat and frozen pangasius (0,005 mg/kg). Slightly higher results than the minimum amounts specified above, were found in fresh carp (0,006 mg/kg) and sand smelt (0,008 mg/kg) (Table 1).

Previous studies have shown that the effect of mercury and contamination by this element is associated with the length, weight, age and sex of fish (Agus *et al.*, 2005; Emami, 2005; Storelli, 2002; De Marco, 2006). Season and place, both greatly affect the level of accumulation of toxic elements in fish (Kagi and Schaffer, 1998). As a result of bioaccumulation and biomagnification, the highest concentrations of mercury in marine organisms, can be recorded in top predators such as swordfish, tuna, shark, whales, etc. Our test results of tuna are in agreement with results reported by Burger and Gochfeld (2005), Kagi and Schaffer (1998), Soylak (2007), Boada (2011), but not in agreement with results reported by Najdek and Bažulić (1983), who found mercury concentrations to be higher than the permissible amount.

Mercury concentrations obtained by this study, 0,006 to 0,256 mg/kg, are lower than the results obtained by Holden (1973) (0,82 to 1,2 mg/kg) and Fricke *et al.*, (1979) (0,04 to 0,44 mg/kg) in canned fish and also lower than the mercury concentrations (0,3 to 0,59 mg/kg), determined by Roxanne et al. (2012). According to Khansari *et al.* (2005), in canned tuna, the mercury content ranged from 0,043 to 0,253 mg/kg, which is in agreement with our results. Concentrations of mercury in canned fish, published by the FDA (2000), ranged between 0,082 to 0,160 mg/kg which is lower than the results obtained in our study. Djinovic *et al.* (2010) demonstrated the presence of mercury in the samples of carp meat, ranging from 0,005 to 0,045 mg/kg, which is similar to our results. Higher concentrations of mercury than those revealed by our examination of river carp samples, were reported by Andreji *et al.* (2006), which proved the presence of mercury from 0,46 to 0,95 mg/kg and Trbovic *et al.*

(2011), which determined average concentration of 0,099 mg/kg. Karadede and Unlu (2000) have not proved presence of mercury in samples of lake carp.
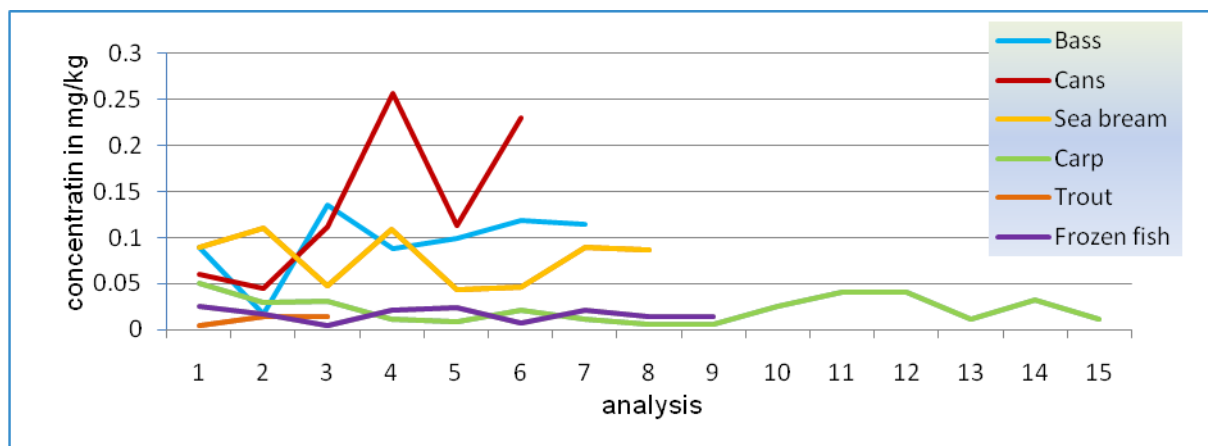
Figure 1. Variations of the concentration of mercury within the same fish species and amongdifferent species of fish

Table 2. Mercury residues (mg/kg) in fish and canned fish samples

Species	Number of samples	Total mercury content (mg/kg of product wet weight)				MRL mg/kg
		average	Std dev.	min	max	
FRESH FISH						
Sea bass	7	0,102	0,022	0,070	0,136	0,50
Sea bream	8	0,078	0,027	0,044	0,110	0,50
Carp	15	0,022	0,014	0,006	0,050	0,50
Trout	3	0,011	0.005	0,005	0,014	0,50
FROZEN FISH						
Salmon	1	0,015	-	0,015	0,015	0,50
Pangasius	1	0,005	-	0,005	0,005	0,50
Hake	2	0,022	0,006	0,017	0,026	0,50
Mackerel	1	0,022	-	0,022	0,022	0,50
Sand smelt	4	0,018	0,008	0,008	0,025	0,50
CANS						
Sardines (in vegetable oil)	1	0,006	-	0,006	0,006	0,50
Mackerel (fillets in vegetable oil)	1	0,045	-	0,045	0,045	0,50
Tuna in vegetable or olive oil	4	0,178	0,076	0,111	0,256	1,0

CONCLUSIONS

1.Concentration of mercury in all samples was below than maximum allowed level.
2.Larger total mercury was found in the muscle meat of fish that are at the end of the food chain.
3.The largest amount of mercury, whose concentration was 0,256 mg/kg, determined in cans of tuna pieces in vegetable oil.
4.Lowest mercury was found in the fresh trout and frozen pangasius (0,005 mg/kg), fresh carp (0,006 mg/kg) and sand smelt (0,007 mg/kg).
5.Based on test results of mercury, there is no serious risk to human health associated with the consumption of fish and fish products in the territory of the Republic of Srpska.

6.It is necessary to carry out more research and evaluation of the quality of fish and fish products in order to provide more data and thus significantly contribute to the preservation of human health.

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PORK MEAT AS A FUNCTIONAL FOOD

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ABSTRACT

The term "functional foods" can include simple foods, technologically treated food products and/or their active components that can be used to prepare fortified foods, or can be consumed separately from foods as supplements. Several epidemiological studies have provided evidence that foods should no longer be considered only for their nutritive value, but also for their potential positive effects in preventing or protecting against serious chronic diseases, especially those associated with a Western lifestyle. These diseases include neoplastic, cardiovascular, or neurodegenerative ailments, cataracts, diabetes, metabolic syndromes, and inflammatory/degenerative processes associated with aging. Accordingly, it is possible that pig diets can be enriched with certain bioactive feed additives or raw materials such as aromatic plants, vitamins, trace elements, chelated substances, probiotics, prebiotics, plant and fish oils, seaweeds, which have antioxidant, antimicrobial, immunomodulatory or anti-inflammatory properties. Therefore, "functional" pork meat and meat products can be produced with enhanced beneficial properties. These new generation products may have a healthier composition and/or compounds that are highly sought by consumers that want or are obliged to follow a specific diet due to health reasons.

Keywords: pork meat, functional foods, bioactive ingredients, health benefits

INTRODUCTION

Recently, "functional foods" have become popular, despite the fact that this term has never been officially defined. An acceptable definition by nutritionists is: "A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to improved state of health and well-being and/or reduction of disease risk" (Zhang *et al.*, 2010).

The first country to develop the idea of functional foods and establish regulations for their uses was Japan in the 1980s (Kwak & Jukes, 2001). The increase of functional food consumption in Japan and USA has also been favored by the respective public authorities, who consider functional foods a possible tool in reducing public health costs (Decker & Park, 2010).

Functional foods can be classified into two main categories according to their expected effects: those aiming to improve physiological functions, and those aiming to reduce the risk of specific pathological conditions (Diplock *et al.*, 1999).

The main ingredients used in functional foods include probiotics, prebiotics, synbiotics, vitamins, minerals, antioxidants, phytobiotics, proteins, peptides, amino acids, and fatty acids such as omega-3 fatty acids and conjugated linoleic acid (CLA).

Nowadays, functional pork meat and meat products can be produced with enhanced beneficial properties. Such novel products are designed to have a modified composition that is highly sought by consumers that want or are obliged to follow a specific diet due to health reasons.

Pork meat and its role as functional food

Chemical composition

Pork meat offers excellent nutritive and dietetic properties for the human consumer. Its proximate composition demonstrates high protein content (19.1 – 23,4%) in lean tissue (Skobrak *et al.* 2011) with high levels of essential amino acids. Mineral content in meat is also constant at around 1.2–1.3 %. The leanest cut of pork meat in the carcass is the loin, with an average lipid content of 1.8 %, whereas the fattest portion of meat tissue has an average lipid content of 10 %. The most quantitatively important cut is the hind leg, and its lipid content is quite low (on average 3.4 %) compared to the other meats. Meat consumption, including pork, has previously been linked with an increased incidence of obesity, type 2 diabetes and cardiovascular diseases, however, fat intake rather than lean meat or protein has been associated to those conditions (Lombardi-Boccia *et al.*, 2005).

Mineral content

Moreover, pork meat is considered as a rather good source of iron and zinc. Heme iron in meat has the advantage of being more biologically available than the iron in plant-based products, with availability ranging from 72 to 87% in red meats, and from 56 to 62% in rabbit and pork meats (Lombardi-Boccia *et al.*, 2005). It is noteworthy that the iron content in meat was found to be higher in local breeds reared extensively compared to the commercial breeds reared conventionally.

Pork meat is characterized by low sodium contents: 37 mg/100 g for loin and 50 mg/100 g for hind leg (Romans *et al.*, 1994). Reduced sodium intake has recently been recommended to limit arterial hypertension, especially in sodium sensitive individuals. Phosphorus is the second-most abundant mineral in meats, and pork meat has a relatively high content (180 mg/100 g for loin). Selenium is an essential trace mineral due to its role in regulating various physiological functions as an integral part of selenoproteins that are part of the body's antioxidant defense system. Selenomethionine is a common source of organic selenium in foods. Since, it is incorporated into general proteins as methionine, animal meat can be feasibly enriched with selenium by feeding to animals supra-nutritional levels of selenomethionine. In pigs the fortification of diets with selenium increased lipid oxidative stability of the muscle tissue (Mahan *et al.*, 1999; Krska *et al.*, 2001). Zinc is also contained in antioxidant enzymes (superoxide dismutase and glutathione peroxidase) in some animal species, and thus the effects of dietary supplementation with zinc on pork meat oxidation have been investigated. A study of Li et al. (2007) showed that exogenous zinc-metallothionein (Zn-MT) had an anti-oxidative function and improved pork quality.

Vitamin content

Meat is an important source of bioavailable B vitamins and especially vitamin B_{12}, which is found only in foods that originating from animals. Their concentrations vary significantly not only among meats of different species but also among different carcass parts; also, heat treatment lowers its content (Lombardi-Boccia *et al.*, 2005). It is reported that a portion of 100 g of pork meat provides up to three times the recommended daily intake of vitamin B_{12} (Williams, 2007).

Folate is essential for normal cell growth and replication. Enriched meat with folate can yield a potentially functional product, since folate deficiency can lead to several health problems, most notably, neural tube defects in developing embryos. Folate like substances may have similar effects to antibiotics and cytotoxic drugs in the treatment of cancer, autoimmune diseases, psoriasis, and bacterial and protozoal infections (Kamen, 1997). Manipulation of the folate level in the pork meat can help supply a substantial portion of the daily recommended consumption levels (Rooke *et al.* 2010).

Vitamin E is essential for growth, immune function enhancement, tissue integrity, reproduction, disease prevention, and antioxidant function in biological systems. Meat products could become even better sources of vitamin E through meat fortification or dietary supplementation with α- tocopheryl acetate. The main benefits in fortifying meat and meat

products with α-tocopheryl acetate are its high antioxidant activity, the fact that its presence in muscle cell membranes reduces lipid oxidation, and also that it is possibly involved in limiting protein oxidation. Vitamin E supplementation extends meat shelf life and also improves quality characteristics like color, flavor, and texture (Boler *et al.*, 2009). Other possible effects of vitamin E fortification have been reported (Zhang *et al.*, 2010), such as increased water holding capacity and reduced pale, soft, exudative meat (PSE) development in poultry and pigs. Like other meats, pork meat can be fortified with vitamin E through dietary supplementation. Studies have shown that the vitamin E content in pork meat can be more than doubled by dietary supplements of 200 mg α- tocopheryl acetate/kg (Boler *et al.*, 2009; Cardenia *et al.*, 2011).

Fatty acid composition
Fatty acid composition has a considerable effect on the diet/ health relationship because each fatty acid affects plasma lipids differently. Pork meat lipids usually contain less than 50% saturated fatty acids (SFA) of which only 25–35% have atherogenic properties, and up to 60% unsaturated fatty acids, monounsaturated or polyunsaturated ones (Decker & Park, 2010). For this reason, meat cannot be described in general as being a highly saturated food, especially when compared to other products (e.g. certain dairy products). In general, SFA increase low density lipoprotein (LDL) cholesterol levels in the plasma and thus increase cardiovascular (CVD) risk, while polyunsaturated fatty acids (PUFA) decrease LDL cholesterol levels (Whitney & Rolfes, 2002). As a result, there is much interest in increasing PUFA, especially long chain n-3 PUFA, which have many known beneficial effects (Harris, 2007) in meat and meat products. Whether dietary fortification with PUFA sources is performed, antioxidant fortification must also be provided for a protective effect.
The fatty acid profile of pork meat can be modified by feeding and, to a limited extent, by selection and housing conditions. Studies on pigs have recently demonstrated a meaningful genetic variation for long-chain PUFA metabolism that only partly depends on the carcass and muscle fat content, and this may allow selection for improved fatty acid composition (Ntawubizi *et al.*, 2010). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the most bioactive form of n-3 fatty acids (Decker & Park, 2010); these two functional food ingredients can be increased in pork meat through animal feeding. Also, linoleic acid (18:2 n-6) is a major ingredient in feeds for all species; derived entirely from the diet, its incorporation into adipose tissue and muscle in proportion to the amount in the diet is greater than that of other fatty acids (Wood *et al.*, 2008).
In many studies, linseed supplementation was performed from weaning up to slaughter pigs; short term dietary supplementation has also been effective. Increasing the n-3 content in animal meats can be achieved by including fish oil/fish meal in the diet (i.e. rich in EPA and DHA), linseed (oil) and/or forages (i.e. rich in linolenic acid (LNA)). Diets rich in LNA result in an increased level of LNA, EPA and docosapentaenoic acid (DPA) in the meat, while in most cases no effect on intramuscular DHA level was observed. Increasing DHA contents in meat was mainly achieved when fish oil/fish meal was included in the animals' diet (Christaki *et al*. 2011). In most studies, increased n-3 content in the intramuscular fat was accompanied with a decreased n-6 deposition, mainly due to a lower n-6 dietary supply between the treatments. This resulted in a more favourable n-6/n-3 ratio in the meat while the polyunsaturated fatty acid/saturated fatty acid (P/S) ratio was less affected (Raes *et al*. 2004).
Monogastrics are unable to synthesize CLA, therefore the CLA present in their meat comes from the diet. CLA concentrations in monogastric animal meats can be increased (Kawahara *et al.*, 2009), for example up to 130 fold in pig *L. dorsi* muscle (Joo *et al.*, 2002), by supplementing diets with synthetic CLA. In addition, in pigs, dietary CLA supplements increased lean tissue deposition and decreased fat deposition (Ostrowska *et al*. 1999). The above researchers report that in pigs fed with CLA supplements the rate of lean tissue deposition was maximized at 5.0 g CLA /kg feed, whereas the depression in fat deposition was linear up to at least 10 g CLA/kg.

Cholesterol content

Feeding can also influence pork meat cholesterol content (Decker & Park, 2010). Considering the potential human health implications of cholesterol intake, this aspect is relevant and all feeding strategies must be directed to achieving the lowest cholesterol content. Martins et al. (2005) found that feeding whole blue lupin seeds to pigs for 3 weeks exerted a marked hypocholesterolemic effect as the consequence of a marked decrease in the intestinal absorption of cholesterol, probably modulated by bile acid reabsorption and blue lupin phytosterols. According to the above researchers, bile acid metabolism was stimulated by blue lupin consumption.

Lipid oxidation and antioxidants

Meat with increased polyunsaturated fatty acids (PUFA) content, shows an increased oxidative rate because of the tendency of unsaturated fatty acids (mainly those with more than two double bonds) to oxidize and reduce meat and meat product shelf-life (Wood *et al.*, 2004). The problem is even more serious when meat is minced, stored for long time, or cooked (Lee *et al.*, 2006). The lipid oxidation rate can be effectively retarded using antioxidant vitamins, such as vitamins A, C and E. These antioxidant vitamins could be used in animal feeding or during meat processing (exogenous addition) for both covering human requirements and enhancing meat lipid stability; having in mind, as well, that they are consumed at less than their recommended intake levels by many consumers.

Some herbs and spices (rosemary, sage, green tea, clove, cinnamon, nutmeg, rose petals) could be efficient food ingredients in improving the shelf life of (mainly processed) meats vulnerable to oxidative changes because they contain many phytochemicals that are potential sources of natural antioxidants, including flavonoids, tannins, phenolic acids, and phenolic diterpenes, while also promoting anti-inflammatory, antimicrobial and anticancer activities (Zhang *et al.*, 2010). Other natural antioxidants extracted from plants, such as soybean, citrus peel, sesame seed, olives, carob pod, and grape skin could also be used for their equivalent or greater effect on lipid oxidation inhibition (Fernández-Ginés *et al.*, 2005). In raw and cooked meat of several species and in meat products like sausages, patties and meatballs, some herbs and spices are added to improve flavor (rosemary extracts, sage, garlic), retard lipid oxidation-induced deterioration (rosemary extracts, green tea leaves, clove, garlic, sage, oregano), inhibit microorganism growth (clove, sage, oregano), and lower the risk of some diseases (green tea). In particular, encouraging results in terms of antioxidant activity were obtained after treating raw and minced pork and beef with oregano and sage essential oils during a 12-day storage period. The effect was greater in cooked than in raw meat (Fasseas *et al.*, 2007).

In a study of Janz et al. (2007) the antioxidant activity of several essential oils from aromatic plants in pig diets has been shown. In this study the performance of finisher pigs on diets containing 0.05% of essential oils or oleoresins of rosemary, garlic, oregano, or ginger, were assessed and the effect of these diets on pork quality was determined. The pigs preferred the garlic-treated diet, and feed intake and average daily gain were significantly increased although no difference in feed efficiency was observed. Carcass and meat quality attributes were unchanged by dietary treatment, although a tendency towards reduction of lipid oxidation was noted in oregano-fed pork.

 Moroney et al. (2012) investigated the effect of supplementation of pig diets with a seaweed extract containing laminarin and fucoidan that was manufactured from brown seaweed (*Laminaria digitata*) and found a significant reduction in lipid oxidation in *Longissimus dorsi* steaks from pigs fed with brown seaweeds compared to controls. Iron-induced lipid oxidation increased in liver, heart, kidney and lung tissue homogenates over the 24 h storage period, whereas dietary seaweed reduced lipid oxidation to the greatest extent in liver tissue homogenates. These results demonstrate potential for the incorporation of marine-derived bioactive antioxidant components into muscle foods via the animal's diet.

CONCLUSIONS

Functional foods are a tool that can possibly be used in reducing public health costs. The consumption of pork meat in regular quantities could provide consumers with bioactive compounds because pig diet manipulation is very effective in increasing levels of PUFA, EPA, DHA, CLA, vitamin E, selenium etc. Also, lowering the n-6/n-3 ratio plays a key role in controlling CVD and other chronic diseases. Compared to meats of other animal species, pork lean meat has lower cholesterol contents and high levels of protein with essential amino acids. Pork meat is a significant source of vitamins of group B and is characterized low sodium content. Dietary fortification with PUFA, long chain n-3 fatty acids, vitamin E, and selenium, etc. meets the criteria required for pork meat to be considered as functional food.

ACKNOWLEDGEMENTS

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OSMOTIC DEHYDRATION OF FISH *(CRASSUS GIBELIO)* IN DIFFERENT SOLUTIONS

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ABSTRACT

This research was conducted in order to examine changes in osmotic dehydrated fish (*Carassius gibelio*) by using hypertonic solutions. The changes were followed in three different osmotic solutions sugar beet molasses (solution 3), the mixed solution of sodium chloride and sucrose(solution 1) and combination of these solutions in a 1:1 ratio (solution 2), under atmospheric pressure, at temperature of 50°C. The effects of osmotic dehydration on mineral composition, water activity and microbiological safety of fish, were investigated in order to determine the usefulness of this technique as pre-treatment for further treatment of fish. Osmotic dehydration of fish meat in sugar beet molasses (solution 3) lead to significant increase of Mg and Fe content, approximately 2 times. Influence of sugar beet molasses on the chemical composition of fish meat could be noticed. Amount of tracked minerals in fish meat increased for Ca and Na, while amount of K did not changed during dehydration in solution with sugar beet molasses. After five hours of immersion in all three solutions content of Hg in fish meat was reduced. Water activity of the fresh fish meat was 0.944±0.007, while the results obtained after the 5 hours of osmotic dehydration process varied from 0.863±0.012 (solution 2) to 0.833±0.009 (solution 3). The reduction of microbial populations in addition to reducing the water activity value was influenced by the temperature treatment, which amounted to 50°C. Bacterial count was reduced from 5 log CFU•g^{-1} to 3 log CFU•g^{-1} which is considered as microbiological limit for good fish meat quality.
Keywords: Osmotic dehydration, fish, mineral and microbiological

INTRODUCTION

Fish is one of the sources of proteins, vitamins and minerals, and it has essential nutrients required for adults diet (Abdullahi *et al.*, 2001). Fresh fish is a very perishable food, as a result of its specific composition and structure (Baltić *et al.*, 2009). Water activity (aw) is a physical property that has a direct implication for microbiological safety of food. Micro-organisms generally grow best between aw values 0.995-0.980, while most microorganisms cease growth at aw<0.900 (Gibbs *et al.*, 2001).

Dehydration of fish meat is one of the key processes in fish processing. The prime reason for fish drying is to reduce the moisture content of the non-aqueous material to such a level that insufficient water remains to support the growth of the microorganisms which feed on it (Oladipo and Bankole, 2013).

One of the potential preservation techniques for producing products with low water content and improved nutritional and functional properties is osmotic treatment. During the osmotic treatment, partial mass transfer is caused by the difference in the osmotic pressure: water outflow from the product to the solution, the solute transfer from the solution to the product, and leaching out of the products own solutes (Koprivca *et al.*, 2001). The main advantages of osmotic treatment are water removal in liquid form, usage of mild temperatures, energy efficiency, providing a stable and quality product (Della Rosa and Giroux, 2001; Pezo *et al.*, 2013). Since osmotic dehydration is more economical than thermal drying, allows microbial quality preservation of food and makes changes in their nutritive quality, it is often used as a pre-treatment for drying of fish (Collignan *et al*, 2001).

The type of the osmotic solution plays a very important role in the osmotic treatment, because has a great influence on the kinetics of water removal and solid uptake (Nićetin *et al.*, 2013). Solutions containing salt and sugar are common osmotic agents for food dehydration (Azoubel and Murr, 2004; Collignan *et al*, 2001). Salt solutions, because of their influence on water activity depression, are widely used in traditional fish meat processing (Barat *et al.*, 2011).

Sugar beet molasses is an excellent medium for osmotic treatment, primarily due to the high dry matter (80%) and specific nutrient content (Koprivica *et al*, 2009; Lević *et al*, 2007). From nutrient point of view, an important advantage of sugar beet molasses, as hypertonic solution, is enrichment of the food material in minerals and vitamins, which penetrate from molasses to the meat tissue (Filipović *et al.,* 2012a).

The Gibel carp(*Carassius gibelio*) is one of the dominant species in stagnant and slow-running waters and may change the flow of nutrients in the entire ecosystem (Paulovits *et al.*, 1998). Generally, *Carassius gibelio* is only freshly consumed. There are not many processing techniques for this fish except smoking (Izci, 2010).

The aim of this work was to examine the influence of three different type of hypertonic solution on the microbiology profile and minerals content of fish meat after osmotic dehydration at temperature of 50°C.

MATERIAL AND METHODS

Raw material and sample preparation

Carassius gibelio samples were purchased in a local market in Novi Sad (Serbia) and stored at 4^oC until use. Prior to the treatment, all working areas and tools were thoroughly washed, cleaned and disinfected with pharmaceutical ethanol (70 vol.%).The fish meat was cut into shapes, dimension of nearly 1x1cm, using a sharp knife.

Osmotic solutions

For the preparation of osmotic solutions were used commercially available sodium chloride and sucrose, and sugar beet molasses from sugar factory Pećinci, Serbia. Then, the samples were immersed in hypertonic solution R_1 (NaCl + sucrose), solution R_2 (NaCl + sucrose+ sugar beet molasses in the ratio 1:1) and solution R_3 (sugar beet molasses). The ratio of raw material and hypertonic solution was 1:5, and the immersion time was 5 hours. Osmotic dehydration was carried out at atmospheric pressure and temperature of 50°C. 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.

Analytical procedures

Water activity (*aw*) was measured using a water activity measurement device (TESTO 650, Germany) with an accuracy of ±0.001 at 25°C.

Determination of the total number of bacteria, *Escherichia coli*, coagulase positive staphylococci and sulphite reducing clostridia was done by the (SRPS EN ISO 4833, 2008); SRPS ISO 16649-2, 2008; SRPS EN ISO 6888-1, 2003; SRPS ISO 15213, 2003). All analyses were performed in triplicates and results will be represented as mean values.

Statistical analysis

Average results of triplicate samples were submitted to statistical analyses. Results were analysed using analysis of variance of the Statistica 10 for Windows, Stat Soft, Tulsa, Oklahoma, USA, 2009. Significant differences between means were determined at p$\leq$0.05.

RESULTS AND DISCUSSION

The changes in the microbial flora of fresh fish meat during dehydration in three different osmotic solutions are shown in Table 1. Results of microbial analysis of fresh and treated fish

by osmotic treatment show the absence of pathogenic bacteria. The total number of bacteria counts was reduced from 5.87 log cfu/g (fresh fish) to 3.04 and 3.18 log cfu/g (fish dehydrated in solution 1 and 2, respectively) and 4.2 log cfu/g (fish dehydrated in solution 3), is considered microbiological limit for good fish meat quality. This result is due to the inhibitory effect caused by the decrease of aw values in dehydrated fish meat (Chabbouh et al., 2011). The reductions of the total number of bacteria in dehydrated samples in comparison to the initial total number of bacteria in the fresh fish meat was 93.66% for samples dehydrated in sugar beet molasses and 89.01% for samples dehydrated in aqueous solution of sodium chloride and sucrose (Lončar et al., 2014).

Table 1. Microbiological analysis of the fresh and dehydrated fish

Parameter (log CFU/g)	Fresh fish	Fish dehydrated in solution 1	Fish dehydrated in solution 2	Fish dehydrated in solution 3
Total number of bacteria	5.87	3.04	3.18	4.20
Escherichia coli	nd[*]	nd	nd	nd
Coagulase positive staphylococci	nd	nd	nd	nd
Sulphite reducing clostridia	nd	nd	nd	nd

Legend: *- not detected

The processes of osmotic dehydration of fish meat drive decreasing aw value. The average aw values and standard deviation of the fresh and dehydrated fish meat in three solutions are shown in Table 2. The lowest aw value 0.833±0.009 was reached after osmotic dehydration in solution 3 (sugar beet molasses). The levels of reached aw values of fish meat after the process of osmotic dehydration in all three solutions, were lower than the limiting value for aw growth of most microorganisms (Huang and Nip, 2001; Feiner, 2006), which indicates a positive effect of the osmotic treatment on microbial profile of dehydrated fish.

Table 2. Average water activity (aw) values and standard deviation of the fresh and dehydrated fish

Parameter	Fresh fish	Fish dehydrated in solution 1	Fish dehydrated in solution 2	Fish dehydrated in solution 3
aw	0.944±0.007	0.847±0.004	0.863±0.001	0.833±0.009

Changes in mineral contents of fresh and dehydrated fish meat in three different osmotic solutions are shown in Figure 1. The quantity of minerals is variable and depends on the types of osmotic solutions. The effect of processing temperature and concentrated solutions on the dehydration of fresh fish resulted in significant increase of Mg and Fe content, approximately 2 times. Significant influence of sugar beet molasses (solution 3) on the mineral composition could be noticed. The Mg content in the fish meat increased significantly ($P < 0.05$) in solution 3, from an average mean of 0.77 to 1.56 g/kg after osmotic dehydration.

Also, Fe content increased considerably in fish meat dehydrated in solution 3, from an average mean of 0.26 to 0.55 g/kg. The concentration of Ca was increased in all three solutions, and the most in fish dehydrated in solution 3 and in range 16.58-18.02 g/kg. The Na content in fish dehydrated in solution 1 and 2 declined in concentration from 6.72 to 3.70 and 2.52 g/kg, respectively. The concentration of Na a slightly increased in fish dehydrated in solution 3 (7.61g/kg). Amount of K slightly increase during dehydration in solution with sugar beet molasses (solution 2 and 3) from 15.53 to 16.00 g/kg. After five hours of immersion in all three solutions content of Hg in fish meat was reduced from 0.11 to 0.08 g/kg.

The present study showed that obtained mineral contents of fresh and dehydrated fish meat in the decreasing order Ca>K>Na>Mg>Fe>Hg .This trend is in agreement with the results of

previous work, except for the Ca content. Akinneye et al. (2007) reported the values of the major elements were obtained in the decreasing order K>Na>Mg>Ca in *Sardinella* (oven dried), *H. niloticus*, (oven dried), *H. niloticus* (smoke dried) and *Sardinella sp* (smoke dried). The significantly increased values of mineral Ca gives priority to application of osmotic dehydration process with the implementation of molasses.
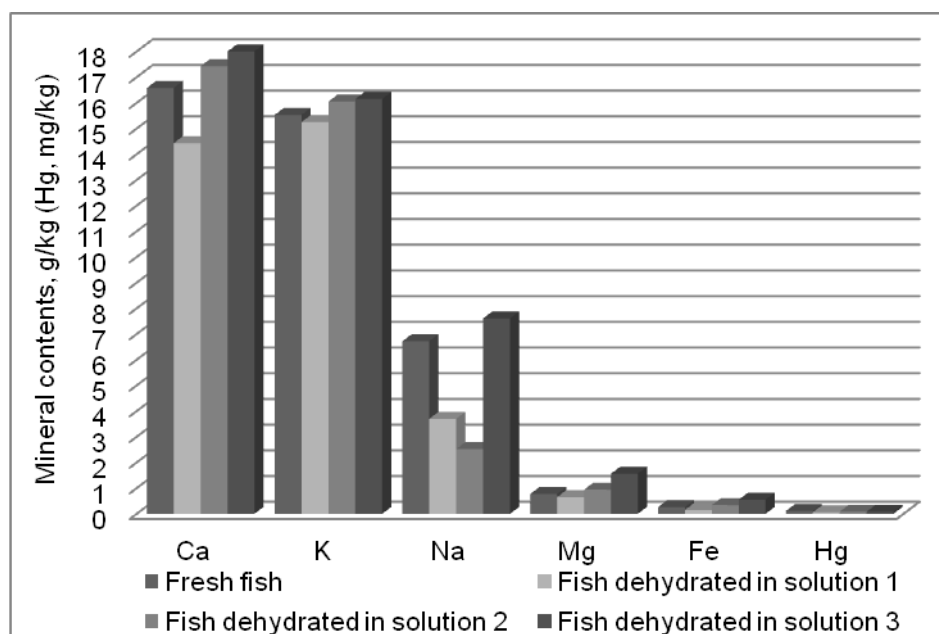
Figure 1. The dynamics of change of mineral elements content in fish meat during osmotic treatment

CONCLUSIONS

The microbiological profile of dehydrated fish in all three osmotic solutions has shown that osmotic dehydration is hygienically and microbiologically safe process regardless to the high processing temperature. Osmotic dehydration treatment had great impact on nutritional improvement of fish meat, especially on the mineral composition when sugar beet molasses was used. The present study showed that obtained mineral contents of fresh and dehydrated fish meat in the decreasing order Ca>K>Na>Mg>Fe>Hg. The lowest aw value 0.833±0.009 was reached after osmotic dehydration in solution 3 (sugar beet molasses).On the basis of presented research it can be concluded that the best results considering the mineral composition and aw value of dehydrated fish were obtained by using sugar beet molasses as an osmotic agent.

ACKNOWLEDGEMENTS

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LAB-SCALE OPTIMIZATION OF BEER FERMENTATION WITH IMMOBILIZED TOP-FERMENTING YEAST

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ABSTRACT

The present work regards batch beer fermentation with immobilized top-fermenting yeast *Saccharomyces cerevisiae* Safbrew S-33. The yeast cells were encapsulated in alginate-chitosan microcapcapsules with a liquid core. The effects of main fermentation temperature (T_{MF}), immobilized cell mass (M_{IC}) and original wort extract (OE) on beer fermentation was investigated using Central Composite Design of type 2^3 with star arm and block structure. The basic beer characteristics, i.e. extract, ethanol, biomass concentration, pH and color, as well as the concentration of aldehydes and vicinal diketones were measured. The results suggested that the process parameters represented a powerful tool in controlling the fermentation time. The differences observed in the main fermentation time and maturation time among the variants were due to the rate of carbonyl compounds production and reduction. The results were used for the development of two mathematical models for beer fermentation with immobilized cells. Subsequently, the optimized process parameters were used to produce beer in laboratory batch fermentation. The system productivity was also investigated and the data was used for the development of another mathematical model.
Keywords: beer, top fermenting yeast, optimization, encapsulation

INTRODUCTION

Fermentation is an important field of interest for system engineering due to its complex biological non-linear phenomena and dynamic processes (Andres-Toro *et al.,* 2010). In recent years, a lot of research has been focused on beer fermentation intensification by the use of immobilized cell systems (for review see Willaert and Nedovic*,* 2006). Beer production with immobilized cells is generally industrialized if the new characteristics acquired result in a more economical system, and the new technology can be readily scaled up (Nedovic *et al.,* 2005). However, it can be successful only on condition that the immobilized cell system produces a competitive final product (Lehnert *et al.,* 2009). Therefore, mathematical model development is an important step towards the determination of suitable fermentation parameters in order to achieve an optimized process (Adeyemo and Enitan, 2011). Moreover, process optimization has been the key issue to maintain operating conditions, increase product yields and ensure product quality (Schmidt, 2005).
The aim of this work was to minimize the beer fermentation time while retaining high product quality with an optimized process control strategy. Therefore, two mathematical models for the effect of main fermentation temperature (T_{MF}), immobilized cell mass (M_{IC}) and original wort extract (OE) on the main fermentation and maturation time were developed. The results on the optimization of primary fermentation model were used for beer production under laboratory conditions. Meanwhile, the system productivity was determined and it could be used for the batch fermentation transfer into a continuous mode.

MATERIALS AND METHODS

Microorganisms and cell Immobilization
The experiments were carried out with commercial dry brewing yeast strain *Saccharomyces cerevisiae* Safbrew S-33, purchased from Fermentis, France. The yeast suspension was

added to a 3% (w/v) solution of sodium alginate and subsequently dropped into a 2% (w/v) $CaCl_2$ solution. The cell concentration in the beads was approximately 10^7 CFU/cm^3 of gel. The beads were left for 30 min in $CaCl_2$ and were then placed into a 0.38% (w/v) chitosan solution in 1% (v/v) acetic acid. The alginate beads stayed in the chitosan solution for 60 min. Afterwards, the chitosan-alginate beads were washed with sterile water. The beads stayed in a 0.05 M sodium citrate solution for 30 min to obtain microcapsules with liquid core (Naydenova *et al.,* 2012, 2013, 2014).

Wort
Wort with OE 17±0.5 °P was supplied by Kamenitza Plc. It was diluted to OE – 8.5±0.5 °P, 10.5±0.5 °P, 13±0.5 °P, 15.5±0.5 °P and 17.5±0.5 °P. All wort types were autoclaved at 120°C for 20 minutes.

Design of experiments and fermentation
Central Composite Design (CCD) of type 2^3 with star arm and block structure (Table 1) was used for the optimization of the fermentation parameters T_{MF}, **OE** and M_{IC}. The mathematical processing of the experimental results was accomplished according to Naydenova *et al.,* 2014. The fermentations (main and secondary) were carried out according to Naydenova *et al.,* 2014.

Analytical Methods
The characterization of wort, green beer and beer was conducted according to the current methods recommended by the European Brewery Convention (*Analytica-EBC, 2004*). The biomass concentration was made according to Parcunev *et al.,* 2012. The aldehyde concentrations were determined according to Marinov, 2010.

Productivity
The system productivity was calculated according to Kopsahelis *et al.,* 2007.

RESULTS AND DISCUSSION

In our previous studies, the fermentation dynamics were found to vary significantly depending on the following parameters: main fermentation temperature (T_{MF}), maturation temperature (T_{MAT}), immobilized cell mass (M_{IC}) and original wort extract (**OE**) (*Naydenova et al., 2012, 2013, 2014*). Therefore, it can be suggested that the combination of these factors increases their influence on the fermentation dynamics. The ranges of variation of these parameters (real and coded) are shown in Table 1.
Figure 1 presents the fermentation dynamics of one of the variants according to Table 1. The data on the other variants are summarized in three-dimensional graphics (Figure 2 and Figure 3). For all the studied variants the dynamic of the fermentation with immobilized cells followed the trends for the conventional beer fermentation, described in (Kunze, 2004).
Table 1 shows that the main fermentation and maturation times were affected differently by T_{MF}, **OE** and M_{IC}. The data on the statistical analysis for the influence of the parameters tested on the main fermentation time are presented in Table 2. The response surface for the influence of T_{MF} and M_{IC} on the main fermentation time is shown on Figure 4.
The non-significant variables were eliminated according to their p-value at a confidence level α = 0.95 (Table 2). The following adequate mathematical model was obtained after the removal of non-significant variables (1):

$$MF = 78.7 - 32.3T_{MF} + 15.13OE - 36.05M_{IC} + 10.44T_{MF}^2 - 10.5OE.M_{IC} + 20,75M_{IC}^2 \quad (1)$$
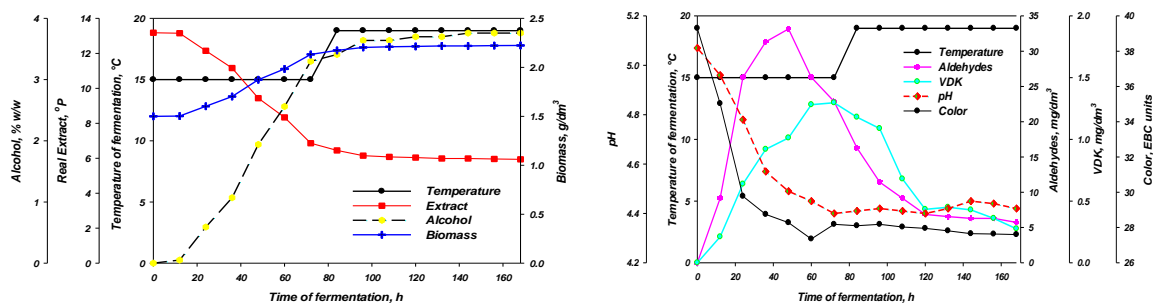
a) extract, ethanol, biomass concentration   b)aldehydes, vicinal diketones (VDK), pH, colour
Fig.1. Fermentation dynamics (variant 6, Tabl.1)

Table 1.Experimental design and results for the modeling and optimization of fermentation parameters

№	Parameters						Time of main fermentation **MF**, h	Maturation time **MatF**, h	Fermentation time, h	Q_{ETH}, g/(dm³.h)		Q, g/(dm³.h)	
	T_{MF}, °C		OE, °P		M_{ic}, g					MF	F	Q_{MF}	Q_F
	coded	real	coded	real	coded	real							
1	-α	10.5	0	13	0	10	180	168	348	0.36	0.43	29.38	33.64
2	-1	12.5	-1	10.5	-1	5	144	84	228	0.37	0.70	19.92	34.01
3	-1	12.5	1	15.5	-1	5	192	108	300	0.38	0.76	40.72	86.14
4	-1	12.5	-1	10.5	1	15	96	132	228	0.53	0.45	28.02	21.68
5	-1	12.5	1	15.5	1	15	108	120	228	0.73	0.72	85.77	76.51
6	0	15	0	13	0	10	72	96	168	0.87	0.74	69.28	57.27
7	0	15	0	13	0	10	76	90	166	0.82	0.79	64.67	60.68
8	0	15	0	13	0	10	74	100	174	0.96	0.71	75.67	54.61
9	0	15	-α	8.5	0	10	72	84	156	0.54	0.54	17.92	17.64
10	0	15	+α	17.5	0	10	120	84	204	0.73	1.13	111.76	158.97
11	0	15	0	13	-α	1.1	228	72	300	0.29	0.87	23.49	68.49
12	0	15	0	13	+α	18.9	72	84	156	0.84	0.83	67.63	63.43
13	0	15	0	13	0	10	68	92	160	0.92	0.77	73.35	59.76
14	1	17.5	-1	10.5	-1	5	72	84	156	0.70	0.68	36.64	34.87
15	1	17.5	1	15.5	-1	5	132	84	216	0.57	1.00	62.99	111.51
16	1	17.5	-1	10.5	1	15	42	114	156	1.08	0.60	50.46	29.82
17	1	17.5	1	15.5	1	15	54	78	132	1.28	0.75	133.35	78.62
18	+α	19.5	0	13	0	10	54	102	156	1.20	0.69	107.42	51.08

T_{MF} - temperature of main fermentation; OE - original extract; Mic - immobilized cell mass; Q_{ETH} - ethanol productivity; Q - beer productivity; MF - for main fermentation; MatF – maturation time; F - for total fermentation time (F=MF+MatF); ±α=1.7885

The model obtained (Equation 1) showed that the main fermentation time decreased with the increase in T_{MF} **and** M_{IC}. On the contrary, the **OE** increase resulted in prolonged primary fermentation. It is interesting to note that the model showed an increase in the main fermentation time with the increase in T_{MF} and M_{IC} when these parameters were squared. It can be assumed that the discrepant effects of T_{MF} and M_{IC} were due to the substrate and product diffusion into and out of the beads. The model obtained (Equation 1) confirmed the suggestion that the combined effect of the factors would be of key importance to the objective function. Therefore, it can be summarized that the reduction of primary fermentation time will occur if the fermentation temperatures are high and used wort is with low original extract. The decrease in T_{MF} below the recommended fermentation temperature for the selected yeast strain led to a very prolonged fermentation. The mathematical model was optimized and the following operational conditions were determined: T_{MF} = 17.24 °C; **OE** = 10.24 °P; M_{IC} = 11.56 g. Under these conditions, the main fermentation time had to be 36 h. It was found that the real main fermentation time was 12 h longer than the main

fermentation time determined by the model. This could be related to the system diffusion resistance which hindered the substrate transfer to the cells.
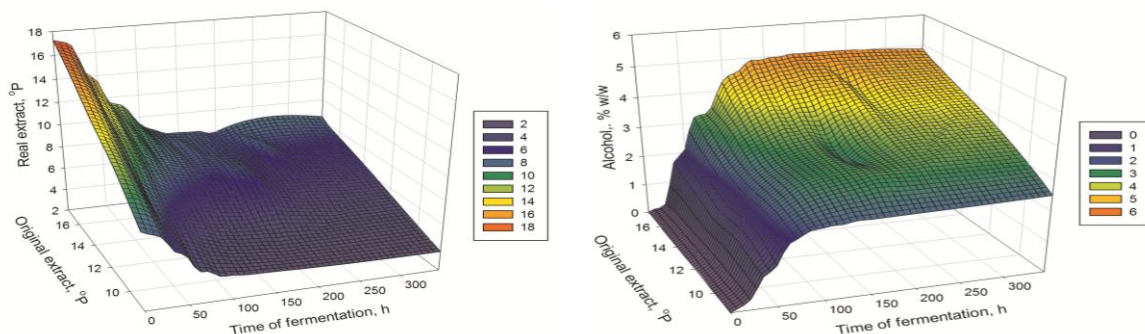
a) real extract   *b) alcohol of beer*
Fig.2 Dynamics of extract and alcohol changes in the factor space for the fermentation time
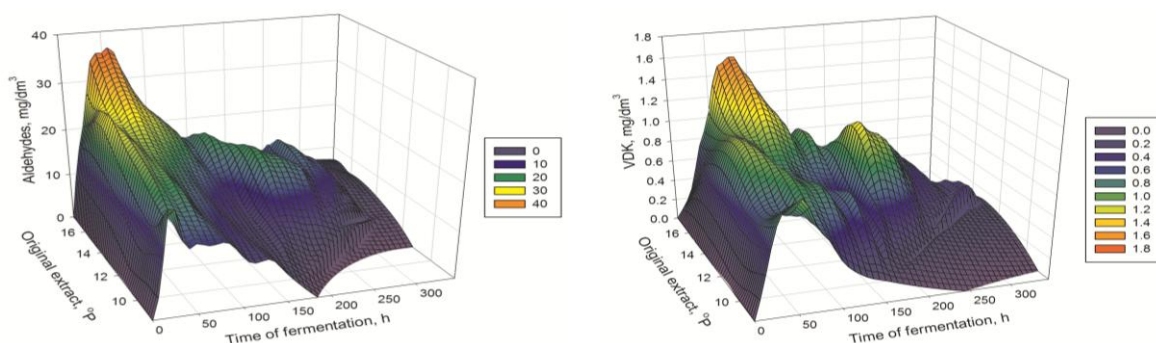
a) aldehydes   *b) vicinal diketones (VDK)*
Fig.3 Dynamics of carbonyl compounds changes in the factor space for the fermentation time

Another adequate mathematical model was developed for the influence of T_{MAT}, OE and M_{IC} on the maturation time:

$$MatF = 87.49 - 14.03T_{MAT} + 7.32M_{IC} + 14.3T_{MAT}^2 - 9OE.M_{IC} \tag{2}$$

The model obtained (Equation 2) showed a controversial effect of T_{MAT} on the maturation time. First, the increase in T_{MAT} affected "negatively" the maturation time. On the other hand, higher temperature led to the synthesis of more carbonyl compounds, which resulted in prolonged maturation. The maturation time also increased with the increase in M_{IC} (Figure 5). Consequently, the maturation was affected not only by the operational conditions, but also by the carbonyl compounds concentration at the beginning of the maturation. Carbonyl compounds which are of importance to beer flavor and aroma are acetaldehyde and some other aldehydes and vicinal diketones. Acetaldehyde has unpleasant "grassy" flavor and aroma. Vicinal diketones – diacetyl and 2,3-pentanedione have "butterscotch" and "toffee" aroma and taste. The carbonyl compounds have low taste threshold, so their concentrations in beer determined the maturation process time (Kunze, 2004). The second mathematical model (Equation 2) can also be optimized. However, this is not necessary, since the model structure is related to the main fermentation model (Equation 1).

One of the purposes of the study was to select the batch fermentation conditions which would be transferred into a continuous fermentation system. According to Kopsahelis *et al.,* 2007, one of the parameters for the comparison between batch fermentations was system productivity, which could be estimated at various stages of fermentation and for various parameters: ethanol, green beer, beer. The productivity depended on the fermentation time and could be used for comparison between batch and continuous fermentation.

It can be summarized that the ethanol productivity increased with the increase in each of the parameters studied (Table 1). It is interesting to note that the highest ethanol productivity during the main fermentation was for variant 17 where all the factors studied were at their upper level. When the temperature was in its star point – 19.5 °C, the system productivity was little lower. This could be related to the OE decrease which led to the production of less ethanol. Therefore, the ethanol productivity determined for the total fermentation time was highest when OE was in its star point – 17.5 °P. The green beer productivity and beer productivity depended on the ethanol productivity, the degree of fermentation and OE. Therefore, the highest green beer productivity was for variant 17 and the highest beer productivity was for variant 10 (Table 1). The green beer productivity can be used for the transfer of a batch fermentation to a continuous mode. Our subsequent research showed that the system productivity in a batch mode was related to the system productivity in a continuous mode.
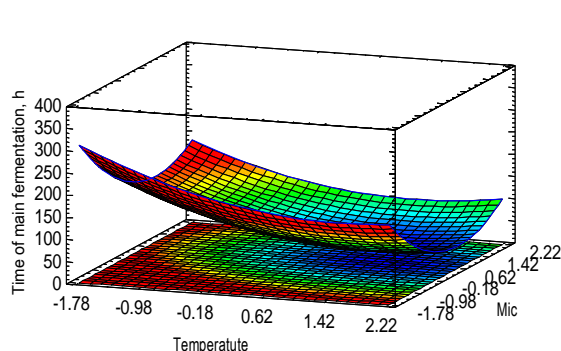
Fig.4 Estimated response surface for the influence of T_{MF} (A) and M_{IC} (C) on "Time of main fermentation" at OE=0 (13 °P) (factors were presented with coded values)
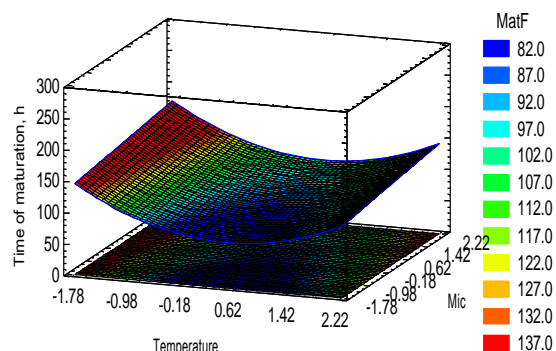
Fig.5 Estimated response surface for the influence of Tmat=TMF+4 (A) and Mic (C) on "Time of maturation" at OE=0 (13 °P) (factors were presented with coded values)

Table 2. ANOVA for „Main fermentation time"

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value	
A:T_{MF}	15041.2	1	15041.2	101.17	0	Significant
B:**OE**	3296.19	1	3296.19	22.17	0.0022	Significant
C:**M_{iC}**	18710	1	18710	125.85	0	Significant
AA	2109.95	1	2109.95	14.19	0.007	Significant
AB	18	1	18	0.12	0.7381	Non-significant
AC	72	1	72	0.48	0.5089	Non-significant
BB	416.955	1	416.955	2.8	0.1379	Non-significant
BC	882	1	882	5.93	0.045	Significant
CC	7400.95	1	7400.95	49.78	0.0002	Significant
blocks	418.191	1	418.191	2.81	0.1374	Significant
Total error	1040.71	7	148.673			
Total (corr.)	47521.8	17				
R^2 = 96.74 %; R^2 (за d.f.) = 94.96 %; Standart error = 12.04; Absolute standart error = 7.84; Durbin-Watson = 1,75;						

A mathematical model for the influence of the fermentation parameters on green beer productivity was developed (equation 3). The model showed that the all tested parameters increase led to an increase in the system productivity. On the one hand it is completely normal because of the use of top fermenting yeast strain. On the other hand, the experimentally reached maximum did not coincide with the mathematical model maximum. The negative sign of the coefficient of M_{IC}^2 showed indirectly that the model did not consider entirely the diffusion resistance in the system. However, the model described with high

accuracy (**R²=96.6%**) the experimental data and can be used for transfer of batch fermentation system to a continuous mode.

$$Q_{MF} = 67.73 + 17.26T_{MF} + 24.7OE + 15.02M_{IC} + 11.68OE.M_{IC} - 7.62M_{IC}^2 \qquad (3)$$

CONCLUSIONS

The influence of fermentation temperature, immobilized cell mass and original wort extract on beer fermentation with alginate-chitosan microcapsules with liquid core was investigated. The results showed that the factors that affected most significantly the fermentation time reduction were temperature and immobilized cell mass. The increase in temperature and immobilized cell mass led to a reduction in the primary fermentation time but did not affect the total fermentation time. This was related to the synthesis of more carbonyl compounds, which caused prolonged maturation. The increase in the wort extract resulted in longer fermentation time. The model parameters were optimized and the data were used for beer production under laboratory conditions. The system productivity was also investigated and the data will be used for the transfer of the optimized fermentation conditions to a continuous beer fermentation system.

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MICROBIOLOGICAL PROFILE OF FISH (*Carassius gibelio*) DEHYDRATED IN SUGAR BEET MOLASSES

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ABSTRACT

Presence of fish in diet is very important for both human health and nutrition, but high water activity (aw) value and high moisture content of fish tissue make fish very perishable food. There are many processing methods to extend fish shelf life. Osmotic dehydration is a water removal process that involves the contact between food material and hypertonic medium. Sugar beet molasses has proved to be very useful osmotic medium. The objectives of the study were to evaluate the effects of two different processing temperatures on microbiological profile of fish (*Carassius gibelio*). Also, aw values, water loss and solid gain of the fresh and processed fish samples were determined and the microbiological profile between the fresh and dehydrated meat was compared. The process was carried out in laboratory jars under atmospheric pressure for 5 hours at 20 and 35°C. Sample to solution ratio was 1:5 (w/w) to neglect the changes of solution concentration during the water removal process. Agitation was performed manually on every 15 minutes. Osmotic dehydration in sugar beet molasses at both temperatures have proved to be efficient for reducing aw and moisture content of fish samples providing quality and safe fish semi product.

Keywords: *Osmotic dehydration, fish, sugar beet molasses*

INTRODUCTION

Meat is very perishable food due to its nutrient composition and high water content that provide a suitable environment for growth of meat spoilage micro-organisms *(*Zhou et *al.,* 2010). Different preservation technics are being used in order to maintain safety and quality of meat, primarily by inhibiting microbiological deterioration (Aymerich *et al.,* 2008*).*

Osmotic dehydration is the process that removes water and reduces aw of food material by immersion in a hypertonic aqueous solution (Tortoe*,* 2010*;* Tsironi *et al.,* 2009).

Many scientists have been researching osmotic dehydration from different aspects and found numerous advantages of this process. The main advantages are preserving the quality of products, water removal in liquid form and low energy consumption (Babić *et al.* 2009).

Temperature, concentration and nature of hypertonic solution have a major influence on the rate of water removal (Sacchetti *et al.,* 2001).

Usually for osmotic treatment of fish samples, sodium chloride and sucrose aqueous solutions are used as osmotic mediums (Oladele *et al.*, 2008).

Recent research (Koprivica *et al.*, 2013; Mišljenović *et al.*, 2011; Filipović *et al.*, 2012) introduced sugar beet molasses as a new osmotic solution efficient for osmotic treatment of plant and animal samples.

As a side product of sugar industry, sugar beet molasses has a significant content of dry matter (about 80%) and specific nutrient composition (about 51% sucrose, 1% raffinose, 0.25% glucose and fructose, 5% proteins, 6% betaine, 1.5% nucleosides, purine and pyramidine bases, organic acids and bases). High dry matter content provides high osmotic pressure, that is the driving force for water removal process, therefor sugar beet molasses appears to be very efficient osmotic medium (Pezo *et al.*, 2013; Mišljenović *et al.* 2011).

The goal of this research was to examine the efficiency of osmotic dehydration process in sugar beet molasses solution, comparing the influence of two different processing temperatures on water removal kinetic parameters and microbiological profile of fish meat.

MATERIAL AND METHODS

The osmotic dehydration was carried out in laboratory jars under atmospheric pressure at constant solution temperatures of 20°C and 35°C. Fish (*Carassius gibelio*) was purchased on a local market in Novi Sad, Serbia, shortly prior to the experiment. The initial moisture content of untreated samples was 75.34%. Fish samples were filleted and cut into shapes (1x1cm) using kitchen slicer and scissors.

Sugar beet molasses was obtained from the sugar factory Crvenka, Serbia with initial dry matter content of 85.04% w/w, with distilled water it was diluted to concentration 80% w/w. After preparation, samples were measured and immersed in hypertonic solution for 5 hours. Sample to solution ratio was 1:5 (w/w) which can be considered high enough to neglect the changes of solution concentration during the process.

After 5 hours, fish samples were taken out from solutions, lightly washed with distilled water, gently blotted with paper to remove excessive water from the surface and weighed.

Dry matter content of the fresh and treated samples was determined by drying the material at 105 °C for 24 hours in a heat chamber (Instrumentaria Sutjeska, Croatia). Water activity of the osmotically dehydrated samples was measured using a water activity measurement device (TESTO 650, Germany) with an accuracy of ±0.001 at 25ºC. Soluble solids content of the molasses solution was measured using Abbe refractometer, Carl Zeis, Jenna, at 20 °C.

All analytical measurements were carried out in accordance to AOAC (2000). In order to describe the mass transfer of osmotic dehydration process, the experimental data for three key process variables are usually used, and these are: the moisture content, the change in the weight, and the change in the soluble solids. Using these, the water loss and solid gain values were calculated as described by Filipović at al. 2014.

Determination of the total number of bacteria, *Escherichia coli*, Sulphite-reducing Clostridia and coagulase-positive Staphylococci was done by the SRPS EN ISO 4833, SRPS ISO 16649-2, ISO 15213 and SRPS EN ISO 6888-1, respectively. All analyses were performed in triplicates and results were represented as mean values.

RESULTS AND DISCUSSION

The osmotic dehydration process of fish meat was studied in terms of kinetics parameters; dry matter content (DM), water loss (WL), solid gain (SG), and aw. Table 1 shows the changes in DM content in the samples of fish meat after osmotic dehydration in sugar beet molasses as a result of different osmotic solution temperatures. The process resulted in increased dry matter content of fish meat samples dehydrated at both temperatures, however slightly higher value was achieved in samples dehydrated at 35°C (64.272±0.647%).

Beside the changes in dry matter content, as a main consequence of the osmotic dehydration process, changes in water content occurred, causing a great amount of water loss from the fish samples. Both processing temperatures of osmotic solution appear to be efficient in water removal process, although a slightly higher WL value (0.539±0.001/g i.s.w.) was noticed in samples dehydrated at 35°C.

SG value shows the degree of penetration of solids from hypertonic solution into the fish meat samples. Because of the complexity of osmotic medium, SG increased after osmotic dehydration in sugar beet molasses, but slightly lower value of SG parameter was obtained in samples dehydrated at 20°C (0.111±0.003 g/g i.s.w.).

Table 1. Average values and standard deviations of kinetic parameters of the dehydrated fish

Kinetic parameter	Fresh fish meat	Fish meat dehydrated at 20°C	Fish meat dehydrated at 35°C
Dry matter content, %	23.975±1.965	58.339±4.471	64.272±0.647
Water loss*, g/g i.s.	0.000±0.000	0.530±0.003	0.539±0.001
Solid gain*, g/g i.s.	0.000±0.000	0.111±0.003	0.124±0.001
a_w	0.944±0.007	0.845±0.023	0.846±0.009

mass in grams of WL or SG per mass in grams of initial sample

Table 1 also shows the average aw values and standard deviation of the fresh and dehydrated fish in sugar beet molasses at 20°C and 35°C. Fresh samples of fish before treatment had average aw value of 0.944±0.007 that is close to the optimum growth level of most microorganisms (Nićetin *et al.*, 2012).
 After the process of osmotic dehydration, lower aw values of fish meat samples dehydrated at both processing temperatures were observed. Obtained aw values of dehydrated samples were 0.845±0.023 for dehydration at 20°C and 0.846±0.009 for dehydration at 35°C. Both solution temperatures were effective in lowering aw of fish samples.
According to these results it can be concluded that process of osmotic dehydration ensures aw values which are within a specified range for fish meat quality and safety, considering that most meat spoiling bacteria do not grow below aw value of 0.91 (Vereš, 1991).

Table 2. Microbiological analysis of the fresh and dehydrated fish meat in two osmotic solutions

Hygiene and food safety criteria	Fresh fish meat	Fish meat at 20°C	Fish meat at 35°C
Total number of bacteria, CFU/g	$7.77 \cdot 10^5 \pm 5.4 \cdot 10^5$	$4.43 \cdot 10^4 \pm 3.6 \cdot 10^3$	$6.03 \cdot 10^4 \pm 1.6 \cdot 10^3$
Escherichia coli (CFU/g)	<10	<10	<10
Sulphite-reducing Clostridia(CFU/g)	<10	<10	<10
Coagulase-positive Staphylococci (CFU/g)	<100	<100	<100

Results of the microbiological analysis of the fresh and dehydrated fish meat are presented in table 2.
Total number of bacteria in fresh fish was $7.77 \cdot 10^5 \pm 5.4 \cdot 10^4$CFU/g. After the osmotic dehydration process, total number of bacteria in dehydrated samples in sugar beet molasses at 20°C was $4.43 \cdot 10^4 \pm 3.6 \cdot 10^3$ and at 35°C was $6.03 \cdot 10^4 \pm 1.6 \cdot 10^3$CFU/g.
 The change in total number of bacteria occurred after the process, leading to a 94.43% reduction in samples dehydrated at 20°C and 92.24% at 35°C.
These results prove that the process of osmotic dehydration has an important influence on reduction of total number of bacteria in the osmotically treated fish.
The number of Escherichia coli, coagulase-positive Staphylococci and sulphite-reducing Clostridia in fresh fish meat samples was in accordance with the hygiene production criteria of the Serbian National Regulation (72/2010). There was no noticable increase in number of these bacteria in dehydrated fish post osmotic treatment.

CONCLUSIONS

According to microbiological profile of dehydrated fish meat samples it can be concluded that osmotic dehydration is hygienically safe process. Better reduction of total number of present microorganisms in dehydrated fish meat was obtained at temperature of 20°C.Both processing temperatures of osmotic solution have proved to be efficient in reducing water

content and aw of samples, providing quality and safe fish semi product. The use of sugar beet molasses as hypertonic agent for osmotic dehydration of fish meat is economically and environmentally reasonable.

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POSSIBILITY OF USE OF POTASSIUM CHLORIDE IN THE FRESH SAUSAGES PRODUCTION

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ABSTRACT

Excessive sodium intake is one of the major causes of human hypertension and because of that there is need to reduce salt/sodium content in meat products. The most common replacer of sodium chloride in meat products is potassium chloride, but its use is limited due to bitter and metallic taste. The aim of this paper was to examine the possibility of use of potassium chloride as replacer of sodium chloride in the production of fresh sausages. Fresh sausages produced in this experiment contained various share of sodium chloride and potassium chloride (first group - 20 g of sodium chloride in 1000 g of mass; second group - 15 g of sodium chloride in 1000 g of mass; third group - 10 g of sodium chloride and 5 g of potassium chloride in 1000 g of mass). Products were sensory evaluated by quantitative descriptive analysis (ISO 6658:2005), using scales with 5 points (5 – the best expressed attribute; 1 – the worst expressed attribute). Sensory evaluation was performed by six assessors, previously trained for detection and recognition of various tastes (ISO 3972:2011) and odours (ISO 5496:2006). Also, products were presented to seven consumers and being evaluated by them. Both of them were performed ranking test for products according to ISO 8587:2006. The analysis of sodium and potassium content was performed by inductively-coupled plasma mass spectrometry (ICP-MS). Products from all experimental groups had acceptable taste for consumption. It can be concluded that the production of sausages with less amount of sodium chloride and with partially replacement of sodium chloride with potassium chloride is possible in the aim of reducing of sodium content in this kind of products.

Keywords: fresh sausages, sensory properties, sodium chloride, potassium chloride

INTRODUCTION

Sodium chloride is the most common ingredient in processed meat products that contributes to the water holding capacity, colour and flavor, especially saltiness. In the ancient time, common salt (sodium chloride) has been used as preservative due to its antimicrobial effects. Nowadays, it is clear that antimicrobial effect of salt is based on the lowering of water activity in meat products. Water activity is important as a hurdle barrier it is one of the main factors only in production of dried meat products, while in other kind of meat products it has no effect (Desmond, 2006).

But excessive dietary intake of sodium originated from sodium chloride is recognized as one of the main factors of risk for appearance of cardiovascular diseases and hypertension. The evidence for adverse cardiovascular effects of sodium, which is supported by a number of observational studies, indicate an association of increased risk of morbidity and mortality from cardiovascular diseases, including coronary heart disease and stroke, with increased sodium intake (Law, 1997). Reduction of sodium intake is widely recommended to prevent hypertension, but this approach is debatable, because some clinical studies showed that sodium restriction is successful only in the one part of population that recognized as sodium sensitive (Morris *et al.*, 1999). Due to this knowledge, it is recommended moderate salt reduction in normotensive people and salt restriction in those with high blood pressure (Chockalingam *et al.*, 1990). Some reports from the European Food Safety Authority (EFSA, 2005) have shown that the general intake of sodium across Europe exceeds the recommended amount. The large amount of sodium originated from industrially processed food (Hermansen, 2000). Meat products are a source of sodium in human diet contributing to

approximately 20% to 30% of the daily intake. Because of that, meat industry has shown a great interest to reduce salt content in the meat products in different ways such as: (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 Matekalo-Sverak, 2007; Matekalo-Sverak *et al.*, 2007); (6) optimisation of the physical form of salt (Angus *et al.*, 2005); and (7) alternative process techniques such as salting of pre-rigor meat and salt injection of intact pre-rigor meat (Claus and Sørheim, 2006).

The most used replacer of sodium chloride is potassium chloride. Many studies recommended potassium chloride as a partial replacer for sodium chloride in meat products (Gou *et al.*, 1996; Gelabert *et al.*, 2003). Even potassium chloride has ionic force properties similar to those of sodium chloride; its use is limited by its bitter and metallic taste (Askar *et al.*, 1994; Desmond, 2006). Using herbs and spices is a promising alternative to improve the quality of meat products when the partial replacement of sodium chloride with potassium chloride occurs (Guàrdia *et al.*, 2006).

The aim of this study was to evaluate the possibility to reduce sodium chloride content in fresh sausages and effect of partially replacement of sodium chloride with potassium chloride on some sensory characteristic and acceptability in small caliber fresh sausages.

MATERIAL AND METHODS

In this experiment three groups of sausages were produced. All sausages were produced from pork and different amount of sodium chloride and potassium chloride (Table 1). Pork was grounded and mixed with different amount of salt. After mixing, meat was stuffed into collagen casings diameter 22 mm. The first experimental group of sausages (control group) contained only sodium chloride in the amount of 20 g per 1000 g of meat that is usually amount of salt added to this kind of product. In the second experimental group of sausages it was added less amount of salt (15 g). In the third group of sausages, one third of sodium chloride was replaced with potassium chloride (10 g of sodium chloride and 5 g of potassium chloride on 1000 g of mass).

Table 1. Sausage composition, g

	Material		
	Group 1	**Group 2**	**Group 3**
Pork (shoulder)	1000	1000	1000
Sodium chloride	20	15	10
Potassium chloride	-	-	5

Sausages were formed in the length of approximately 20 cm and roasted on electric grill. Products were sensory evaluated by quantitative descriptive analysis (ISO 6658:2005), using scales with 5 points (5 – the best expressed attribute; 1 – the worst expressed attribute). Sensory evaluation was performed by six assessors, previously trained for detection and recognition of various tastes (ISO 3972:2011) and odours (ISO 5496:2006). Also, products were presented to seven consumers and being evaluated by them. Both of them were performed ranking test for products according to ISO 8587:2006.

In roasted sausages were determined sodium and potassium content. Homogenized sample (approximately 0.3 g) was digested in microwave oven with nitric acid (p.a. SIGMA) and hydrogen peroxide (30%, p.a., MERCK). The analysis was performed by inductively-coupled plasma mass spectrometry (ICP-MS) using the instrument "iCap Q" (Thermo Scientific, Bremen, Germany), equipped with collision cell and operating in kinetic energy discrimination (KED) mode. The following isotopes were measured: ^{39}K and ^{23}Na. The quality of the analytical process was controlled by the analysis of the standard reference material (NIST

SRM 1577c). Measured concentrations were within the range of the certified values for all isotopes.

Statistical analysis was performed using Microsoft Excel 2010 by method of descriptive statistics, analysis of variance and student t-test with expression of differences between arithmetic means of each group at the two levels of significance ($p \leq 0.05$ and $p \leq 0.01$). Different letters in superscript of average values presents the significant statistical difference.

RESULTS AND DISCUSSION

Sensory evaluation was performed by six trained assessors and seven consumers. To most assessors, sausages from the first group were too salty in the relation to products from two other groups in significant level ($p \leq 0.05$), (Table 2). Due to that, taste acceptability was also evaluated for sausages from the first group with less evaluation than in other two groups and level of statistically difference was the same ($p \leq 0.05$). Opposite them, consumers did not recognize the differences in the saltiness and taste acceptability, even products from the first group in both sensory attribute less evaluated, but without significant difference ($p \geq 0.05$). Although determined differences from assessors, overall impression was evaluated as equal from assessors and consumers as well as consistency ($p \geq 0.05$).

Table 2. Sensory evaluation of sausages

Group	M	Sx	Sd	Cv	Group	M	Sx	Sd	Cv
Taste acceptability									
Assessors, n = 6					**Consumers**, n = 7				
1st	3,33 [a]	0,37	0,94	28,28	1st	3,57	0,40	0,49	13,86
2nd	4,67 [b]	0,44	0,47	10,10	2nd	4,29	0,43	0,70	16,33
3rd	4,50 [b]	0,59	0,50	11,11	3rd	3,43	0,50	0,90	26,35
Saltiness									
Assessors, n = 6					**Consumers**, n = 7				
1st	2,50 [a]	0,32	1,38	55,38	1st	3,57	0,41	0,90	25,30
2nd	4,33 [b]	0,41	0,75	17,20	2nd	4,29	0,43	0,70	16,33
3rd	4,33 [b]	0,57	0,47	10,88	3rd	3,57	0,53	1,18	32,98
Consistency									
Assessors, n = 6					**Consumers**, n = 7				
1st	3,33	0,38	1,25	37,42	1st	4,29	0,48	0,70	16,33
2nd	4,17	0,40	0,69	16,49	2nd	4,57	0,45	0,49	10,83
3rd	4,58	0,60	0,45	9,79	3rd	4,29	0,61	0,70	16,33
Overall impression									
Assessors, n = 6					**Consumers**, n = 7				
1st	2,50	0,30	1,12	44,72	1st	3,86	0,43	0,64	16,56
2nd	3,83	0,39	1,34	35,05	2nd	4,43	0,44	0,49	11,17
3rd	3,75	0,50	0,69	18,46	3rd	4,29	0,61	0,70	16,33

[a, b] $p \leq 0.05$

Most assessors (83.3%) put the sausages from the first group, as too salty, on the third place in the ranking test (Table 3) and 50% of them the products from the third group on the first place and on the second place, respectively. As assessors, most of consumers gave the last place in the ranking to products from the first group (71.4%), and one of them the first and the second place (14.3%), respectively. In the case of third group of sausages, consumers were not so consistent and three of them (42.9%) gave the first place to products from the first group. Two of them gave the third and the second place to products from the third group (28.6%), respectively.

Table 3. Ranking of sausges

Rank	Assessors, n = 6			Rank	Consumers, n = 7		
	1st group	2nd group	3rd group		1st group	2nd group	3rd group
1	1 (16.7%)	2 (33.3%)	3 (50%)	1	1 (14.3%)	3 (42.9%)	3 (42.9%)
2	0	3 (50%)	3 (50%)	2	1 (14.3%)	4 (57.1%)	2 (28.6%)
3	5 (83.3%)	1 (16.7%)	0	3	5 (71.4%)	0	2 (28.6%)

Obtained results shows the highest sodium content in the sausages from the first group (1123 mg/100 g) and the lowest content in the products from the third group (560 mg/100 g), according to the added amount of sodium chloride (Table 4). The potassium content was highest in the sausages from the third group, where is added. Amount of potassium in the two first groups of products is the result of potassium content originated from meat.

Table 4. Sodium and potassium content in 100 g of products, mg

	Group		
	I	II	III
Sodium	1123	770	560
Potassium	616	563	860

The results obtained in this investigation open the possibility to reduce sodium content in fresh sausages. Commonly, mass of meat for production of fresh sausages contents around 2% of sodium chloride. Fresh sausages in Serbia are consumed mostly as a part of barbeque with other grilled meat and liver, salad and bread. This experiment showed that, if it is consumed separately, sausages with 2% of sodium chloride is too salty. According to obtained results it can be concluded there is possibility to reduce sodium chloride in fresh sausages for 25%. If we take in the consideration the partial replacement of sodium chloride with potassium chloride, it is clear that is possible to get the fresh sausages with lowest sodium content and better ration of potassium and sodium which should be approximately 4:1 to improve human health.

CONCLUSIONS

Sausages from all three groups had acceptable taste that was better in the sausages from the second and from the third group ($p \leq 0.05$) than sausages in the first group.
Roasted fresh sausages with the largest amount of added sodium chloride are recognized as too salty from assessors and more salty than sausages from the other two groups ($p \leq 0.05$).
Most of assessors ranked sausages with the largest amount of sodium chloride on the third place (83.3%). Sausages from the third group, with partially replacement of sodium chloride with potassium chloride ranked on the first and the second position from assessors, respectively.
There is great possibility to reduce sodium content in the production of fresh sausages by reducing of amount of added sodium chloride as well as by partially replacement of sodium chloride with potassium chloride without so important consequences to the taste acceptability, saltiness and overall impression of products.

ACKNOWLEDGEMENTS

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THE EFFECT OF SODIUM REDUCTION ON THE PHYSICO-CHEMICAL QUALITY AND SAFETY OF HOT DOGS

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ABSTRACT

The aim of this study is to evaluate the effects of replacing nitrite curing salt with sodium nitrite with the finished mixture of sodium and potassium chloride for human consumption (Na-max 27 g/kg-min K 16 g/kg) at a rate of 25 %, 50 %, 75 % and 100 % on the physico-chemical quality and microbiological safety of the cooked sausages - hot dogs. Five different production batches (PB) were manufactured, each 10 kg in weight. The first PB (control) was prepared according to the manufacturer's original recipe. Experimental PB I was made by adding 50 g of mixed sodium-potassium salt (25 %) and 150 g nitrite salt into 10 kg of stuffing. PB II was prepared with 100 g of combined sodium-potassium salt (50 %) and 100 g nitrite salt, while PB III was made with 150 g of combined sodium-potassium salt (75 %) and 50 g nitrite salt. Hot-dogs of PB IV were made by adding 200 g (100 %) of combined sodium-potassium salt into 10 kg of stuffing.

The results obtained clearly indicate a continuous decrease of sodium content in samples originating from the control group to the experimental samples PB IV, in which the nitrite curing salt with sodium nitrite was fully replaced with a mixture of potassium-sodium salt. Replacing nitrite curing salt with the finished mixture of sodium and potassium chloride did not significantly affected examined chemical quality (content of moisture, proteins, fat, ash) of experimentally prepared hot-dogs.

Tested samples originating from all experimental batches were microbiologically safe, according to the criteria laid down by Serbian food safety legislative.

Keywords: *sodium nitrite salt, potassium chloride salt, physico-chemical quality, microbiological safety, hot dogs*

INTRODUCTION

Many of scientific reports linking excessive sodium intake to the incidence of hypertension (Dahl, 1972; Law *et al*., 1991a, 1991b; Xiaosong, 2007) is the main reason for reducing the sodium content of processed meats. Vandendriessche (2008) is today's meat processing characterized as a period of improving the quality, food security and nutrition/health.

From the earliest period of human society development salt has been used as a preservative for meat and one of the most common ingredients we use in meat processing in a wide range of products. Sodium and chloride are essential for life and health, for stabilizing the internal fluid, electrolytes and blood pressure in human body. They provide adequate function of muscles and nerves and allow absorption of sodium nutrients, such as glucose (sugar) and amino acids. Salt has a strong influence on the formation of desirable properties of meat products and their safety, through a different line of activities: it is essential for achieving an adequate taste, bacteriostatic (reduces the water activity), it activates protein and improves water-binding capacity and hydration, enable meat proteins participating in the processing of meat and connected meat to be more soluble, renders water and fat in the desirable structure of gel formation to improve texture, increase the viscosity of the meat, emulsified product salts separate protein myofibrils and contribute to their ability to emulsify fat, increase the pH of meat or meat system.

Salt reduction in meat products thus has adverse effects on such technological functions as water and fat binding, impairing overall texture and increasing cooking loss, shelf life and also on sensory quality, especially taste (Ruusunen *et al.*, 2005; Desmond, 2006).

Food manufacturers are faced with a dilemma: "How to reduce sodium content in foods without excessive modification of its flavor"? Trends show that consumers are increasingly opting for "healthy" food, and the taste remains the most critical factor for the purchase. Manufacturers can choose to simply reduce the NaCl content without changing the taste. Toldrá and Barat (2009) published an overview of innovative patents to reduce salt in food.

Sodium enters the meat and meat products (12 to 20% of the total food intake), and therefore the meat and meat products is one of the priority products that need to contribute to the reduction of its content (Desmond, 2006).

Reducing the content of sodium in meat products can be achieved in the following manner: (1) by reducing the amount of sodium chloride added (Lilić, 2000); (2) by replacing part of NaCl with other salts (Lilić *et al.*, 2008).; (3) by using flavor/aroma enhancers and masking agents (Desmond, 2006); (4) combination of mentioned procedures (Terell, 1983); (5) adding of spice herbs and spice extracts to meat products (Lilić and Matekalo- Sverak, 2007;); (6) optimization of the physical form of salt (Angus *et al.*, 2005) and (7) alternative process techniques (Claus and Sørheim, 2006).

The aim of this study was to evaluate the effects of replacing nitrite curing salt with sodium nitrite with the finished mixture of sodium and potassium chloride for human consumption (Na-max 27 g/kg-min K 16 g/kg) at a rate of 25 %, 50 %, 75 % and 100 % on the physico-chemical quality and microbiological safety of the cooked sausages - hot dogs.

MATERIAL AND METHODS

Sausage formulation and processing

As part of our examination of the hot dogs on the possibility of replacing Nitrite curing salt with sodium nitrite originating from mixed sodium potassium salt five different production batches (PB) manufactured, each weight by 10 kg. The first PB (control) was prepared according to the manufacturer's original recipe (Table 1).

Table 1. Hot dog: Recipe of the standard control sample - control group (C-1)

Ingredients	Amount (%)
Beef	20
Pork	20
Emulsion of pig skin	15
Firmly pork fatty tissue	13
Ice	22
Spice mixture for frankfurters (Raps GmbH, Austria)	0.4
Adifos (E450, E451)	0.5
Miocolor VS (Ireks Aroma Ltd, Zagreb, Croatia)	0.4
Corn Starch	2.25
Rubysin *Rouge* (Lay Gewürze OHG 98631, Germany)	0.05
Nitrite salt	2
Soy protein isolates for emulsification, WDF PRO950E	4.4

Experimental PB I at 10 kg stuffing added 50 g of mixed sodium-potassium salt (Na-max 27 g/kg-min K 16 g/kg) (25%) and 150 g nitrite salt. In PB II to 10 kg stuffing added 100 g of the above mentioned combined sodium-potassium salt (50%) and 100 g nitrite salt. In PB III to 10 kg stuffing added 150 g of combined sodium-potassium salt (75%) and 50 g nitrite salt. In hot-dogs of PB IV added 200 g (100%) combined sodium-potassium salt on 10 kg of stuffing. Beef, pork and firm fatty tissue were ground in the Wolf, using grid of 3 mm, and then further processed by fragmentation in cutter. Fragmentation of the mass in the cutter lasted approximately 7 minutes at 2.000 revolutions/minute (Rex-Maschinen, Type: HYDRO100N,

Wilh. Duker GmbH&Co, Werk Laufach). First, in the cutter chopped beef and pork, pork skin emulsion and half of the total amount of ice were added. Then additives other than soy isolate had been added, and treatment in the cutter lasted about 3 minutes. After addition of soy isolate (an emulsifier), and the other half of the ice, the treatment continued for another 3 minutes. During last minute of processing ground firmly fat tissue was added in the cutter and emulsion was completely homogenized. Stuffing is done in hopes impermeable layer (Mineralen 24 light brown; 42 strand (s)/27.5 m - 1 155m ; LOT-CASE: 202303123/038) with vacuum fillers (VEMAG, model ROBBY-2). Filled with stuffing envelopes were hand-paired, and sausages hanging on metal rods which were placed in the appropriate frame trolley, where transported to the conditioned chamber (athmos furnace) for heat treatment (boiling). Temperature during the boiling in the chamber was $85°C$ and the process lasted until $72°C$ had been achieved in the geometric center of the product.

Determination of the hot dogs Proximate Composition
The main chemical composition was evaluated by determining of the moisture content (SRPS ISO 1442), total protein (SRPS ISO 937), total fat (SRPS ISO 1443) and total ash (SRPS ISO 936).

Determination of Na and K content
After cooling, samples were transferred into a 50 mL volumetric flask with de-ionized water. Analyses were carried out on atomic absorption spectrometer "SpectrAA 220" (Varian, Palo Alto, California, USA) according to Varian AAS Analytical methods (Flame Atomic Absorption Spectrometry Analytical Methods). All reagents used were of analytical grade and equipment which was pre-calibrated appropriately with standard solutions prior to measurement. Samples were prepared by microwave digestion (ETHOS TC, Milestone S.r.l., Sorisole, Italy) according to manufacturer's recommendations (Tips and Techniques for ETHOS Series Microwave Lab Stations, an Operations Overview). 0.5 g of the sausage sample was treated with 8 mL of nitric acid (HNO_3) and 2 mL of hydrogen peroxide (30 % H_2O_2); temperature program was as follows: 5 min from room temperature to 18ºC then 10 min hold at $180°C$.

Microbiological examination
Three hot dogs per batch were used to evaluate the microbiological quality of the treatments. Coagulase-positive staphylococci, *Salmonella spp.*, *Listeria monocytogenes*, sulphite-reducing clostridia, coliforms, total viable count and mesophilic lactic acid bacteria were quantified according to the standard methodology (SRPS EN ISO 6888-1 and 2:2009; SRPS EN ISO 6579:2008; SRPS ISO 11290-2:2010; SRPS ISO 15213:2011; SRPS ISO 16649-2:2008; SRPS EN ISO 4833-1:2014; ISO 15214:1998).

RESULTS AND DISCUSSION

The chemical characteristics of hot dogs formulated according producer's recipe (control group C-1) and with replacement of 25%, 50%, 75% and 100% nitrite curing salt by mixed sodium-potassium salt are presented in Table 2. Ash content was not significantly different between the samples of hot dog originating from the control and experimental groups. The treatments with the replacement of nitrite curing salt by mixed sodium-potassium salt presented a significant reduction in the content of sodium and a significant increase in that of potassium. Ruusunen *et al.* (2002) examined the sodium reduction of cooked bologna-type sausage by replacing sodium phosphate with potassium phosphate. Target sodium content in two different formulations was 0.55 g Na/100 g, and in next two formulations was 0.63 g Na/100 g. Sodium content in our investigation was in descendent order 4950.74 mg/kg, 4871.96 mg/kg, 4223.08 mg/kg, 3898.65 mg/kg and 3422.44 mg/kg, respectively. The control formulation (C-1) presented a sodium content considered common for cooked emulsion products. The replacement of 25%, 50%, 75% and 100% nitrite curing salt by mixed sodium-potassium salt generated a reduction of approximately 1.59%, 14.70%, 21.25% and 30.87%

in the sodium content in relation to the control formulation, respectively. This reduction in the sodium content provides the modified products with a healthier appeal since the decrease of sodium intake in our diet is seen as a way to reduce risk factors for hypertension and, consequently, heart diseases (Antonios and Macgregor, 1997).

Table 2. Physico-chemical quality of the hot dogs with standard control sample (C-1), replacement of 25% (PB I), 50% (PB II), 75% (PB III) and 100 % (PB IV) Nitrite salt by mixed sodium-potassium salt

Group	Descriptive statistic	Moisture (%)	Protein (%)	Fat (%)	Ashes (%)	Sodium (mg/kg)	Potassium (mg/kg)
C-1	$\overline{X}$	55.10	11.13	28.14	2.85	4950.74	777.57
	$S_{\overline{X}}$	0.15	0.07	0.42	0.03	148.47	27334,00
	Sd	0.26	0.12	0.73	0.06	257.16	20.34
	Cv (%)	0.47	1.08	2.59	2.10	5.19	2.61
	min	54.83	11.01	27.63	2.79	4718.10	759.53
	max	55.35	11.26	28.98	2.91	5226.87	799.62
PB I	$\overline{X}$	55.84	12.05	27.01	2.94	4871.96	950.00
	$S_{\overline{X}}$	0.01	0.06	0.07	0.03	24.35	17.98
	Sd	0.17	0.11	0.13	0.05	42.17	31.15
	Cv (%)	0.30	0.91	0.48	1.70	0.86	46813,00
	min	55.69	11.96	26.87	2.88	4825.25	916.17
	max	56.03	12.17	27.13	2.98	4907.25	977.50
PB II	$\overline{X}$	55.44	11.81	27.44	2.86	4223.08	1741.26
	$S_{\overline{X}}$	0.06	0.08	0.10	0.06	19.61	9.11
	Sd	0.11	0.14	0.17	0.10	33.97	15.78
	Cv (%)	0.20	1.18	0.62	3.50	2.78	0.91
	min	55.31	11.66	27.29	2.77	4195.44	1728.56
	max	55.51	11.92	27.63	2.97	4261.00	1758.92
PB III	$\overline{X}$	53.69	12.35	28.63	2,99	3898.65	2379.38
	$S_{\overline{X}}$	0.07	0.10	0.06	0.08	29.45	27.68
	Sd	0.12	0.18	0.10	0.14	51.02	47.92
	Cv (%)	0.22	1.46	0.35	4.68	1.31	2,02
	min	53.56	12.13	28.52	2,88	3841.40	2325.25
	max	53.80	12.48	28.70	3.15	3939.30	2416.39
PB IV	$\overline{X}$	56.45	12.31	25.87	2.94	3422.44	1741.32
	$S_{\overline{X}}$	0.07	0.15	0.11	0.04	43.96	214.26
	Sd	0.11	0.26	0.20	0.07	76.14	829.82
	Cv (%)	0.19	2,11	0.77	2,38	2,22	47.65
	min	56.36	12.02	25.65	2.87	3377.41	759.53
	max	56.58	12.52	26.04	3.00	3510.35	2900.83

$\overline{X}$ – *mean*
Sd – standard deviation
Cv – coefficient of variation

Handling and processing of meat are likely to generate conditions for a more intense microbial contamination. The presence of aerobic bacteria in our samples tested indicates possible contamination of hot dogs caused by poor hygiene of employees, surfaces or equipment or usage of additives. Increase of temperature during certain stages of the technological process and separation of meat juice contributes to the rapid propagation of the present microflora. In order to produce high-quality meat products, it is necessary that the raw material is high-quality and microbiologically safe, and that control is exercised during all stages of production and processing of the finished product.

Results of microbiological testing indicated that none of the experimental sausages had been contaminated with *L. monocytogenes* which renders sausages to meet criteria laid down by Regulation on the general and special conditions of hygiene of food at any stage of production, processing and trade (Official Gazette of RS, 72/10). Furthermore, none of the

tested samples had been contaminated with *Salmonella*, coagulase-positive staphylococci, sulfite-reducing *Clostridia*, and *Escherichia coli*.

Table 3. Results of microbiological examination of tested samples of hot dogs

Group	Coagulase-positive Staphylococci	Salmonella spp.	Listeria monocytogenes	Sulfite-reducing bacteria	β-glucuronidase-positive *Escherichia coli*	No. of aerobic bacteria	Mesophilic lactic acid bacteria
C - 1	/	/	/	/	/	350	300
PB I	/	/	/	/	/	1000	/
PB II	/	/	/	/	/	850	/
PB III	/	/	/	/	/	2000	/
PB IV	/	/	/	/	/	300	/

CONCLUSIONS

The replacement of 25%, 50%, 75% and 100% nitrite salt by mixed sodium-potassium salt promote healthier characteristics to hot dogs produced with low levels of sodium and high potassium level. The physicochemical quality varied, and statistical differences were observed in the moisture, protein and fat content, pH and water activity values, sodium and potassium content among the treatment. Ash content was not significantly different between the samples of hot dog originating from the control and experimental groups. Microbiological safety of the hot dogs produced with replacement of nitrite salt by mixed sodium-potassium salt not altered by this substitution. Further studies are necessary to assess the impact of this technological strategy on the hot dogs shelf life regarding their sensory quality.

ACKNOWLEDGEMENTS

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SUGAR BEET JUICE CLARIFICATION USING CALCIUM SULFATE, COPPER SULFATE AND ALUMINUM SULFATE

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ABSTRACT

In the sugar industry, chemical processing of raw sugar beet juice and molasses are important operations that include disposal of non sucrose substances in order to obtain higher quality confectionery product- white sugar. Calcium ions (in the form of CaO) that are commonly used to eliminate these compounds from beet juice have a relatively low binding affinity and the quantities of used lime are very large (1 – 3 % w/w, calculated on the beet). In order to reduce the amount of waste materials, the possible application of alternative coagulants with divalent and trivalent cations, $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$ was studied. These compounds cause the process of charge neutralization beet juice macromolecules which creates conditions for coagulation and sedimentation. Mechanism of discharge of macromolecules compounds was suggested. Model - pectin solutions (50 cm^3 and 0.1 % wt.) were treated with different concentration of $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$ ranging of 50 – 450 mg/dm^3. The pH of solutions were regulated at 7. In order to monitor coagulation and sedimentation process of solution, a method of measuring the zeta potential of solution was used. Zeta potential was determined by electrophoretic method. Optimal amounts of applied coagulants were: 410 mg/dm^3 (610 mg/g_{pectin}) for $CaSO_4$; 100 mg/dm^3 (151,5 mg/g_{pectin}) for $CuSO_4$ and 110 mg/dm^3 (162 mg/g_{pectin}) for $Al_2(SO_4)_3$. These values are significantly less than the average amount of CaO used in classical process of beet juice purification (about 9 g/g_{pectin}).
Keywords: sugar beet juice, $CaSO_4$, $CuSO_4$, $Al_2(SO_4)_3$, zeta potential

INTRODUCTION

Processing of raw sugar beet juice and molasses are important operations which are used to remove pectin and protein substances in order to obtain the final product - white sugar. Separation of these compounds in our country is mostly done by compounds with calcium ion. Calcium ions (in the form of CaO) have a relatively low binding affinity and the quantities of used lime are very large (1 – 3 % w/w, calculated on the beet). Also, due to its absorption features CaO can cause unwanted alkalization process of soil in the near environment of the sugar factory (Haapala *et al.*,1996).

In sugar beet macromolecules, primarily pectin and proteins, the negative charge on the surface of particles is formed by dissociation of functional groups and adsorption of ions from the surrounding solution (Koper, 2007). This is the main reason for the stability of colloidal solution such us sugar beet juice. By adding two- and trivalent cations, a decrease of negative charge of macromolecules and conditions for the coagulation and sedimentation process occur. In this case occurring electrostatic and sometimes chemical forces (specific adsorption mechanism) (Axelos *et al.*,1996; Schneider *et al.,* 2011; Duan and Gregory, 2003). The charge neutralization of these macromolecules can be monitored by electrokinetic or zeta potential (ζ). Zeta potential is easily measurable property of colloidal particles. Bringing zeta potential near the zero value, colloidal particles will discharge and the conditions for effective coagulation and sedimentation are achieved (Koper, 2007).

Dronnet et al. (1996) and Garnier et al. (1994) defined the selectivity order of the binding affinity of various divalent cations by citrus and sugar-beet pectins:

$$Cu^{2+} \sim Pb^{2+} >> Zn^{2+} > Cd^{2+} \sim Ni^{2+} > Ca^{2+} > Mg^{2+} \tag{1}$$

The binding affinity of divalent metal ions on protein macromolecules has been also studied (Philippe *et al.,* 2005). It is evident that Cu^{2+} ions are more efficient than Ca^{2+} ions because of marked surface complexation ability (mechanisam of specific adsorption). In the presence of Ca^{2+} ions are performed mainly electrostatic interactions between the negatively charged side chains of pectin polysaccharide or the negatively charged surface of protein macromolecules (Kuljanin, 2008; Kuljanin *et al.,* 2012a).

Sugar beet juice has a concentration of undesirable colloids as most of the waste and contaminated water (about 1 wt %). For treatment of water, Al^{3+} ions are often used in the form of hydrolyzing salts $Al_2(SO_4)_3$ (Duan and Gregory, 2003). The bonding strength Al ions with humic materials in water compared with divalent metal ions are studied. The order of ion binding are shown (Kinniburgh *et al.,* 1999):

$$Al^{3+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Ca^{2+} \tag{2}$$

In accordance with this selectivity order, it is clearly that applying of Al^{3+} ions will be much efficient in sugar beet juice clarification in relation to classical process by Ca^{2+} ions originating from the CaO (Kuljanin *et al.,* 2012b).

Comparing the activity of coagulants with the same cation and different anions under the same experiment conditions, $CaSO_4$ showed a slight difference compared to the effect of $CaCl_2$. Investigation performed in paper (Lević *et al.*, 2007) suggest that three salts: $CaCl_2$, $CuSO_4$ and $AlCl_3$&$NaHCO_3$ are more efficient in pectin precipitation than commonly used CaO.

The aim of this study was to investigate the effect of $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$ concentration on sugar beet juice clarification. These results were compare with the influence of Ca ions originating from the CaO.

MATERIAL AND METHODS

Pectin preparation was extracted from the pressed sugar beet cossettes obtained in industrial processing of sugar beet (sugar factory "Šajkaška" Žabalj). Calcium, copper and aluminium sulphate were used in a crystal- hydrated form ($CaSO_4$ x $7H_2O$; $CuSO_4$ x $5 H_2O$ and $Al_2(SO_4)_3$ x $18H_2O$) in the form of aqueous solutions, (manufacturer's *Zorka Pharma*, Šabac, Serbia). To correct the pH value in presence of $Al_2(SO_4)_3$, an equivalent amount of Na_2CO_3 was used.

Pectin preparation was isolated by extraction in acidic condition by standard laboratory procedure AOAC (2000). The extraction was conducted discontinually in an extractor (volume 2 dm^3) using aqueous solution of HCl. The mass ratio of sugar beet cossettes to solvent was 1:10. The extraction was performed at pH 3.5 and $85^{\circ}C$ during 2.5 h. High molecular colloidal fraction was isolated from extract by multistage precipitation with 70 % ethanol. The precipitated colloids were left overnight to deposit and the obtained sediment was washed out with 70 % ethanol. Pectin preparation, after precipitation and cleaning were dried in a vacuum drier for 12 hours at 70 °C (Lević *et al.,* 2007; Kuljanin *et al.,* 2012a). Procedure was repeated several times and basic parameters of pectin preparation were determined according to standard methods of AOAC (2000).

The degree of esterification of pectin preparation was calculated over the equivalents of free (X) and the esterified carboxyl groups (Y), using equation:

$$DE = \frac{Y}{X+Y} \cdot 100 \tag{3}$$

Mean molar mass of the pectin preparation was determined using spectrophotometry and the refractometer, by the method of Kar and Arslan (Kuljanin, 2008).

The experiment has tested the model-solutions of pectin preparations concentrations of 0.1 % (mass). Working solutions were prepared by dissolving 1 g of pectin preparation in 250 cm^3 of distilled water and left over night to swallow. After that, distilled water was added up to

1 dm^3, and for every measurement 50 cm^3 was separated. After dissolution of 1 g Al$_2$(SO$_4$)$_3$, CuSO$_4$ and CaSO$_4$ in 200 cm^3 of distilled water, an appropriate amount was added to 50 cm^3 of pectin solution (0.1 mass. %). The obtained concentrations of CaSO$_4$, CuSO$_4$ and Al$_2$(SO$_4$)$_3$ were in the range of 50 to 450 mg/dm^3. All measurements were performed at pH = 7. At this pH, ions Ca^{2+}, Cu^{2+} and Al^{3+} have limited solubility. To obtained desired solution alcality (pH=7), Na$_2$CO$_3$ was added to Al$_2$(SO$_4$)$_3$ (mass ratio of Na$_2$CO$_3$ to Al$_2$(SO$_4$)$_3$ was 1:1.07, calculated on pure Al$_2$(SO$_4$)$_3$ (Kuljanin *et al.,* 2012a).

After the coagulants CaSO$_4$, CuSO$_4$ and Al$_2$(SO$_4$)$_3$ were added to the tested preparation, pH was adjusted and the solution was stirred for 30 min on a high-speed magnetic stirrer (stirring speed 500 o/min). After aging the solution for 5 min, zeta potential of clear part the solution was measured. Zeta potential was determined by electrophoretic method using a commercial apparatus ZETA-METER ZM 77 (Riddick, 1975). The measurements were performed at room temperature as described by Kuljanin *et al.,* 2012a. Results represent an average value of 3 measurements.

RESULTS AND DISCUSSION

Results related to the composition of pectin preparation are given in Table 1.

Table 1. Basic physico-chemical composition of pectin preparation

Solid content (g/100g)	Equivalent of free COOH groups X · 10^5	Equivalent of esterif. COOH groups Y · 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

The content of galacturonic acid (degree of purity) and the degree of esterification (DE) in the test preparation corresponds to the mean value of the pectin contents of the degree of esterification of sugar beet raw juice. Obtained preparation is one within group of less esterified pectin (DE < 50). This means a greater ability to bind cations to macromolecules of sugar beet juice due to the greater presence of the free carboxyl groups (COO$^-$). Changes of mean values of Zeta potential (mV) of the tested pectin preparation after adding various quantities of coagulants CaSO$_4$, CuSO$_4$ and Al$_2$(SO$_4$)$_3$ in the form of pure salts (mg/dm^3) are shown in Figure 1.

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 Ca^{2+}, 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.
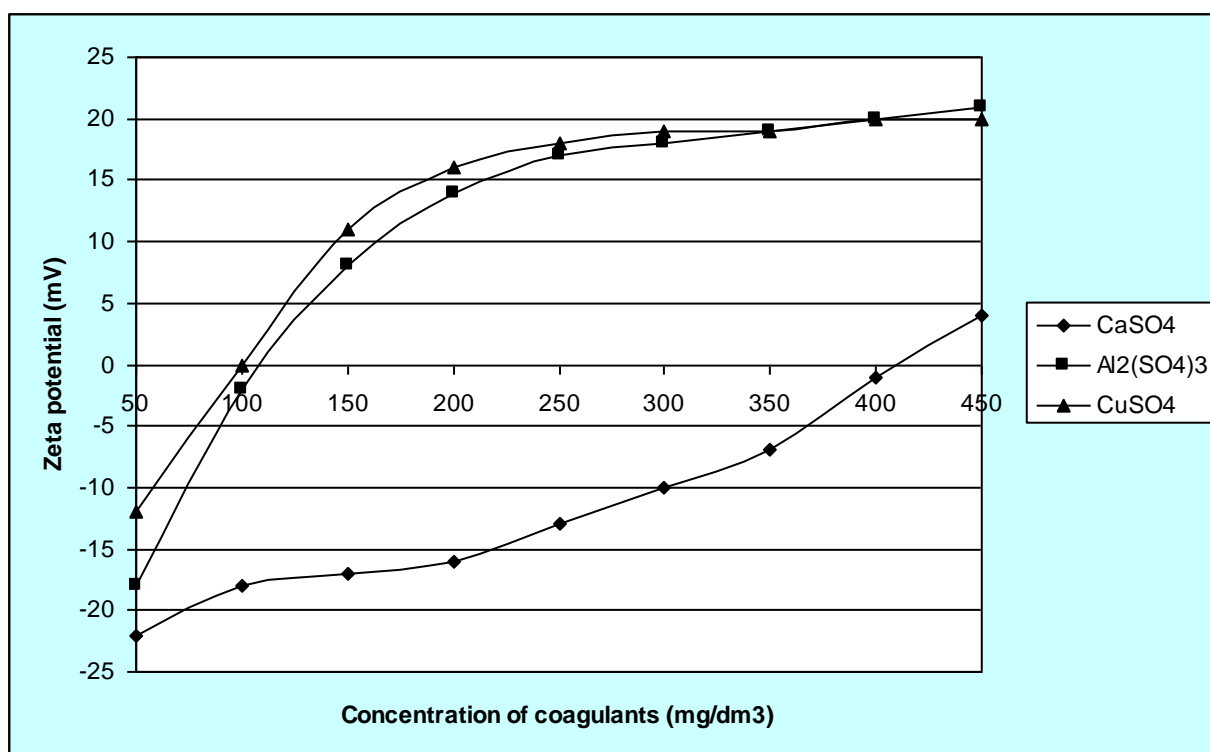
Figure 1. Influence of $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$ on change in the zeta potential pectin solution

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 $CuSO_4$ (100 mg/dm^3) compared to $Al_2(SO_4)_3$ (110 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 (1). The amounts of Ca^{2+} ions originating from $CaSO_4$ were compared with the amounts of Al^{3+} ions from $Al_2(SO_4)_3$ and Cu^{2+} ions from $CuSO_4$ to achieve zero zeta potential values. It takes about 3.5 times as much of Ca^{2+} ions in comparison with Al^{3+} and 4 times as much of Ca^{2+} ions in comparison with Cu^{2+} ions for zeta potential to reach zero value. These values are: 410 mg/dm^3 $CaSO_4$ (610 mg $CaSO_4$/g$_{pectin}$), 110 mg/dm^3 $Al_2(SO_4)_3$ (162 mg $Al_2(SO_4)_3$/g$_{pectin)}$ and 100 mg/dm^3 $CuSO_4$ ($151,5$ mg $CuSO_4$/g$_{pectin}$). This can be explained by weaker, electrostatic binding of Ca^{2+} ions. Ca^{2+} ions are in the last place in the previously given binding scale of divalent ions with citrus and sugar-beet pectins (1). The binding of Ca^{+2} ions from $CaSO_4$ appeared to be regulated by mechanism of electrostatic bonding (Coulombic attractions) with a small share of specific adsorption. The binding of Cu^{+2} and Al^{+3} ions appeared to be regulated by mechanism surface complexation, solely through the mechanisam of specific adsorption, with a small portion of electrostatic bonding.
Compared with conventional process of sugar beet juice purification where is approximately used 9 g CaO per g of pectin, the amount of studied metal salts $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$ were significantly lower, ranging in the interval of $151,5 - 610$ mg per g pectin.
Control of the zeta potential and appropriate dosing of $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$, could efficiently remove the pectin from sugar beet juice. The significance of this method is that it achieves the lower consumption of coagulants and higher cleaning efficiency of sugar beet juice compared to the conventional method. However, reliable comparison between the proposed coagulants and traditional coagulant CaO, require additional testing under industrial conditions of sugar beet juice purification.

CONCLUSIONS

The amounts of $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$ were compared with the amount of CaO to achieve zero zeta potential values when creating the optimal conditions for separation pectin from solution. Ca^{2+}, Cu^{2+} and Al^{3+} ions originating from the $CaSO_4$, $CuSO_4$, and $Al_2(SO_4)_3$ possess a greater bonding strength with sugar beet pectin compared to Ca^{2+} ions originating from the CaO.

The conventional purification process of sugar beet juice requires approximately 9 g CaO per g of pectin. The amount of studied metal salts $CaSO_4$, $CuSO_4$ and $Al_2(SO_4)_3$ were significantly lower, ranging in the interval of 151,5 - 610 mg per g pectin. That would further reduce the cost of sugar beet juice purification while protecting the environment.

ACKNOWLEDGMENTS

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CRYOPROTECTIVE EFFECT OF OAT B – GLUCANS ON CHICKEN SURIMI

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ABSTRACT

Differential scanning calorimetry (DSC) was used to study cryoprotective effects of oat β – glucans on chicken surimi after frozen storage. Also influence of frozen storage on texture profile analysis (TPA), instrumental colour parameters and cooking loss of chicken surimi was investigated. Chicken surimi samples were prepared from broiler meat, mixed with oat β – glucans ($w = 0 - 6\%$), quickly frozen and stored for 30 days on -30 °C. Onset temperature of transition (T_o), peak thermal transition (T_p), and endset temperature of transition (T_e), and denaturation enthalpy (ΔH), was evaluated. Peak (T_p) thermal transition temperatures of chicken myosin showed shift to higher values with the increase of mass fraction of β – glucans. Denaturation enthalpies (ΔH) of chicken myofibrillar proteins showed increased values with increase of mass fraction of oat β – glucans. Instrumental colour parameters (Lightness (L*), redness (a*), yellowness (b*) and whiteness (L* − 3b*)) of chicken surimi gels were significantly (P<0.05) affected by addition oat β – glucans. Hardness and chewiness also increased significantly (P<0.05) and cooking loss decreased significantly (P<0.05) by addition of oat β – glucans. Cohesiveness and springiness of chicken surimi gels were not significantly (P>0.05) affected by addition of oat β – glucans. The Increase in peak thermal transition (T_p), denaturation enthalpies (ΔH), some TPA and instrumental colour parameters indicates possible cryostabilsation of chicken myofibrillar proteins with addition of oat β – glucans.

Keywords: *cryoprotection, chicken myofibrillar proteins, DSC, β – glucans, texture (TPA), instrumental colour (L*, a*, b*)*

INTRODUCTION

Chicken myofibrillar protein concentrate, produced with modified technology used for fish surimi (Dawson *et al.*,1988), is characterized by very good technological properties, such as high water holding capacity and high ability to form strong gels after being heated. Freezing has become the most frequently used preservation method for meat and meat products. To protect myofibrillar proteins from denaturation during frozen storage and maintain its possible high processability, some cryoprotectants (i. e. disaccharides, polysaccharides, polyalcohols, organic acids and polyphosphates) are generally added (MacDonald *et al.,* 2005). Most commonly used instrumental methods for determination cryoprotective effects of added substances are measurement of myofibrillar protein solubility SEP (Salt extractable protein), Ca^{2+}ATP-ase activity, unfrozen water by Nuclear Magnet Resonance (NMR), transition temperatures and denaturation enthalpies of myofibrillar proteins by Differential Scanning Calorimetry (DSC) (Sych *et al.*, 1990). β -glucans are composed of glucose molecules, which are linked with β - (1,3), (1,4) and (1,6) glycosidic bonds. (1,3), (1,4) – β – D - glucans are commonly isolated from wheat, barley and oat. Although found in all grains, their concentration is highest in oat (4.6 - 4.9 %) and barley (1.8 to 6 %). β-glucans from various sources are used in the food industry as a thickening agent, dietary fibers, emulsifiers, etc. (Brennan and Cleary, 2005). Studies have shown that the addition of β - glucans to meat batter increases the denaturation enthalpy of myofibrillar proteins, which suggests that β - glucans interact with meat proteins and stabilize them (Morin *et al.*, 2004). Differential scanning calorimetry (DSC) is a useful technique used for studying thermal behaviour of muscle proteins (Finday and Barbut, 1990). Changes in the protein structure during heating in DSC analysis are referred to as protein denaturation, and peak temperatures of these transitions are used to represent denaturation temperatures.

The objective of this study was to determine cryoprotective effects of oat β – glucans on chicken myofibrillar proteins using Differential Scanning Calorimetry (DSC), as well as to determine texture profile *(*TPA) and instrumental colour parameters (L*, a*, b*) of chicken surimi.

MATERIAL AND METHODS

Sample preparation
Chicken surimi samples were prepared in the laboratory from broiler meat (mainly lat. *Pectoralis major M. and Pectoralis minor M.*) using the modified procedure of Yang and Froning (1992) since washing and leaching was performed with distilled water, instead of with tap water. Samples were mixed with oat β – glucans in mass fractions of 2, 4 and 6 %. Mass fractions were determined as percent of total mass. The pH level was measured in a homogenate of the sample with distilled water (1:10, p/v) with pH/Ion 510 – Bench pH/Ion/mV Meter (Eutech Instruments Pte Ltd/ Oakton Instruments, USA). Water activity (a_w) was determined using a Rotronic Hygrolab 3 (Rotronic AG, Bassersdorf, Switzerland) at a room temperature (20 ± 2 ºC). The FoodScan Meat Analyser was used to determine moisture, total protein share, total fat share and collagen content according to the AOAC 2007. 04.

DSC measurements
Differential scanning calorimetry (DSC) was performed using Mettler Toledo DSC 822e differential scanning calorimeter equipped with STARe software. After defrosting in a refrigerator (4 °C, over night), samples of cca. 15 mg (± 1 mg) were weighed and sealed into standard aluminium pans (40 µl) and scanned over the rage from 25 to 95 °C at a heating rate of 10 °C min^{-1}, using empty standard aluminium pan as a reference. The peak temperatures (T_p) were determined from DSC curves. The changes in enthalpy (ΔH J g^{-1}), associated with the denaturation of proteins, were determined by measuring the area under the DSC curves using STARe software.

Textural analysis (TPA) and cooking loss
Samples of chicken surimi were placed into plastic test tubes with an inside diameter of 10 mm. After defrosting, test tubes with their content were heated for 25 min in a water bath at 80 ºC. Test tubes with produced gels were cooled in ice water until the temperature of approx. 20 ºC was obtained inside the sample. After that they were stored at 4 – 6 ºC until the next day. Cooking loss was calculated as a weight difference of the sample prior to the cooking and after the removal of the cooked gel from the test tubes. Cooking loss was expressed as a percent of the fresh sample weight. Texture profile analysis (TPA) tests were performed using a TA.XT2i SMS Stable Micro Systems Texture Analyzer (Stable Microsystems Ltd., Surrey, England) equipped with a cylindrical probe P/75. This involved cutting samples into 1.5 cm thick slices, compressed twice to 60 % of their thickness. Force-time curves were recorded at across-head speed of 5 mms^{-1} and the recording speed was also 5 mms^{-1}. The following parameters were quantified (Bourne 1978): hardness (g), maximum force required to compress the sample, springiness (ratio), the ability of the sample to recover its original form after the deforming force was removed, cohesiveness, the extent to which the sample could be deformed prior to rupture (ratio) and chewiness (g). Also, the work required to masticate the sample before swallowing, which is calculated hardness · cohesiveness · springiness, was measured.

Determination of colour
Colour 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 correspond to lightness, greenness (-a*) or redness (+a*), and blueness (-b*) or yellowness (+b*), respectively. The whiteness (W) was

calculated: L* - 3 b*. The colour measurements were performed on chicken surimi at a room temperature (20 ± 2 ºC).

Statistical analysis

Three determinations for basic chemical composition, cooking loss, pH, a_w, onset (T_o), peak (T) and endset (T_e) temperatures and transition enthalpies (ΔH J g^{-1}), seven for TPA and colour parameters were measured from each sample. Experimental data were analyzed by the analysis of variance (ANOVA) and Fisher's least significant difference (LSD), with significance defined at $p < 0.05$. Statistical analysis was carried out with Statistica ver. 8.0 StatSoft Inc. Tulsa, OK. USA.

RESULTS AND DISCUSSION

The mean basic chemical composition, pH and a_w values of individual chicken surimi samples did not vary significantly and are presented in Table 1.

Differential scanning calorimetry thermogram's of chicken surimi samples for each treatment after 30 days of frozen storage without addition of oat β – glucans contained two endothermic transitions. Referring to previous DSC studies of similar samples (Kijowski and Mast, 1988; Fernandez-Martin, 2007), it can be assumed that two peaks in this study are related to the thermal denaturation of myosin and actin. Values of peak thermal temperatures (T_p) of myosin and actin (Table 2) were different then values of raw chicken breast meat reported by Bircan and Barringer (2002). Similar results were reported by Kijowski and Richardson (1996) for washed mechanically deboned poultry meat. This could be explained by the concentration of myofibrillar proteins by washing and different pH and ionic environment when compared to the raw state of muscle (Lesiow and Xiong, 2001).

Variance analysis of myosin T_p showed that myosin's T_p varied significantly ($p < 0.05$) as a function of mass fraction of oat β – glucans (Table 2). Addition of oat β – glucans ($w = 2 – 6$ %) caused shift of myosin's T_p to higher values. These shifts in T_p of myosin to the higher values as the mass fraction of oat β – glucans increases can be interpreted as a stabilization of myofibrillar proteins since a higher temperature was required to denature these proteins (Sych *et al.*, 1990). Actin's T_p did not vary significantly ($p > 0.05$) as a function of mass fraction of oat β – glucans (Table 3). Highest value of actin T_p showed the sample without addition of oat β – glucans (Table 3).

The method of expressing peak enthalpies ΔH was adopted to provide an estimate of the quantity of native proteins (Sych *et al.*, 1990; Herrera *et al.*, 2001). Enthalpies of myosin and actin denaturation for chicken surimi with addition of oat β – glucans ($w = 2 - 6\%$) are shown in tables 2 and 3. Values of ΔH for myosin and actin showed an increase with the increase of mass fraction of oat β – glucans ($w = 2 - 6$ %). Since the value of denaturation enthalpy is directly related to the amount of native proteins, higher values of ΔH indicate possible stabilization of chicken myofibrillar proteins with addition of oat β – glucans ($w = 2 - 6$ %).

Table 1. Basic chemical composition, a_w and pH of chicken surimi samples

Water w (%)	Proteins w (%)	Fat	Collagen	pH	a_w
84.75 ± 0.28	13.06 ± 0.58	0.73 ± 0.07	0.79 ± 0.01	6.95 ± 0.04	0.98 ± 0.01

Values are means ± Standard deviation of triplicate.

The cooking loss of chicken surimi mixed with different mass fracion of oat β – glucans after 30 days of frozen storage are presented in Figure 1. The additon of oat β – glucans ($w = 2 – 6\%$) caused a
significant reduction ($p < 0.05$) of cooking loss in the obtained gels. Similar results were reported by Stangierski and Kijowski (2004) for mechanically recovered washed and frozen stored poultry meat with the addition of Cremodan and Pork Stock.

Table 2. Values of denaturation temperatures (T_o, T_p, T_e) and denaturation enthalpies (ΔH) of chicken surimi myosin mixed with different mass fracion of oat β – glucans after 30 days of frozen storage.

w (%)	T_o (ºC)	T_p (ºC)	T_e (ºC)	$ΔH_m$ (J g^{-1})
0	58.48a ± 0.09	60.86c ± 0.03	65.36d± 0.05	0.37b ± 0.03
2	58.15a ± 0.23	60.99c ± 0.79	67.51c ± 0.08	0.36b ± 0.02
4	56.09b ± 0.49	61.53b ±0.30	66.59b ± 0.25	0.37b ± 0.01
6	54.73c ± 0.02	62.11c ± 0.03	71.65d ± 0.25	0.49a ± 0.01

Values are means ± Standard deviation of triplicate. Values in the same row with different letters (a-d) are significantly different (p < 0.05).

Table 3 Values of denaturation temperatures (T_o, T_p, T_e) and denaturation enthalpies (ΔH) of chicken surimi samples actin mixed with different mass fracion of oat β – glucans after 30 days of frozen storage.

w (%)	T_o (ºC)	T_p (ºC)	T_e (ºC)	$ΔH_a$ (J g^{-1})
0	72.08b ± 0.04	70.32a ± 0.11	82.63a ± 0.04	0.09a ± 0.01
2	72.26ab ± 0.15	77.14a ± 0.19	80.77c ± 0.11	0.08ab ± 0.01
4	72.47ab ± 0.35	77.19a ± 0.17	80.14d ± 0.03	0.08ab ± 0.01
6	72.65b ± 0.02	77.30a ± 0.15	81.45b ± 0.05	0.09a ± 0.01

Values are means ± Standard deviation of triplicate. Values in the same row with different letters (a-d) are significantly different (p < 0.05).
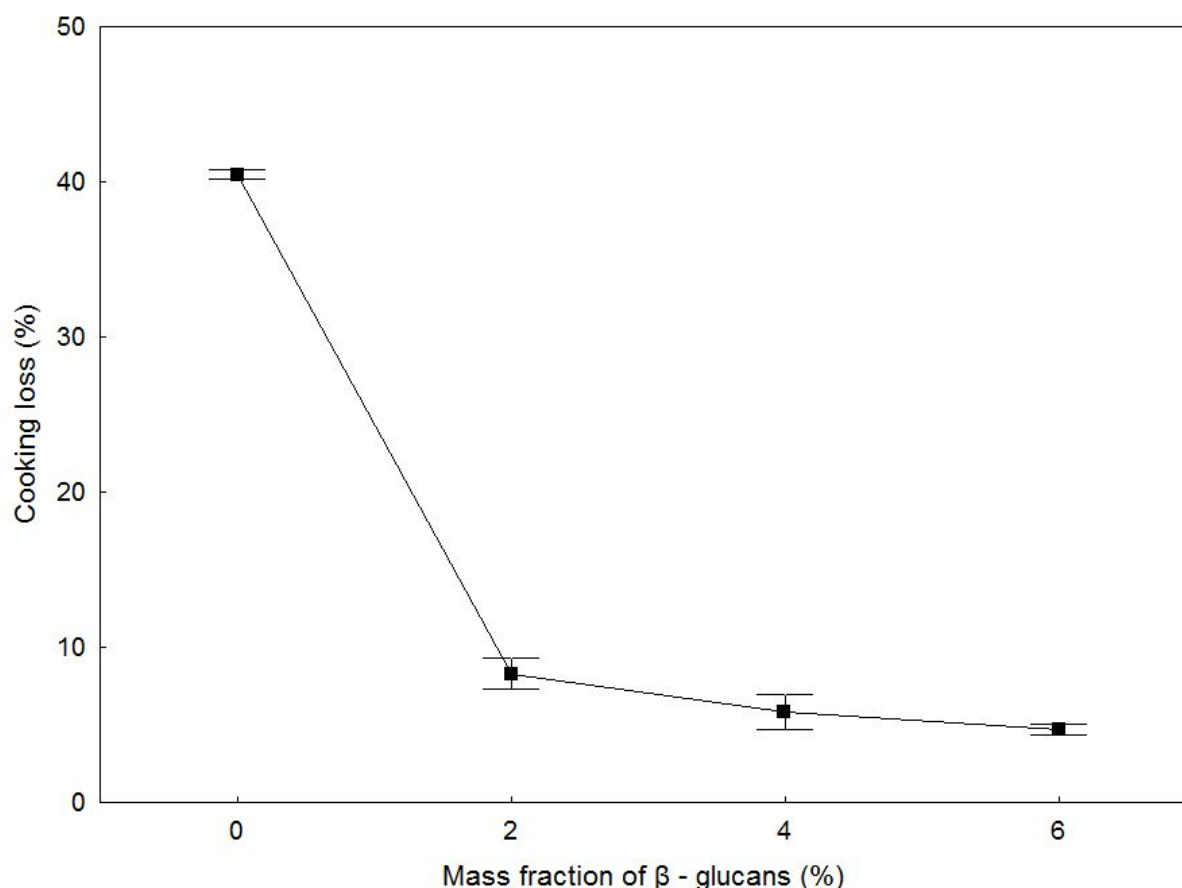
Figure 1. The Cooking loss of chicken surimi samples mixed with different mass fracion of oat β – glucans after 30 days of frozen storage.

Table 4. Texture profile of chicken surimi mixed with different mass fracion of oat β – glucans after 30 days of frozen storage.

w (%)	Hardness (g)	Springiness	Cohesiveness	Chewiness (g)
0	843.59b ± 114.31	0.92ab ± 0.06	0.64a ± 0.04	541.24ab ± 172.41
2	352.34c ± 21.2	0.83b ± 0.11	0.55c ± 0.04	159.92c ± 12.57
4	1081.23ab ± 205.49	0.94a ± 0.04	0.47b ± 0.03	712.73a ± 107.59
6	1323.19a ± 232.98	0.89ab ± 0.06	0.33b ± 0.03	441.44b ± 79.05

Values are means ± Standard deviation of seven measurements. Values in the same row with different letters (a-c) are significantly different (p < 0.05)

Texture profile analysis parametrs of chicken surimi mixed with different mass fracion of oat β – glucans after 30 days of frozen storage are shown in Table 4. The hardness of chicken surimi sampels incresed significantly (p < 0.05) with the increase of mass fraction of oat β – glucans. The cohesiveness and springiness were not affected with the increase of β – glucans mass fraction (Table 4.)

Table 5. Instrumental colour parameters of chicken surimi mixed with different mass fracion of oat β – glucans after 30 days of frozen storage.

w (%)	L*	a*	b*	W
0	77.31b ± 1.13	2.69a ± 0.86	19.75a ± 0.51	18.06b ± 0,27
2	79.06b ± 1.12	1.93b ± 0.63	18.48b ± 0.54	23.62b ± 0,14
4	79.81ab ± 1.23	1.87b ± 0.63	18.76b ± 0.53	23.53b ± 0,33
6	80.71a ± 1.06	1.82b ± 0.78	18.79b ± 0.82	24.34a ± 0.62

Values are means ±Standard deviation of seven measurements. Values in the same row with different letters (a-c) are significantly different (p < 0.05)

Instrumental colour parameters of chicken surimi with addion of oat β – glucans are presented in Table 5. Generally, the demand is higher for surimi gels with high lightness (L*), low yellowness (b*) and high whiteness (W). The addition of β – glucans significantly increased (p < 0.05) lightness and whiteness of chicken surimi samples. This is in agreement with the study that investigated the addition of potato starch and egg white to Alaska Pollock surimi (Tabilo-Munizaga and Barbosa-Canovas, 2004).

CONCLUSIONS

Differential scanning calorimetry (DSC) revealed a shift in peak thermal transition temperature (T_p) of myosin to higher temperature in chicken surimi samples mixed with different mass fraction of oat β – glucans. Shift in thermal transition temperature of myosin to higher temperature, the increase of myosin transition enthalpies and some TPA (hardness, chewiness) and colour parameters (L* and W) with the mass fraction oat β – glucans increase, indicate that oat β – glucans are acting as cryoprotectants and interact with chicken myofibrillar proteins.

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COMPARISON OF TWO MICROBIOLOGICAL METHODS AFTER SWABBING IN SURFACE HYGIENE CONTROL

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ABSTRACT

The important part of food producing and processing is microbiological hygiene. Hygiene monitoring of process surface is necessary for assuring of food quality and protection of consumers.

The swab method is standardized surface sampling method. Otherwise, the total count of bacteria and total count of *Enterobacteriaceae* estimate the level of contamination risk during food processing.

In this study was performed artificial contamination of previously sterilized glass surface. For contamination was prepared suspension of *Escherichia coli* ATCC 25922 adjusted to 3.7 on McFarland scale. Pour plates were made by pipetting of prepared bacterial suspension into an empty petri dish and adding of Tryptone Soya Agar (TSA). After incubation of petri plates at 37 ± 1 °C for 72 ± 3 hours, results were used for estimation of total count bacteria in 1ml of suspension. Each of 20 test surface with an artificially soiled area of 10x10 cm^2 was sampled with the swab and the solutions were sealed with Plate count agar (PCA). Inoculated PCA plates were incubated at 37 ± 1 °C for 72 ± 3 hours. Another 20 test surfaces with an artificially soiled area were swabbed and the solutions were sealed with Violet red bile glucose agar (VRBGA). Inoculated VRBGA plates were incubated at 37 ± 1 °C for 24 ± 2 hours.

Results show that recovery was less than 50% for the count of *Escherichia coli* on PCA as well as on VRBGA.

Keywords: surface, sampling method, swab, Escherichia coli

INTRODUCTION

The important part of food producing and processing is microbiological hygiene. For food processors the hygiene monitoring programs of process surface is necessary for assuring of food quality and protection of consumers. The obtained results are often used for verification of cleaning and sanitation as well as in investigation of foodborne disease outbreak (Sudheesh *et al.*, 2013).

Sampling programs should include the collection of samples during production and the samples should be taken from the sites after sanitizing. In some cases sampling should not only be conducted on food contact surfaces, but the evaluation of non-food contact surfaces (walls, rollers, conveyors,…) could be also important because of aerosols as a way of microorganisms migration. Also microorganisms can be transferred from contaminated hands of food workers (Montville *et al.*, 2002).

The swab method is standardized surface sampling method. Salo et al. (2000) reported that swabbing method gave similar results comparing to contact plate and Hygicult TPC dipslides methods at three microbial levels tested.

Most of food contact surfaces are commonly encountered wet, which can provide a good environment for bacterial growth (Buckalew,1996) and provide a higher bacterial transfer rate than dry surfaces (Marples and Towers, 1979). In case that surface is dry, swabs must be moist with suitable diluents in the prior of use. Because samples should be taken from the site after sanitising, it is usually that the diluent contains one or more neutralizers. The

recommendation of SRPS ISO 18593:2010 (Institute for Standardization of Serbia, 2010) is to use Tween 80, lecithin, sodium thiosulfate, L-histidine and saponin as neutralizers.

The present study was carried out with the aim of ascertaining the efficiency of swabbing as sampling method performed in the enumeration of *Escherichia coli* with the method for a total count of microorganisms and the method for *Enterobacteriaceae.*

MATERIAL AND METHODS

Meterial
- *Escherichia coli* ATCC 25922 (Microbiologics, USA)
- Physiological solution, 8.5g sodium chloride in 1000mL distilled water, autoclaved at 121±1 °C for 15 min,
- Buffered Peptone Water (ISO) (LabM, Bury, UK) containing: enzymatic digest of casein 10.0g, sodium chloride 5.0g, disodium hydrogen phosphate (anhydrous) 3.6g, potassium dihydrogen phosphate 1.5g in 1000mL distilled water, autoclaved at 121±1 °C for 15 min,
- Tryptone Soy Agar (U.S.P) (LabM, Bury, UK) containing: tryptone 15.0g, soy peptone 5.0g, sodium chloride 5.0g, agar no.2 12.0g in 1000mL distilled water, autoclaved at 121±1 °C for 15 min,
- Plate count agar (A.P.H.A) (LabM, Bury, UK) containing: tryptone 5.0g, yeast extract 2.5g, glucose 1.0g, agar no.1 15.0g in 1000mL distilled water, autoclaved at 121±1 °C for 15 min,
- Violet red bile glucose agar (HiMedia, India) containing: peptic digest of animal tissue 7.00g, yeast extract 3.0g, sodium chlodride 5.0g, bile salts mixture 1.5g, glucose 10.0g, neutral red 0.03g, crystal violet 0.002g, agar 12.0g in 1000mL distilled water; heated and boiled, not autoclaved.
- Sterile Petri dishes, ⌀90 mm (Noex, Polad)
- Sterile cottonwool swab sticks (Deltalab, Spain)
- Frames of stainless steel, 100 cm^2 (10x10cm) and 20 cm^2 (4x5 cm)
- Sterile glass Z-shaped rod
- Laboratory glassware – test tubes and bottles
- Laboratory equipment – autoclave, water bath, incubators, vortex mixer, colony counter, pipets, pH meter

Methods
For contamination was prepared suspension of *Escherichia coli* ATCC 25922 in physiological solution and adjusted to 3.7 of McFarland scale. From bacterial suspension were prepared a logarithmic dilution series up to 10^{-8} dilution in tubes with 9 ml buffered peptone water without added neutralizers. Pour plates were made by pipetting of prepared dilutions 10^{-6}, 10^{-7} and 10^{-8} into an empty petri dishes in duplicate and adding of Tryptone Soya Agar (TSA). After incubation of petri plates at 37±1 °C for 72±3 hours results used for estimation of total count bacteria in 1ml of bacterial suspension.

Test glass plates used for surface contamination previously were washed with a neutral detergent and rinsed in distilled water. After drying in oven at 70 °C, glass plates were wrapped in aluminium foil and wet sterilized at 121 °C for 15 minutes.

The frame of stainless steel was put on each of 20 sterilized test glass plates and enclosing an area of 100 cm^2 (10x10 cm) was used for surface contamination. Dilution 10^{-4} of prepared bacterial soil suspension was pipetted onto the enclosed test glass plate in amounts of 300 µm and evenly spread on the surface with a z-shaped rod. Contaminated surface was dried for 5 min, after which sampling was performed using swab. From each contaminated surface was sampled area of 20 cm^2 using a small stainless steel frame (4x5 cm). Each of the swabs was inserted into the test tubes with 10 ml of physiological solution and the sticks were cut off under sterile conditions. The test tubes were mixed well, than a dilution 10^{-1} was prepared for each sample. The undiluted solution and dilution 10^{-1} were sealed using the pour plate technique with Plate count agar (PCA) and Violet red bile glucose agar (VRBGA) in duplicate. According to the method SRPS EN ISO 4833-1:2014 inoculated PCA plates were

incubated at 37±1 °C for 72±3 hours for enumeration of total microorganisms. Another 20 test surface with an artificially soiled area were swabbed and the solutions were sealed with violet red bile glucose agar (VRBGA) for enumeration of *Enterobacteriaceae*. According method SRPS ISO 21528-2:2009 inoculated VRBGA plates were incubated at 37±1 °C for 24±2 hours.

Number of colony-forming units (cfu) was calculated per square centimeter of investigated surface (20 cm^2) as is stated in method SRPS ISO 18593:2010 (Institute for Standardization of Serbia, 2010)

RESULTS AND DISCUSSION

Results of enumeration of prepared bacterial suspension are presented in Table 1. According estimated number of *Escherichia coli* in suspension.

Table 1. Enumeration of Escherichia coli ATCC 25922 from Suspension

Dilution	Number of colony forming units (cfu)	
	Petri Plate 1	**Petri Plate 2**
10^{-6}	278	269
10^{-7}	28	25
10^{-8}	1	1
Estimated count of *Eschrichia coli* ATCC 25922: 2.7 x 10^8 cfu/ml suspension		

Results obtained after performing swabbing of contaminated glass surface and inoculation in PCA and VRBGA are present in Table 2.

Table 2. Results of enumeration of Escherichi coli after swabbing

Colony count of *Escherichia coli* per analysed surface (cfu/cm^2)	
SRPS EN ISO 4833-1:2014	**SRPS ISO 21528-2:2009**
33.0	43.5
28.5	45.0
22.5	44.5
31.5	40.0
36.0	38.0
32.5	39.5
23.0	41.0
32.0	34.0
30.0	46.0
27.5	40.0
27.0	44.0
30.5	43.0
30.0	39.0
23.5	33.0
26.5	40.0
25.0	37.5
25.5	37.5
27.5	39.0
32.0	40.0
30.0	40.5
AV: 28.7	AV: 40.3

AV-average

According to the estimated count of *Escherichia coli* in prepared suspension (Table 1), theoretical expected result after performed experimental design described in Material and Methods is 90 cfu/cm$^{2.}$

Obtained experimental results show higher colony count on VRBGA comparing with PCA, 40.3 cfu/cm^2 and 28.7 cfu/cm^2, respectively. It is obviously that gained results are more than 50% less comparing to theoretical estimation. Reasons for such low recovery can be different and they are including the loss through bacterial suspension spreading with Z-shaped rode and also the influence of the cotton swab. Otherwise, surviving of bacteria on this kind of surface in real condition with various influencing factors is very difficult to predict and could be the direction to serious study. As a requirement of their survival and growth, bacteria attach to the surfaces and form biofilm which is more resistant to cleaning agents and disinfectants than suspension cells. The attachment and biofilm-forming capabilities of bacteria depends on multiple factors including the attachment surface, the presence of other bacteria, the temperature, the availability of nutrients and pH (Van Houdt and Michiels, 2010). Bacterial colonization process is enhanced when nutrients availability is high (Sanin *et al.*, 2003; Myszka *et al*, 2007). Nutrient-rich condition sets the stage for initial surface colonization, while starvation promotes the biofilm maturation process (Myszka *et al.*, 2007; Myszka and Czaczyk, 2009). Several studies have shown that *Escherichia coli* has the capacity to attach to and form biofilms on various surface materials (Castonguay *et al.*, 2006; Rivas *et al*, 2007). Also our experiments used monoculture without considering the possible influence of resident organisms from food-processing environments on the surface colonization of *E. coli*. One recent study showed that resident microflora increased *E. coli* O157:H7 colonization on solid surfaces under static conditions (Marouani-Gadri *et al.*, 2009). Taking into account above discussion, artificial contamination of surface in laboratory condition could not provide real environmental condition of food processing plant and on that way gained results are only direction to better understanding of the efficiency of the swabs as a sampling method.

CONCLUSIONS

The control of surface in food processing plants have to be connected to influence of different environmental factors on the survival and growth of bacteria as well as the research of the efficiency of applied sampling methods. Artificial contamination of surface in laboratory condition could not provide real environmental condition and experimental design has to give attention to the formation of biofilm. However, our performed experiments showed a difference in enumeration of *Escherichia coli* with two different methods after surface swabbing and recovery was less than 50% for each of them.

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THE INFLUENCE OF PARTIAL REPLACEMENT OF MECHANICALLY DEBONED CHICKEN MEAT WITH CHICKEN LIVER ON PROXIMATE COMPOSITION AND COLOUR OF COOKED SAUSAGES

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ABSTRACT

The effects of partial replacement of mechanically deboned chicken meat - MDCM with chicken liver on proximate composition (moisture, protein, total fat, total ash) and colour characteristics (CIE *L*a*b**) of cooked sausages were investigated. The control sausage was formulated with 60% of MDCM. In experimental cooked sausage samples tree levels of chicken liver were incorporated 5, 10 and 15%, as partial replacement of MDCM. Regarding proximate composition, control was numerically (P>0.05) the highest in moisture content, lowest in protein content, and not different in fat content, comparing with experimental sausages. Different contents of liver addition did not significantly (P>0.05) affect protein content; moisture content was significantly (P<0.05) the lowest in sample with 5% of liver, fat content was significantly different (P<0.05) between samples with 5% and 15% of liver addition, and total ash content was significantly different (P<0.05) among all three experimental sausages. Regarding colour characteristics, control had significantly (P<0.05) the highest lightness (*L**), and lowest redness (*a**) and yellowness (*b**), comparing with experimental sausages. Different contents of liver addition did not significantly (P>0.05) affect lightness (*L**), while the significant (P<0.05) differences were noted for redness (*a**) among all three sausages, and for yellowness (*b**) between samples with 5 and 10% of liver.

Keywords: cooked sausage, mechanically deboned chicken meat, liver, proximate composition, colour

INTRODUCTION

Consumption of poultry meat and poultry meat products is growing all over the world (Mielnik *et al.*, 2002). Poultry is the world's second most consumed type of meat, with chicken meat dominating the world poultry consumption over 70%. Currently, the annual worldwide growth rate is about 5% (Somsen *et al.,* 2004). For several reasons, people prefer this kind of meat to beef and pork, at least partly due to the desirable flavour of poultry products and its comparative low price (Mielnik *et al.*, 2002; Sallam, 2007). The preparation of various chicken products, such as special cuts, sausages and others, is followed by substantial increase in residues such as bones or cut-up chicken parts with adhering meat that still generate the raw material, suitable for mechanical deboning (Daros *et al.*, 2005; Savadkoohi *et al.*, 2013). The mechanical deboning process is an efficient method of harvesting meat from parts left after hand deboning and from poor quality poultry (Pereira *et al.*, 2011). This process allows recovery of most of the residual meat that is otherwise wasted and increases costs (Savadkoohi *et al.*, 2013). The most used raw materials for mechanically deboned poultry meat (MDPM) are back, neck and thighs either from the initial carcass or after removal of most of the meat (Daros *et al.*, 2005). Yield of MDPM ranges from 55 to 80% depending on the part deboned and deboner settings. MDPM has good nutritional and functional properties, of which most interested are jelly consistency, water retention and ability to emulsify fat, what makes is suitable for the formulation of many meat products (Mielnik *et al.*, 2002). Due to these good technological characteristics (fine consistency) and relatively low cost it is frequently used in the formulation of comminuted meat products

(Mielnik *et al.*, 2002). The use of MDPM in the sausage formulation is considered recent in the food industry since it only started in the 60's. The main applications of MDPM are in sausages and salamis, which demand emulsion stability, and also benefit from the MDPM natural colour (Daros *et al.*, 2005).

Today, economic, modern technology and industrial concern for the environment results in maximum salvage and utilization of all by-product materials (Romans *et al.*, 1994). In the last few decades, the amount of available meat by-products from slaughterhouses, meat processors and wholesalers has increased considerably (Darine *et al.*, 2010). From a general perspective, food processors face increasing demands to improve their raw material yield, so as broiler processing companies, because the raw material costs are a considerable part of the overall business costs (Somsen *et al.*, 2004). Preparation of mechanically deboned meat presents one way of efficient economical use of animal products and reduction of the amount of biological wastes (Pussa *et al.*, 2009). On the other hand, many meat edible by-products are down-graded because of the lack of a profitable market. Since the yield of edible by-products for chickens is from 5 to 6% of the live weight; more attention should be given to these products, especially because the majority of by-products offer a range of foods which are nutritionally attractive, with high protein content and good nutritional properties due to the presence of many essential nutrients, and have a wide variety of flavors and textures (Darine *et al.* 2010; Ockerman and Hansen 1988; Spooncer, 1988). Additionally, chicken by-products are low in cost, have low content in fat and the short period of time needed in preparation (Alvarez-Astorga *et al.*, 2002).

Chicken liver, an edible meat by-product which presents main part of red giblets which constitute about 4.36% of live weight in the chicken, is not processed and many consumers have a negative perception, considering it an inferior source of protein as compared to lean chicken meat. As a result, chicken meat has been underutilized and low-priced compared to beef and lamb livers (Hasapidou and Savvaidis, 2011; Somsen *et al.*, 2004).

Sensory properties, such as colour and texture are important for consumer acceptance, choice of food products and, consequently, the manufacturer. Therefore, many studies were taken to optimize and improve these characteristics in various foods (Daros *et al.*, 2005). Some of these studies indicate that mechanically deboned meat (MDM) could be used to manufacture meat formulated sausages with a positive effect on the desired colour, leading to improved attractiveness to consumers. Also, addition up to 30% of MDM to salami results in a better colour and texture of the product (Savadkoohi *et al.*, 2013). Process of mechanical deboning generally releases heme and lipid components from bone marrow and this may increase the content of hemoprotein pigments in MDPM up to three times higher than those in manually deboned meats. Higher heme contents affect the colour of MDCM, thus making it both redder and darker (Shahidi *et al.*, 1992).

Thus, the objective of this study was to investigate the effect of partial replacement of mechanically deboned chicken meat with an equal weight (5, 10 and 15 kg) of chicken liver on proximate composition and colour characteristics of emulsion-type cooked sausages formulated from MDCM.

MATERIAL AND METHODS

Preparation of Sausages
MDCM was produced from breasts (30%), after removal of most meat, backs (50%), necks (10%) and thighs (10%) in a commercial processing plant. Yield of MDCM was 67%. Cooked sausages (emulsion-type product) were prepared according to standard methodology and techniques. The main mixture consisted of 60 kg MDCM, 10 kg vegetable fat, 25 kg ice/water, 3 kg maize starch, 2 kg textured soy protein, 1.6 kg nitrite salt, 0.1 kg dextrose, 0.3 kg polyphosphate, 0.05 kg antioxidant and 0.5 kg spice mix. Experimental sausages were prepared by replacing 5, 10 and 15 kg of MDCM, in main mixture, with equal weight of chicken liver (L–5, L–10 and L–15 sausages, respectively). The emulsified materials were stuffed into artificial cellulose casings (diameter of 65 mm) and were then cooked until an internal temperature of 71 °C was reached. Immediately after the heating process sausages

were cooled with combination of water/air cooling for 45 min (till internal sausage temperature was reduced to 25 °C), followed by air cooling in the chamber. The sausages were stored at +3 °C until analysis.

Proximate Composition
Moisture, protein, total fat and total ash contents of cooked sausages were determined according to methods recommended by International Organization for Standardization (ISO 1442; ISO 937; ISO 1443; ISO 936, respectively). All analyses were performed in duplicate. A strict analytical quality control programme was applied and the results of the analytical quality control programme for proximate composition are presented in Table 1.

Table 1. Results of the Analytical Quality Programme (N=8) Used in the Determination of the Proximate Composition of Cooked Sausages

Quality control	Moisture	Nitrogen	Fat	Ash
Certified concentration (g/kg)	688 ± 2.6	16.3 ± 0.6	143 ± 5.0	26.5 ±1.0
Recovery (%)	99.6	100.4	99.7	100

Colour
The surface colour of fresh sausage cut was measured using a tristimulus colorimeter Minolta Chroma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan) using D-65 lighting, a 2° standard observer angle and an 8mm aperture in the measuring head. Colour characteristics are expressed by *CIE L*a*b** system (lightness-*L**, redness and greenness-*a**; yellowness and blueness-*b**) (CIE, 1976).

Statistical analysis
All data are presented as average and standard deviation. Analysis of variance (Duncan's multiple range test) were used to test the hypothesis about differences between two or more average values. The software package STATISTICA 12.0 was used for analysis (StatSoft, Inc., 2012).

RESULTS AND DISCUSSION

Proximate compositions of the control and experimental sausages are presented in Table 2. Comparing with the control sausage replacement of MDCM with equal weight of chicken liver only slightly affected the proximate composition of the final product. Control sausage was numerically the highest in moisture content (71.46 g/100g), and only significantly ($P<0.05$) higher comparing to L–5 (69.76 g/100g). Protein content was numerically the lowest for control sausage (9.21 g/100g), and significantly lower ($P<0.05$) comparing to L–5 (10.04 g/100g) and L–10 (10.36 g/100g) sausages. Content of total fat determined in control sausage (12.99 g/100g) didn't significantly differ ($P>0.05$) from experimental sausages. Control sausage was significantly higher in total ash content (2.82 g/100g) comparing to L–10 (2.27 g/100g) sausage. Regarding different amounts of chicken liver used as replacement for MDCM in sausage production L–5 sausage was significantly the lowest in moisture content comparing to L–10 and L–15, and significantly higher in total fat content comparing to L–15. Protein content didn't significantly differ ($P>0.05$) between experimental sausages, while total ash content significantly ($P<0.05$) differ between them. Mechanical deboning of poultry affects the proximate composition of resulting meat. Depending on the raw material used for deboning moisture content can varied from 60.0 to 72.2 %, protein content from 8.5 to 13.4% and total fat content from 14.4 to 30.4%. Moreover, the proximate composition can vary according to the settings and type of machine used for the mechanical separation, but generally the lipid content of the MDCM is really higher and the protein content is lower in comparison to fillets (Trindade *et al.*, 2004). Higher moisture content in control sausages could possible resulted from the fact that MDCM has higher ability to retain water (Daros *et al*, 2005). Results of Daros *et al.* (2005) and Pereira *et al.* (2011) showed that moisture

content of cooked sausages, produced from beef and pork and with different percent of MDCM addition, increased with increased content of MDCM, as a consequence of the higher ability of the MDPM, than meat, to retain water. On the other hand, the increase of protein content in experimental sausages could be a consequence of the chicken liver inclusion, since poultry liver is high in protein content (17.97% for chicken liver and 21.90 for turkey liver), and represents an important source of protein with functional characteristics that are related with different protein fractions and with physicochemical conditions (Zouari *et al.*, 2011, Hasapidou and Savvaidis, 2011).

Table 2. Proximate composition of control and experimental sausages

Component	Treatment			
(g/100g)	Control	L–5	L–10	L–15
Moisture	71.46 ±0.07^A	69.76 ±0.23^{B,b}	70.93 ±0.08^{A,a}	71.35 ±0.70^{A,a}
Protein	9.21 ±0.04^C	10.04 ±0.38^{AB,ns}	10.36 ±0.87^{A,ns}	9.42 ±0.23^{BC,ns}
Total fat	12.99 ±0.42^{AB}	13.31 ±0.52^{A,a}	13.02 ±0.04^{AB,ab}	12.27 ±0.51^{B,b}
Total ash	2.82 ±0.12^{AB}	2.84 ±0.07^{A,a}	2.27 ±0.20^{C,c}	2.62 ±0.09^{B,b}

^{A,B,C} indicates significant difference within raw at P<0.05 (including control in the statistical analyses).
^{a,b,c} indicates significant difference within raw at P<0.05 (excluding control in the statistical analyses).

Results obtained for proximate composition of control and experimental sausages were in agreement with results for emulsion-type sausages produced from 100% MDPM reported by Mielnik *et al.* (2002), Daros *et al.* (2005) and Pereira *et al.* (2011).

Table 3 shows the changes in colour characteristics of control and experimental sausages containing different amounts of chicken liver. The colour of cooked sausages is influenced by fat content, added water and pigmentation of the meat with which they are made (Pereira *et al.*, 2011). Comparing with the control sausage replacement of MDCM with equal weight of chicken liver significantly affected the colour of the final product. Control was significantly the highest (*P*<0.05) in *L** value, i.e. control had the lightest colour comparing to experimental sausages. According to Pereira *et al.* (2011) results for *L** values could be due to fat–protein interactions and the emulsion stability during meat emulsion manufacturing, becouse low emulsion stability can be accompanied with reduced *L** values during emulsification process. On the other hand, MDCM has a high pH value (around 6.2–6.5) and, therefore, has a strong negative impact on the lightness of product colour comparing to sausages made from meat (Pereira *et al.*, 2011). Further, control was significantly the lowest in *a** value (15.20). Thus, the redness (*a**) values increased with the addition of chicken liver resulting in a darker red colour. Also, control was significantly the lowest in *b** values (11.64), comparing to experimental sausages.

Table 3. Colour characteristics of control and experimental sausages presented in CIE L*a*b* system

Colour	Treatment			
characteristics	Control	L–5	L–10	L–15
L* (D65)	68.75± 0.29^A	66.52±0.85^{B,ns}	66.83±0.32^{B,ns}	66.66±0.69^{B,ns}
a* (D65)	15.20±0.09^D	15.74±0.12^{B,b}	15.85±0.12^{A,a}	15.60±0.21^{C,c}
b* (D65)	11.64±0.07^C	12.73±0.41^{A,a}	12.53±0.07^{B,b}	12.66±0.17^{AB,ab}

^{A,B,C,D} indicates significant difference within raw at P<0.05 (including control in the statistical analyses).
^{a,b,c} indicates significant difference within raw at P<0.05 (excluding control in the statistical analyses).

Different amounts of chicken liver used as replacement for MDCM in sausage formulation didn't significantly (*P*>0.05) affect the *L** value of the final product, but significantly affected *a** and *b** values. Redness of the experimental sausages ranged from 15.60 (L–15) to 15.85 (L–10) with significant (*P*<0.05) differences between samples. Sausages L–5 and L–10 differed significantly in *b** value (12.73 and 12.53, respectively). Results obtained for colour characteristics showed that control and experimental sausages were darker and redder than low fat chicken sausages produced from breast meat as reported by Andres et al. (2006)

(from 81.2 to 83.2 for *L** value; from 2.66 to 3.47 for *a** value), while the *b** value were on the same level (11.4 to 12.3). Further, results obtained for *L** value were at the same level comparing to fresh sausages produced from chicken tenderloin, and *a** values were higher than reported by Liu et al. (2009).

CONCLUSIONS

Different amounts of chicken liver (5, 10 and 15 kg) addition, as replacement for equal weight of MDCM, in cooked sausages formulated from MDCM, affected proximate composition and colour characteristics of final product. Regarding proximate composition protein content was increased, and moisture content decreased with liver addition. Regarding colour characteristics differences were notable between control and experimental sausages, as well as between experimental sausages with different amount of added liver. At the liver addition weight of 10 kg, studied in this experiment, sausages tended to be significantly ($P<0.05$) the highest in *L** and *a** values, comparing to other two experimental sausages.

ACKNOWLEDGEMENTS

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THE GROWTH RATE DISPERSION OF SUCROSE CRYSTALS

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ABSTRACT

Crystallization is a complex kinetic process aimed at the separation of the sucrose crystal from a liquid mixture. It is an exothermic process, which consists of nucleation and crystal growth. In industrial sugar crystallization, nucleation is avoided by introducing crystalline centers by seed crystals produced in different ways.

In this study, the measurement of size distribution was applied for determination of sucrose crystal growth rate in pure solutions in a batch cooling crystallizer. The sucrose crystal growth rate was estimated using the theory of molecular diffusion according to the two-step model.

Under the same conditions of supersaturation, temperature and hydrodynamics of solution, different crystals grew at different rates. This phenomenon was designated as growth rate dispersion. It was shown that the dependence of the crystal mass growth rate on the crystal size followed linear dependency. It was in accordance with the size-dependent growth model. During cooling crystallization maximum of mass distribution was shifted to higher values of crystal size and increased the distribution widths. Findings indicated that spread of sucrose crystal size distribution during cooling was a consequence of growth rate dispersion. The Rosin-Rammler-Sperling-Bennet model fitted the crystal size distribution data well. The achievement of a narrower size distribution in the final product is of great practical importance in industrial sugar crystallization.

Keywords*: sucrose crystal, cooling crystallization, size distribution, growth rate dispersion*

INTRODUCTION

Crystallization from solution is one of the oldest industrial separation processes widely developed in chemical, pharmaceutical and food industries. It is carried out as a large scale continuous or batch operation in sugar beet and sugar cane industry. The aim of the crystallization process is to achieve the highest sugar quality and yield, the highest exhaustion of molasses, the lowest energy consumption together with the most effective use of equipment and time.

Crystallization is an exothermic process, which consists of nucleation and crystal growth. In industrial sugar crystallization, nucleation is avoided by introducing crystalline centers by slurry as material for seeding (Wittenberg *et al.*, 2000).

A unified crystal growth theory does not exist. Complementary theories were developed, each dealing with a single aspect of the crystal growth (Sgualdino at al., 1996). According to the diffusion theory, sucrose crystal growth includes a diffusion process of molecules through the stagnant layer and the reaction process corresponding to the integration of molecules in the crystal structure through the adsorption layer. Classical crystallization theory considers the sucrose concentration gradient as the driving force for crystal growth [Poel *et al.*, 1998].

The process of cooling crystallization is based on the decreasing sucrose solubility in solutions as the temperature drops. Thus, the supersaturation required for crystallization is produced by removing heat from the crystallizing solution.

The current work is targeted towards investigation of crystal growth rate dispersion in pure sucrose solutions during batch cooling crystallization. The understanding of crystal size distribution variation is an important aspect of crystallization processes.

MATERIAL AND METHODS

The growth of crystals from pure sucrose solutions was studied in a batch cooling crystallizer using a Coulter Counter technique. Solutions were prepared by dissolving granulated refined sugar in distilled water. Solutions were kept for one hour undersaturated at 85 °C before cooling. The growth process was initiated by the addition of fixed amount of sucrose seeds i.e. slurry, which was prepared by wet-milling sugar/isopropanol mixture in a ball mill. The cooling crystallization was conducted from the point of seeding at 50 °C to the final temperature of 25 °C, according to the defined program.

The quality of solutions was determined according to the methods published in the manual for laboratory control of the process in sugar factories (Milić *et al.*, 1992). Changes in crystal number and size were followed by a Coulter Counter, model ZM, England. It is a precise dual threshold, particle counting and sizing instrument for materials in the range of 0,4 – 800 μm, based on measurement of electrical conductivity (Grbić *et al*, 2008).

The Rosin-Rammler-Sperling-Bennet (RRSB) method was applied for evaluation of the main distribution parameters: mean size and uniformity of crystals (ICUMSA, 2007). It is based on an exponential function to describe the particle size distribution of sugar that does not have a normal distribution. The RRSB function is given by:

$$D(d) = 1 - \exp\left[- (d/d')^n\right] \tag{1}$$

where is: $D(d)$ – cumulative mass fraction transmitted as function of particle equivalent diameter in g/g; d – particle equivalent diameter in mm; d' – sieve aperture in mm corresponding to $D = 0,632$; n – slope of RRSB function.

Crystal growth rate was determined as a mass growth rate based on measuring crystal size distributions in solutions. The mathematical model considered two mass transfer steps which can be described as:

$$R_G = dm / A \cdot dt \tag{2}$$

where is: R_G – mass growth rate (mg/m^{2}min), m – crystal mass (mg), A – crystal surface area (m^2), t – time (min).

RESULTS AND DISCUSSION

Parameters of pure sucrose solutions are presented in Table 1. Supersaturation of all solutions was in metastable zone. Seeding was carried out by slurry in amount of 0,2 % (w/w). Particle size distribution of slurry is presented in Figure 1.

Table 1. Quality parameters of pure sucrose solutions

Parameters	Temperature (°C)			
	50	**45**	**35**	**25**
Dry substance (%)	73,60	73,00	71,60	70,60
Supersaturation	1,07	1,09	1,11	1,15
Cooling rate (°C/min)	-	0,05	0,17	0,17
Mean crystal size (μm)	17,5	57	97	120
Uniformity coefficient	1,35	2,00	2,05	1,80
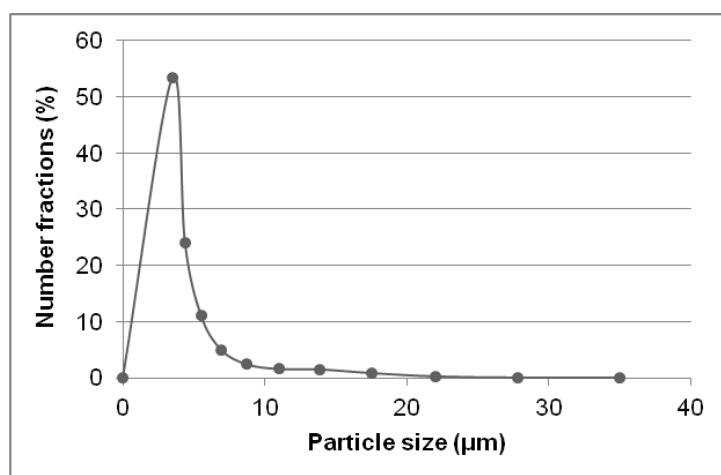
Figure 1. Number distribution of particles size in slurry

Determination of sucrose crystal growth rate showed that: crystals of different size grew at different rate and the growth rate increased with the crystal size under the same external conditions. The dependence of the crystal mass growth rate on the crystal size during cooling process followed linear dependency (Grbić *et al.*, 2014). The linear coefficients of correlation between the growth rate and the crystal size were 1 in all systems. Crystal size distributions followed the Rosin-Rammler-Sperling-Bennet function. Figure 2 presents the growth rate dispersion at the different temperatures.
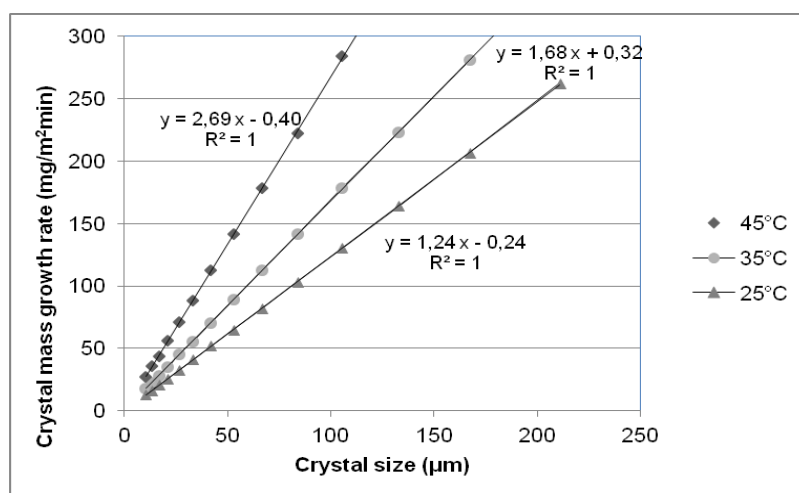
Figure 2. Sucrose crystal mass growth rate as a function of crystal size

Variation in mass distributions of sucrose crystal size during cooling crystallization is presented in Figure 3. Mass distribution maximum was shifted to higher values of crystal size and the distribution width was increased. The increasing spread of the crystal size distribution during time of cooling was a predictable consequence of the growth rate dispersion and size dependent growth. Growth rate dispersion has a large effect on the formation of crystal size distribution (Iswanto *et al.*, 2007). The changes of mass fractions of crystals size in range: 9 - 37 µm, 37 – 93 µm and > 93 µm are presented in Figure 4.
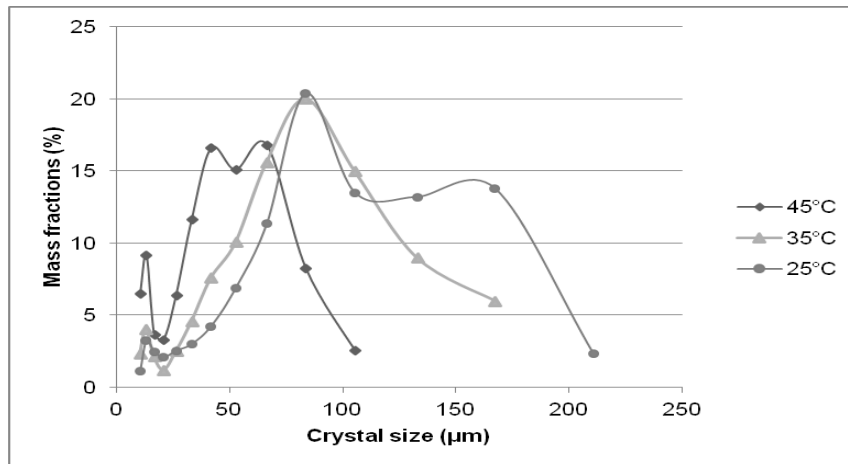
Figure 3. Mass distributions of sucrose crystal size
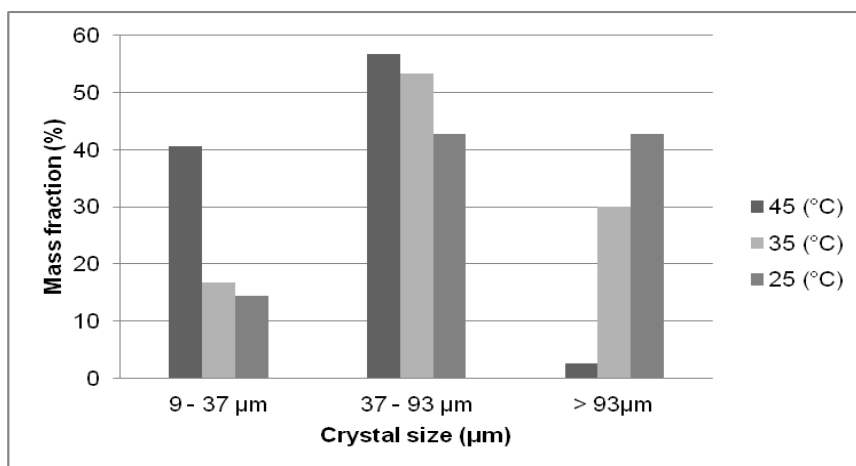
Figure 4. Histogram of sucrose mass distribution

Figure 5 presents cumulative mass distribution curves of sucrose crystal. These curves are useful tools for crystal size evaluation. It is presumed that 0 % of cumulative mass fraction can be achieved for the largest crystals and 100 % for the smallest crystals, which is similar to the sieving analysis applications. Results show changes in the crystal size, their shifting to higher values and increasing the distribution widths during time of cooling. Distributions of crystals followed the Rosin-Rammler-Sperling-Bennet function.
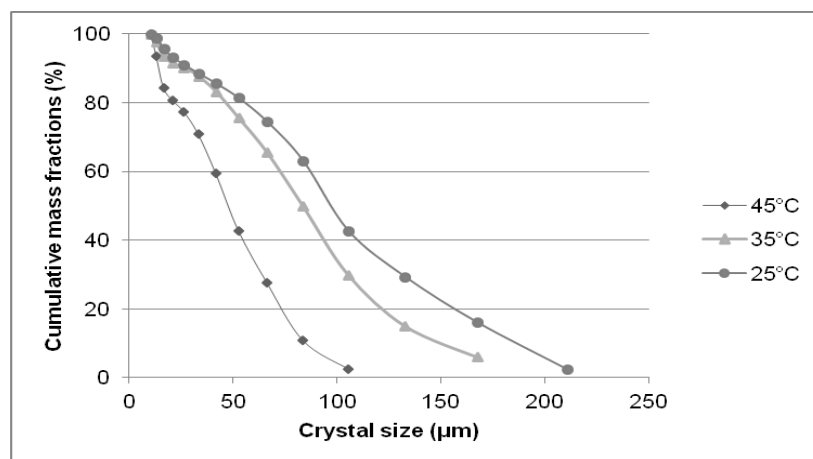
Figure 5. Cumulative mass distribution curves of sucrose crystal

CONCLUSIONS

In the present work, the sucrose crystal growth, obtained by cooling crystallization of pure solutions, was investigated. Based on the results of this study, the following conclusions were made:
- The crystal growth rate increased with the crystal size under the same external conditions.
- The dependence of the crystal mass growth rate on the crystal size followed linear dependency which was confirmed by high values of correlation coefficients in all systems.
- The shifting of mass distribution maximum to higher crystal values were followed by distribution spread from point of seeding to the final cooling temperature.
- Crystal size distributions followed the Rosin-Rammler-Sperling-Bennet function.

ACKNOWLEDGEMENTS

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OSMOTIC DEHYDRATION OF CHICKEN MEAT IN SUGAR BEET MOLASSES

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ABSTRACT

Osmotic dehydration is an environmentally acceptable, material gentle drying method, which received considerable attention because of the low processing temperature, base waste material and low energy requirement. The use of sugar beet molasses as an osmotic medium has proven to be good solution for several plant and animal materials, but there is no literature data of dehydrating chicken meat in molasses.
The goal of this research is to investigate the effectiveness of the process of osmotic dehydration of chicken meat in sugar beet molasses.
The investigated parameters of the process were: time and temperature of the osmotic process and concentration of the sugar beet molasses, while the monitored responses of the osmotic dehydration process were: dry matter content, water loss, solid gain, dehydration efficiency index and water activity.
The highest results obtained of DMC 68.69%, WL 0.5810 $g/g_{i.s.}$, SG 0.1492 $g/g_{i.s.}$ and a_w 0.809, were at maximal process parameters of time, temperature and concentration of the process of osmotic dehydration, since the higher values of process parameters induced the higher mass transfers between molasses as an osmotic solution and chicken meat as a dehydrating medium.
Keywords: osmotic dehydration, sugar beet molasses, chicken meat

INTRODUCTION

Many traditional techniques and their combinations, such as salting, drying, cooking, smoking and marinating are used to prevent spoilage of meat and its products by reducing its water content. A common step in these processes is placing product (meat) in contact with a concentrated solution (salt, sugar, acids, seasonings etc) (Colignan *et al.*, 2001).
One of the potential preservation techniques for producing products with low water content and improved nutritional, sensorial and functional properties is osmotic dehydration. This technology promotes partial removal of water from food by immersion in a concentrated hypertonic solution. The driving force for the diffusion of water from the plant tissue into the concentrated solution is provided by the high osmotic pressure of the solution. The diffusion of water, as the primary mass transfer, is accompanied by the simultaneous counter-diffusion of solute(s) from the osmotic solution into the meat tissue, which is considered as the secondary mass transfer. 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).
Osmotic dehydration is recognized as a pre-treatment step to meat drying processes such asair-drying, microwave or freeze-drying, to improve the nutritional, sensorial and functional properties of meats, reduce heat damage and minimize their colour and flavour changes (Rastogi *et al.,* 2002).
Osmotic dehydration is an environmentally acceptable method, with its ultimate aim for keeping the initial characteristics of the final product, also material gentle drying method, which received considerable attention because of the low processing temperature, low waste material and low energy requirements (Panagiotou *et al.*, 1999; Waliszewiski, *et al.*, 1999).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 (Della Rosa and Giroux, 2001;Torreggiani, 1993).

Sugar beet molasses is an excellent medium for osmotic dehydration, primarily due to the high dry matter (d.m.) of 80 % and specific nutrient content. From nutrient point of view, an important advantage of sugar beet molasses use as hypertonic solution is enrichment of the food material in minerals and vitamins, which penetrate from molasses into the plant tissue (Koprivica *et al.,* 2008; Koprivica *et al.,* 2009). The presence of complex solute compositions maintains a high transfer potential favourable to water loss, and at the same time by the presence of sugar, salt impregnation is hindered (Santchurn *et al.,* 2007). High salt concentrations decrease the water holding capacity, which contributes to meat dehydration and shrinkage while there is no swelling of muscle fibres or myofibrils (Barat *et al,.* 2009; Alino *et al.,* 2010).

Sensory analysis of the previously researched dehydrated pork meat in molasses has shown that meat had satisfactory sensory characteristics, with the positive impact on taste and natural colour of meat. Alteration of the aroma and taste was not too expressed, so it could be corrected in further steps of technological operations (Nićetin *et al.,* 2012).

There has been no previous research of the process of osmotic dehydration of chicken meat in sugar beet molasses.

The goal of this research was to investigate the effectiveness of the process of osmotic dehydration of chicken meat in sugar beet molasses as an osmotic solution of different concentrations, at different temperatures and different times of the process.

MATERIAL AND METHODS

Raw skinless chicken breast was purchased at the lokal shop in Novi Sad, just before use. Before the osmotic treatment, whole muscle, (24h post mortem,with removed fat tissue), was cut into cubes, dimension 1x1x1 cm, and then homogenized before the samples were taken for the process. Sugar beet molasses, with inital dry matter content of 85.04 %, was obtained from the sugar factory Crvenka, Serbia. Distilled water was used for dilution of molasses. The diluded molasses concentrations were 60, 70 and 80 % w/w. The sample to molasses ratio was 1:5 (w/w). The process was performed in laboratory jars at temperature of 20, 35 and 50 °C under atmospheric pressure, in constant temperature chamber (KMF 115 l, Binder, Germany). Process temperatures were chosen according to osmotic dehydration temperatures for other animal raw materials (Filipović, 2013; Filipović *et al.*, 2013; Ćurčić *et al.*, 2014). Meat samples were stirred every 15 minutes. Processing conditions regarding stirring, intesity, duration and frequency, were the same for all concentrations of molasses at all temperatures, so the results could be comparable.

After 1, 3 and 5 hours the samples were taken out from molasses to be lightly washed with water and gently blotted to remove excessive water. Times of the process were chosen according to osmotic dehydration times for other animal raw materials (Filipović, 2013; Filipović *et al.*, 2013; Ćurčić *et al.*, 2014). Dry matter contents of the fresh and treated samples, and molasses were determined by drying at 105 ºC in a heat chamber until constant mass was achived (Instrumentaria Sutjeska, Srbija). All analytical measurements were carried out in accordance to AOAC (2000).

Water activity (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 25 °C.

In order to describe the effectiveness of the mass transfer of the osmotic dehydration process, dry matter content (DMC), water loss (WL), solid gain (SG) and dehydration efficiency index (DEI) were calculated for different temperatures (T), processing times (τ) and concentration (C) of sugar beet molasses and presented as mean values and standard deviation of three parallel runs:

$$DMC = \frac{m_d}{m_i} \cdot 100\% \tag{1}$$

$$WL = \frac{m_i z_i - m_f z_f}{m_i} \left[\frac{g}{g_{\text{initial sample (i.s.)}}} \right] \tag{2}$$

$$SG = \frac{m_f s_f - m_i s_i}{m_i} \left[\frac{g}{g_{\text{i.s.}}} \right] \tag{3}$$

$$DEI = \frac{WL}{SG} \tag{4}$$

where m_i and m_f are the initial and final mass (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 (Filipović *et al.*, 2013).

RESULTS AND DISCUSSION

In the table 1 the results of the process responces (DMC, WL, SG, DEI and a_w) at three different T, т and C of the process of osmotic dehydration of chicken meat in molasses are shown.

DMC and a_w values of the raw chicken meat were: 24.92±0,48% and 0.936±0.01 respectively.

The increase of T of the process has influenced statistically significant increase in DMC values of osmodehydrated meat.

The increase of т of the process while other process parameters were constant has also statistically significantly increased obtained DMC values of osmodehydrated chicken meat.

Increase of molasses concentration has shown statistically significant effect on increase of the DMC values of dehydrating chicken meat.

Maximal obtained DMC values were 68.69±0,62 % at T of 50°C, after 5h process in concentrated molasses of 80%. The level of the maximal obtained DMC values were the same as in case of other osmotic dehydrations of different types of meats (71.11% of osmodehydrated pork meat and 66.30% of osmodehydrated fish meat) (Pezo *et al.* 2013; Ćurčić *et al.* 2014).

Increasing all three process parameters, both mass transfers between dehydrating chicken meat and molasses as an osmotic solution has intensified, allowing higher obtained DMC values.

The increase of T of the process has statistically significantly influenced on the increase of WL values of osmodehydrated meat, the same as in case of DMC.

The increase of т of the process while other process parameters were constant has also statistically significantly increased obtained WL values of osmodehydrated chicken meat, again the same as in case of DMC.

Increase of molasses concentration has shown statistically significant effect on increase of the WL values of dehydrating chicken meat.

Maximal obtained WL values were 0.5810±0.0034 g/g_{i.s.} at the same process parameters as in case of DMC: T of 50°C, т of 5h, C of 80%. The level of the maximal obtained WL values were, again, the same as in case of other osmotic dehydrations of different types of meats (0.58 g/g_{i.s.} of osmodehydrated pork meat and 0.57 g/g_{i.s.} of osmodehydrated fish meat) (Pezo *et al.* 2013; Ćurčić *et al.* 2014).

By increasing all three process parameters, the main mass transfer of water between dehydrating chicken meat and molasses has intensified, providing higher achived WL values.

The increase of T of the process has led to the increase of SG values of osmodehydrated meat, but not statistically significantly.

The increase of т of the process while other process parameters were constant has statistically significantly increased obtained SG values of osmodehydrated chicken meat, the same as in case of DMC and WL.

Table 1. Values of the osmotic dehydration process responses of chicken meat in molasses

T (°C)	τ (h)	C (% d.m.)	DMC (%)	WL (g/g$_{i.s.}$)	SG (g/g$_{i.s.}$)	DEI	a$_w$
20	1	60	40.77 ±1.76^a	0.2856 ±0.0140^a	0.0905 ±0.0025^{ab}	3.15 ±0.07^{abc}	0.904 ±0.009^a
20	3	60	51.08 ±0.98^b	0.4147 ±0.0068^b	0.1129 ±0.0110^{bcde}	3.69 ±0.30^{bcdefg}	0.897 ±0.011^a
20	5	60	54.58 ±0.24^{bc}	0.4506 ±0.0016^c	0.1225 ±0.0108^{cdefg}	3.70 ±0.31^{bcdefg}	0.885 ±0.006^{ab}
35	1	60	42.03 ±0.59^{ad}	0.2815 ±0.0048^a	0.1033 ±0.0044^{abcd}	2.73 ±0.07^{ad}	0.912 ±0.0012^a
35	3	60	52.29 ±3.03^{be}	0.4009 ±0.0220^{bd}	0.1176 ±0.0046^{bcdef}	3.41 ±0.05^{abcdef}	0.897 ±0.001^{ac}
35	5	60	56.84 ±0.04^{cf}	0.4549 ±0.0002^c	0.1183 ±0.0006^{bcdef}	3.85 ±0.02^{bcdefg}	0.880 ±0.014^{ad}
50	1	60	45.06 ±1.00^{dg}	0.3286 ±0.0077^e	0.0802 ±0.0140^{ag}	4.17 ±0.64^{fg}	0.900 ±0.006^a
50	3	60	56.93 ±0.63^{ch}	0.4603 ±0.0042^{cf}	0.1306 ±0.0068^{defg}	3.53 ±0.15^{bcdefg}	0.890 ±0.007^{ae}
50	5	60	59.87 ±0.38^{fhi}	0.4840 ±0.0025^{fg}	0.1389 ±0.0016^{efg}	3.48 ±0.02^{bcdefg}	0.873 ±0.003^{bdef}
20	1	70	42.03 ±0.56^{agj}	0.2955 ±0.0044^{ah}	0.0802 ±0.0167^a	3.72 ±0.23^{bcdefg}	0.897 ±0.009^{afg}
20	3	70	54.28 ±0.68^{bfhk}	0.4478 ±0.0046^c	0.1130 ±0.0107^{bcde}	3.96 ±0.03^{defg}	0.873 ±0.007^{bcdegh}
20	5	70	56.93 ±0.09^{cikl}	0.4864 ±0.0006^{fi}	0.1236 ±0.0025^{cdefg}	3.94 ±0.07^{defg}	0.872 ±0.011^{bdei}
35	1	70	42.09 ±1.13^{agm}	0.2888 ±0.0090^{aj}	0.0931 ±0.0090^{ab}	3.11 ±0.21^{ab}	0.901 ±0.004^a
35	3	70	55.75 ±2.12^{cekn}	0.4362 ±0.0149^{bc}	0.1289 ±0.0149^{defg}	3.41 ±0.28^{abcdef}	0.872 ±0.010^{bdej}
35	5	70	59.91 ±1.06^{fhlo}	0.4905 ±0.0068^{gi}	0.1290 ±0.0068^{defg}	3.81 ±0.15^{bcdefg}	0.850 ±0.008^{fhijk}
50	1	70	46.28 ±1.03^{gp}	0.3496 ±0.0077^e	0.0847 ±0.0048^a	4.13 ±0.14^{fg}	0.893 ±0.003^{afhijl}
50	3	70	61.24 ±0.61^{ior}	0.5086 ±0.0038^{gik}	0.1265 ±0.0220^{cdefg}	4.10 ±0.69^{efg}	0.871 ±0.008^{bdeklm}
50	5	70	65.02 ±0.36^{rs}	0.5445 ±0.0021^l	0.1319 ±0.0002^{efg}	4.13 ±0.05^{fg}	0.842 ±0.009^{kn}
20	1	80	44.27 ±0.32^{agt}	0.3247 ±0.0025^e	0.0991 ±0.0028^{abc}	3.28 ±0.07^{abcd}	0.895 ±0.010^{afhij}
20	3	80	55.77 ±1.66^{ceku}	0.4634 ±0.0110^{cgi}	0.1256 ±0.0109^{cdefg}	3.70 ±0.23^{bcdefg}	0.854 ±0.011^{fhijno}
20	5	80	58.86 ±1.71^{fhlnruv}	0.4963 ±0.0108^{gim}	0.1283 ±0.0033^{defg}	3.87 ±0.02^{cdefg}	0.836 ±0.003^{kop}
35	1	80	45.18 ±2.12^{djmpt}	0.3149 ±0.0167^{ehj}	0.0938 ±0.0077^{ab}	3.36 ±0.10^{abcdefg}	0.898 ±0.005^a
35	3	80	58.69 ±1.64^{fhlnruw}	0.4758 ±0.0108^{cgi}	0.1306 ±0.0038^{defg}	3.64 ±0.02^{bcdefg}	0.849 ±0.004^{fhijmnpr}
35	5	80	64.76 ±0.40^{rx}	0.5283 ±0.0025^{kl}	0.1443 ±0.0021^{fg}	3.66 ±0.04^{bcdefg}	0.812 ±0.001^{ps}
50	1	80	48.82 ±0.36^{bgop}	0.3776 ±0.0028^d	0.1032 ±0.0077^{abcd}	3.67 ±0.25^{bcdefg}	0.890 ±0.009^{afhijm}
50	3	80	62.15 ±1.84^{isvwx}	0.5241 ±0.0109^{klm}	0.1325 ±0.0042^{efg}	3.96 ±0.04^{defg}	0.841 ±0.008^{kor}
50	5	80	68.69 ±0.62^{sx}	0.5810 ±0.0034^n	0.1492 ±0.0025^g	3.89 ±0.04^{cdefg}	0.809 ±0.004^s

^{abcdefghijklmnopqrstuvwx}*Different letters in the superscript in the same column of the table indicate on statistical significant difference between values at the level of significance of p<0.05 (based on post-hoc Tukey HSD test)*

Increase of molasses concentration has shown statistically insignificant increase of the SG values of dehydrating chicken meat.

Maximal obtained SG values were 0.1492 ± 0.0025 g/$g_{i.s.}$ at the same process parameters as in case of DMC and WL: T of 50°C, τ of 5h, C of 80%. The level of the maximal obtained SG values was the same as in case of osmotic dehydration of fish (0.14 g/$g_{i.s}$), (Ćurčić *et al.* 2014), while it was significantly lower than SG values of osmodehydrated pork meat ($0,21$ g/$g_{i.s.}$) (Pezo *et al.* 2013).

By increasing all three process parameters, the secondary mass transfer of solutes from molasses as an osmotic medim to dehydrating chicken meat has intensified, providing higher achived SG values.

DEI indicates on the efficiency of the osmotic dehydration process, since the higher values are characterised by high WL and low SG values.

The increase of T and τ of the process and C of osmotic solution provided complex effect on DEI values. Increase of these process parameters has intensified both mass transfers responsibille for increasing WL and SG values. Combined effect of these increases is noticable in complex increase of DEI values.

DEI values ranged from 2.73 to 4.17 depending of the applied process parameters, which are at the same level as DEI values of osmodehydrated pork meat (ranged from 2.78 3.61) (Filipović, 2013).

The increase of each process parameter: T and τ of the process and C of osmotic solution has statistically significantly decreased obtained a_w valued of dehydrated chicken meat. The results of the obtained a_w values are in direct corelation with DMC values of osmodehydrated chicken meat.

Minimal obtained a_w value of osmodehydrated chicken meat was 0.809 ± 0.004 at process parameters of 50°C, after 5h process in molasses of concentration of 80%. This level of achived a_w values is lower than inhibiting levels for growth of most microorganisms exept some molds (Huang and Nip, 2001; Feiner, 2006).

CONCLUSIONS

From the presented results it can be concluded that osmotic dehydration is effective alternative method for lowering water content of raw chicken meat producing intermediate-product of increased DMC, up to 68.69%, and lowered a_w values, down to 0.809.

Osmotic dehydration in molasses as an osmotic solution is very effective, obtaining high WL values of up to 0.5810 g/$g_{i.s.}$ and SG values of 0.1492 g/$g_{i.s.}$.

The highest results obtained were at maximal process parameters of time, temperature and concentration of the process of osmotic dehydration.

Alternative use of molasses has provided a new environmentally friendly value to the by-product of sugar beet production, also allowing incorporation of beneficial nutritive composition of sugar beet molasses in human diet.

ACKNOWLEDGEMENTS

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EFFECT OF HOT SMOKING PROCESS ON PROCESSING YIELD AND MICROBIOLOGICAL SAFETY OF COMMON CARP

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ABSTRACT

Fish processing industry is still underdeveloped in Republic of Serbia. Manufacturing fish products and development of new fish products could increase offer of that type of food and to contribute to better sale of fish, not only in traditional fish markets, but also in retail stores and supermarkets. Therefore, the main objective of this study was to describe the proper technological process of hot smoking of common carp as well as to determine the yield of final product and also microbiological safety of hot-smoked common carp meat. The whole manufacturing cycle was described. Smoking process yield was determined by using 21 fish. Microbiological tests were conducted according to the national legislation. Based on presented data, it can be concluded that it could be useful for promoting smoked carp. These results also may be helpful in making different strategies for the manufacturing industry. Data on yield of final products are very important when we talk about any kind of fish processing and analysis of economic feasibility of production and processing. Microbiological analysis of hot smoked common carp showed the absence of pathogenic bacteria and confirmed that the obtained product is safe and suitable for human consumption. Appropriate technological process of manufacturing smoked carp provides high quality of final product. Obtained results could help to develop similar products from different fish species, which would complete the current offer of fish and fish products in the market.

Keywords: common carp, hot-smoking, yield, microbiological safety, manufacturing

INTRODUCTION

Common carp is economically the most significant fish species on the fish farms in Serbia (Ćirkovic *et al.*, 2012), and the cyprinids are the most common species in the total world production of freshwater fishes (71.9%, 24.2 million tons in 2010) (FAO, 2012). Consumption of fish meat is increasing worldwide, primarily due to the fact that fish meat is recommended as an important component in healthy human nutrition. According to the latest FAO (2012) report, Serbia belongs to the group of the countries where the average consumption of fish ranges between 5-10 kg per capita per year, which is significantly below European and world average consumption. Baltić *et al.* (2009) suggested that poor standard is the main reason for this situation, but also weak and undiversified offer of fish and fish meat products in the local market significantly contribute to the low fish consumption. Fish processing industry is still underdeveloped. Manufacturing fish products and development of new fish products could increase offer of that type of food and to contribute to better sale of fish, not only in traditional fish markets, but also in retail stores and supermarkets. Technological processes of processing, preservation and storage are different for fish meat and for the mammalian meat. For proper manufacturing of fish products, knowledge about chemical composition and characteristics of raw fish meat is very important in order to apply the most appropriate technology procedures adjusted to certain fish species. Recently fish sausages of common carp meat and also of meat of other cyprinid fish species were developed (Okanović *et al.*, 2013 a, b) which represents a great contribution to this branch of food processing, but there is still a need for developing new products and improving the existing. In Republic of Serbia, there is growing market demand for smoked trout and smoked common carp (*Cyprinus*

carpio) and popularity of these products is increased particularly (Pavlićević *et al.*, 2013). Common carp is usually sold as a raw fish. The application of smoking for the extension of the shelf-life is a process of great interest, given that fish meat is generally spoils fast and easily. As far as we know, there is no information in the literature about the procedure of traditional hot smoking of common carp. Therefore, the main objective of this study was to describe the proper technological process of hot smoking of common carp as well as to determine the yield of final product and also microbiological safety of hot-smoked common carp meat.

MATERIAL AND METHODS

Fresh whole common carp (average weight of 2850 g) were obtained from the fishery farm Ečka, Lukino Selo, Republic of Serbia and transported to the fish processing facilities (Agropapuk, Kuzmin, Republic of Serbia) in refrigerator, at 4°C. The fish were slaughtered and the scales and guts were removed for common carp and fish were then washed in clean fresh water. The fish were dry salted on a room temperature using refined NaCl. The ratio between fish and salt weight was approximately 20. After finishing dry salting, excess salt was taken off. Then the fish were kept at room temperature for 30 minutes and after that the fish were smoked. Fish were subsequently washed in clean water and dried. The fish were then hot smoked for 12 h at temperatures above 60°C. For the hot smoking hard wood as well as wood sawdust were used in order to produce enough smoke. After smoking, fish was cooled (at 4°C, for 4 h), and then packed. Figure 1 presents flow diagram of the production of hot-smoked common carp. The samples were stored at a temperature of 4°C until the end of the analysis. Analyses of hot smoked common carp were carried out at the Institute of Meat Hygiene and Technology in Belgrade. Smoking process yield was determined by using 21 fish. Calculation of eviscerating, descaling, dressing percentage, smoking yields and losses of total smoking process were based on fish live weight.

Microbiological tests were conducted according to the national legislation (Sl. glasnik RS 72/2010) by determining: the total number of microorganisms (EN ISO 4833), the presence of *Salmonella* species (EN ISO 6579), *Escherichia coli* (ISO 16 649), *L. monocytogenes* (EN ISO 11290-1) and *Bacillus cereus* in g /ml (EN ISO 7932).

RESULTS AND DISCUSSION

Average yield of smoked common carp was 82.19 % based on weight of eviscerated and descaled fish or 47% based on weight of live fish (Table 1). Hot - smoking method affected weight losses of smoked fillet as also noted by Franco *et al.* (2010). The mentioned authors found out that the process losses were significantly higher in hot-smoking process in comparison with the process of cold smoking. Sigurgisladottir *et al.* (2000) suggested that the weight loss is due to dehydration during smoking and it varies between 10 and 25% depending on several factors, including origin of raw material, characteristics of final products and parameters used in the process, especially time and temperature.

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┌─────────────────┐     ┌─────────────────┐     ┌─────────────────┐
│  RECEIVING AND  │ ──> │  SLAUGHTERING   │ ──> │   WASHING AND   │
│    TRANSPORT    │     │ AND EVISCERATION│     │     DRYING      │
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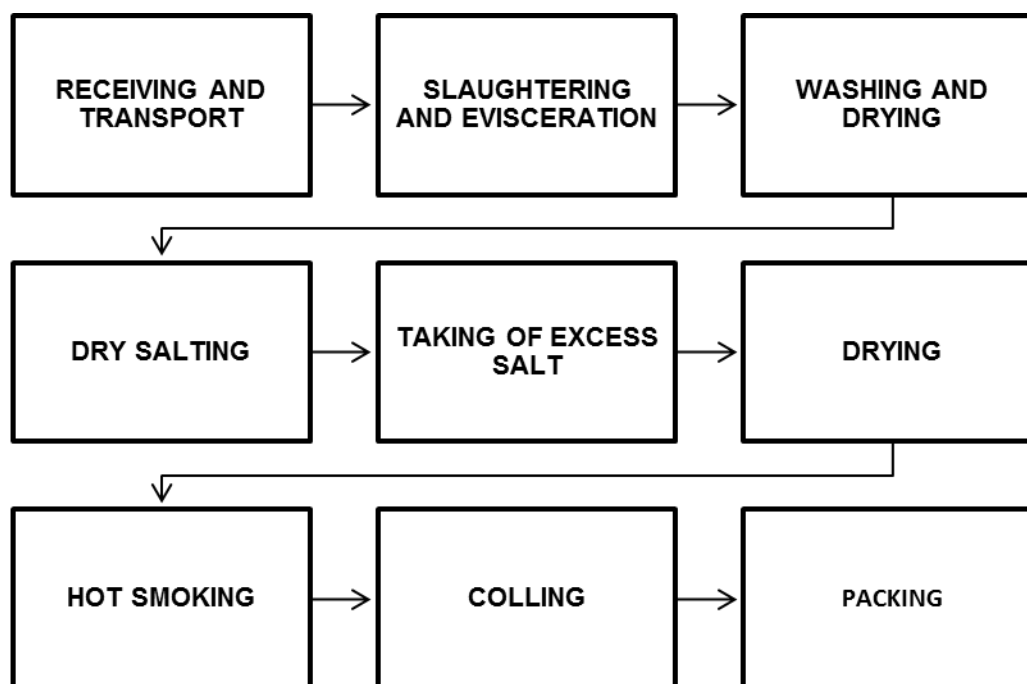
Figure 1. Flow diagram of the production of hot-smoked common carp

Ljubojević *et al.* (2012) and Okanović *et al.* (2013 ab) reported that quality of raw fish is highly important for yield and quality of the final product. Martinez *et al.* (2010) noted that type of raw material, manual processing method, method of salting, and smoking conditions (temperature, moisture, air flow rate, drying) are determinant factors on the weight variation during smoking. Yield of final product is of great technological and economical importance. Nutrition, age, weight, seasonal variation and physiological phase have great influence on yield (Ljubojević *et al.*, 2012). Therefore, those factors that might affect analysis could be avoided. Fish were of the same weight class, origin and fed on the same diet.

Table 1. - Average values of fish weight and yield, losses occurred during the smoking process of common carp (Cyprinus carpio) (n=21)

	$\dot{x}$	Sd	Se	Iv	Cv
Weight of whole fish (g)	2850	64.65	14.11	2700 - 2980	2.28
Weight of eviscerated and descaled fish (g)	1852.9	74.82	16.33	1701-1966.8	4.04
Dressing percentage (%)	65	1.64	0.36	62-68	2.52
Weight of hot smoked fish (g)	1523.1	67.45	14.72	1374.4-1632.4	4.43
Yield of smoked fish (%)	82.19	0.93	0.2	80.5-84	1.13
Smoking process loss (%)	17.81	0.93	0.2	16-19.5	5.23
Total loss (%)	53.42	1.53	0.33	50.53-56.1	2.87

Legend: $\dot{x}$ - mean value; Sd –standard deviation; Se- standard error; Cv – coefficient of variation; Iv – interval of variation

Results of microbiological analysis of hot smoked common carp (Table 2) showed that the minimal number of aerobic bacteria (3000) was found in the examined products. This suggests that the carp meat was microbiologicaly safe and that proper manufacturing and hygiene conditions in the processing facilities Agropapuk were presented during all production cycle. Also, a thermal treatment was carried out in a proper way, which resulted in destroying of most microorganisms. Proper salt content also contributed to the microbiological safety of final product. It is significant that the presence of any pathogen bacteria was no found.

Table 2. - Results of microbiological analysis of hot smoked common carp

Microorganisms	Number of microorganisms
L.monocytogenes in 25 g/mL	ND
E.coli in g/mL	ND
Number of aerobic colony in g/mL	3000
Salmonella species in 25 g/mL	ND
Bacillus cereus in g/ml	ND

Legend: ND- not determined

CONCLUSIONS

Based on presented data, it can be concluded that it could be useful for promoting smoked carp. These results also may be helpful in making different strategies for the manufacturing industry. Data on yield of final products are very important when we talk about any kind of fish processing and analysis of economic feasibility of production and processing. Microbiological analysis of hot smoked common carp showed the absence of pathogenic bacteria and confirmed that the obtained product is safe and suitable for human consumption. Appropriate technological process of manufacturing smoked carp provides high quality of final product. Obtained results could help to develop similar products from different fish species, which would complete the current offer of fish and fish products in the market.

ACKNOWLEDGEMENTS

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CONTRIBUTION OF MEAT INSPECTION AND ABATTOIR PROCESS HYGIENE TO BIOLOGICAL SAFETY ASSURANCE OF POULTRY CARCASSES

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ABSTRACT

The aim of the study was to compare performances of the current official meat inspection and abattoir process hygiene in ensuring biological safety of poultry carcasses through qualitative risk ranking of hazards associated with poultry that each of these risk management strategies can control at abattoir level. Available literature was used, and where no literature was available, international experts were consulted. The results indicate high risk of *Salmonella enterica* and thermophilic *Campylobacter* spp., and low risk of human pathogenic *Escherichia coli*, *Yersinia enterocolitica, Listeria monocytogenes, Clostridium* spp., *Toxoplasma gondii* and numerous other identified meat-borne biological hazards. Analysis of the current two main risk management strategies in poultry abattoirs indicated that abattoir process hygiene has a higher public health protection potential than official meat inspection. For many hazards, data gaps were identified; further studies and zoonoses monitoring should be addressed to fill those gaps so to have more accurate inputs for risk assessment and selection of appropriate risk management measures for those hazards.
Keywords: poultry, biohazards, meat inspection, abattoir process hygiene

INTRODUCTION

Many biological, chemical and physical hazards affect the safety of poultry meat; however, it is generally accepted that the biological hazards pose the highest threat to meat consumers (Norrung and Buncic, 2008). These biological hazards are mainly zoonotic - originating from slaughtered poultry for - and can be divided into i) hazards that can cause macroscopically visible lesions, and ii) hazards that usually do not cause macroscopically visible lesions in slaughtered birds but are often present in/on the alimentary tract/skin/feathers. Lesions due to the hazards in the first group can be detected with the current official meat inspection (EC, 2004) and removed from the meat chain. Hazards from the second group can be excreted by any bird (healthy or diseased) and, even if causing lesions, these are normally not detected by the current meat inspection. Therefore, control of the later group of hazards in abattoirs is based on prevention/reduction of their transfer from skin/guts to meat – i.e. abattoir process hygiene (FAO, 2004).

Risk management process within the food safety risk analysis, i.e. selection and implementation of measures for public health risk reduction to acceptable level, shall take into account the results of risk assessment (FAO/WHO, 2006). Given the potentially high resource costs associated with conducting risk assessments and/or implementing risk management decisions, risk ranking has been recognized as the proper starting point for risk-based priority setting and resource allocation. The effectiveness of the risk management options need to be reviewed regularly with an aim of continual improvement in public health (CAC, 2007). The performances of meat inspection and process hygiene in ensuring the overall biological safety of poultry meat are not assessed to-date. Therefore, the aim of this study was to assess those performances based on a previously developed model (Blagojevic and Antic, 2014) for assessment of meat inspection and process hygiene in cattle and pig abattoirs.

MATERIAL AND METHODS

Hazard identification
Hazard identification was based on literature review. To be included, hazards had to meet the following criteria: to be biological, zoonotic and associated with poultry. Meat-borne hazards were ranked further, and those that are not proven to be meat-borne were excluded from ranking (risks are considered negligible).

Risk ranking
A qualitative risk ranking of identified hazards was conducted using modified decision tree developed by the European Food Safety Authority (EFSA, 2012; Figure 1). Risks for public health were assessed qualitatively at the point of chilled carcasses as a proxy for meat consumers' exposure.

Incidence of disease: Average incidence of human diseases reported in the European Union (EU) for the period 2010-2012 was determined based on the data from the EU zoonoses monitoring reports (EFSA/ECDC, 2012, 2013, 2014) and was assigned as: low (<1 confirmed cases/100,000 inhabitants), medium (1-10/100,000) or high (>10/100,000).

Severity of disease: Severity of disease was determined based on available scientific literature and expert elicitation (in the case of lacking or controversial literature data). Twelve international experts in veterinary public health and food safety gave a response regarding meat-borne hazards: i.e. are the respective consequences of disease in humans severe or not. The description of "severe consequences of disease" was: high mortality and/or severe symptoms and/or high likelihood of hospitalization and/or high likelihood of sequelae.

Source attribution: Attribution to poultry of human disease caused with each identified hazard (whether the attribution to poultry is high or not) was determined on the basis of EFSA's opinion on poultry meat inspection (EFSA, 2012).

Hazard prevalence in/on chilled carcasses: Prevalence of hazards in/on chilled poultry carcasses in the EU for the period 2010-2012 was determined on the basis of EU zoonoses monitoring reports (EFSA/ECDC, 2012, 2013, 2014), and was assigned in one of three categories: low (<0.1%), medium (0.1-5%) or high (>5%).
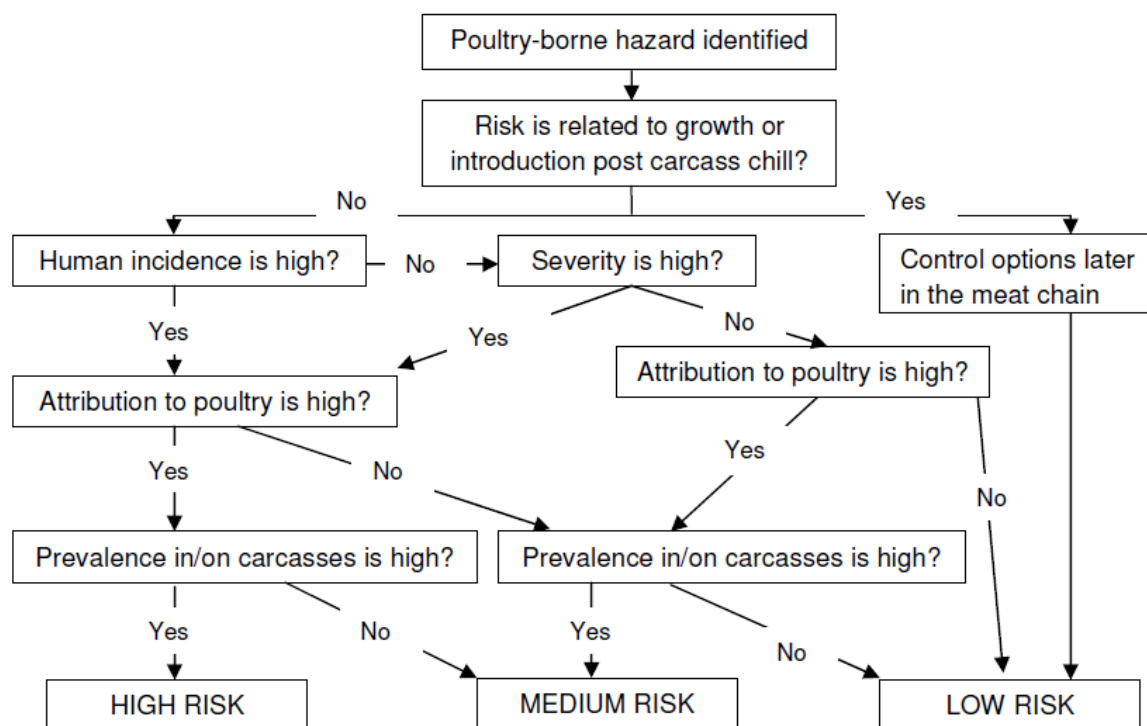
Figure 1. Flowchart for risk ranking of poultry-borne hazards (EFSA, 2012)

Assessment of performances of the risk management strategies at abattoirs
Performances of the meat inspection and abattoir process hygiene to protect public health are defined as their capacities for reducing risk of meat-borne hazards. These performances were expressed through the number of identified meat-borne hazards that can be controlled with each strategy and the level of risk those hazards pose to public health.

RESULTS AND DISCUSSION

Hazard identification
Identified hazards that are associated with poultry (Table 1) do not represent all biological hazards with zoonotic potential; rather, the most common hazards, occurring in the Europe primarily, are outlined. Public health risks of some ubiquitous hazards (*Clostridium* spp., *L. monocytogenes* and *S. aureus*) are mainly related to their growth and/or entering the meat chain after carcass chilling in abattoirs (Norrung *et al.*, 2009). Given that the aim of this study was to evaluate performances of risk management strategies at abattoir level, risks of these hazards were assessed as "low". Identified hazards that are not meat-borne (transmission to humans occurs through contact, aerosol or consumption of water, other food, etc.) are also shown in Table 1. As this study is focused on risk analysis related to meat consumption, the related risks are assessed as "negligible".

Table 1. Hazard identification

Hazard	Meatborne?	Detectable through meat inspection (examples of detectable lesions*)?
Campylobacter spp. (termophilic)	Yes	No
Yersinia enterocolitica	Yes	No
Escherichia coli (human pathogenic)	Yes	No
Salmonella enterica	Yes	No
Listeria monocytogenes	Yes	No
Staphylococcus aureus	Yes	Yes (arthritis, liver/spleen/kidney necrosis, abscesses)
Clostridium perfringens	Yes	Yes (haemorrhagic enteritis, hyperaemic organs)
Clostridium botulinum	Yes	No
Clostridium difficile	Yes	No
Toxoplasma gondii	Yes	Yes (spleen and liver enlargement, necrotic hepatitis, pericarditis, enteritis, lung congestion)
Zoonotic *Mycobacterium* spp.	No	Yes (yellow nodules with caseous necrosis in liver, kidney, lungs, heart)
Streptococcus spp. (haemolytic)	No	Yes (spleen, kidney and liver enlargement; peritonitis, congestion of internal organs)
Erysipelothrix rhusiopathiae	No	Yes (general septicaemia, congestions, haemorrhagic myocardium, enteritis)
Chlamydia psittaci	No	Yes (fibrous pericarditis, liver enlargement, conjunctivitis)
Pasteurella multocida	No	Yes (comb and craw cyanosis, intestinal haemorrhages, abscesses, arthritis)
Highly pathogenic avian influenza virus	No	Yes (lung congestion and oedema, comb congestions and cyanosis, atrophy of thymus and bursa Fabricii, haemorrhage)
Newcastle disease virus	No	Yes (haemorrhagic lesions in intestinal tract, conjunctivitis)
Cryptococcus neoformans	No	Yes (granuloma and necrosis of liver, intestines, lungs and spleen)
Aeromonas hydrophila	No	Yes (intestinal inflammation and haemorrhage, aerosaculitis, salphingitis)

According to FAO, 2004

Drawing a clear line between the hazards that "cause" and "do not cause" detectable lesions is very difficult. For instance, although *Salmonella* is grouped into "not causing lesions" hazards, these organisms can sometimes cause lesions that are recognizable by meat inspection (e.g. emaciation, septicaemia, enteritis; Poppe, 2000). Therefore, it is also difficult to draw a distinct line between hazards that are controlled by meat inspection or by process hygiene. Nevertheless, this hazard (like some others) is here arbitrarily grouped into hazards that do not cause lesions and are controllable by process hygiene, as it is more often present in faeces than it causes detectable lesions in infected birds.

Risk ranking of meat-borne hazards
Parameters used for risk ranking of poultry-borne hazards are shown in Table 2 and the results of the ranking including risk management options are shown in Table 3. *Salmonella enterica* and thermophilic *Campylobacter* spp. represent the high-risk hazards. There are no hazards in the medium risk category, and other meat-borne hazards are of low risk. High risk of *Salmonella* and *Campylobacter* in poultry meat is a consequence of high incidence, high attribution to poultry and high prevalence of each of those hazards on poultry carcasses. Salmonellosis is the second most reported zoonosis in the EU with 95-100,000 reported human cases per year (EFSA/ECDC, 2012, 2013, 2014), while it has been estimated that real number is around 6 million (Havelaar *et al.*, 2012). Infection is foodborne and poultry meat is considered as the second most important source (after eggs). This study found that the prevalence on chilled carcasses is 8.71%; however, EU baseline study in 2008 found 16% of contaminated broiler carcasses (EFSA, 2010). Campylobacteriosis is the most commonly reported zoonotic disease in humans in the EU - roughly with 200,000 cases per year (EFSA/ECDC, 2012, 2013, 2014), although it has been estimated that true number is around 9 million (Havelaar *et al.*, 2012). Poultry meat is considered as the most important source for humans. Beside that, this study found prevalence of around 28%, but EU baseline study found that 76% of contaminated dressed broiler carcasses in 2008 (EFSA, 2010).
It should be kept in mind that the risk ranking results are related to the EU as a whole and for the period 2010-2012. It is necessary to re-visit and update the risk ranking periodically (when newer data are available), as well as to perform regional assessments which would be more of practical use.

Table 2. Parameters used for ranking of meat-borne hazards

Hazard	Incidence of disease		Severity of disease is high?	Attribution to poultry is high?	Prevalence in/on chilled carcasses	
	confirmed cases /100,000	risk category			% of cont. carcasses	risk category
Campylobacter spp. (thermophylic)	51.5	high	no	yes	28.67	high
Yersinia enterocolitica	1.61	medium	no	no	no data	low
E. coli (human pathogenic)	1.3	medium	yes	no	1.56	medium
Salmonella spp. (nontyphoidal)	21.5	high	no	yes	8.71	high
Toxoplasma gondii	0.1	low	no	no	no data	low

Assessment of performances of the main risk management strategies at abattoirs
Analysis of the risk ranking results (Table 3) indicates that abattoir process hygiene has a higher potential for protection of public health that official meat inspection. This is mainly due to the fact that both high risk hazards are controlled with adequate process hygiene that is in practice achieved through successful implementation of Good Manufacturing/Hygienic Practice and Hazard Analysis and Critical Control Points systems (FAO, 2004).The current meat inspection was found to be of no relevance in ensuring biological safety of meat as it

addresses only zoonotic hazards that are not proven to be meat-borne (Table 1). Weaknesses of the current meat inspection are well recognized; concerns have been expressed that meat inspection can no longer be considered adequate to protect public health as it is ineffective in controlling the hazards that currently pose highest public health burden such as *Salmonella*, *Campylobacter*, pathogenic *Yersinia* and verotoxigenic *Escherichia coli*. In the EU significant actions have been initiated in order to review and modernise meat inspection moving towards a more risk-based approach (EFSA, 2012).

Although the risks of non-meat-borne hazards are assessed as negligible for consumers, these hazards can pose serious threat for workers at abattoirs as occupational hazards. Also, to increase the effectiveness of process hygiene for control of hazards present in/on faeces, skin and feathers, previous assessment of cleanliness of birds, as an important part of the *ante-mortem* inspection, is ultimate precondition. Thus, in the case of excessively dirty birds, logistical slaughter can be applied, resulting in better microbiological status of dressed carcasses. Also, analysis of the food chain information (FCI) within meat inspection system can reveal higher risk of specific pathogens' contamination (e.g. *Salmonella*) associated with incoming birds and a need for logistical slaughter. A visual *post-mortem* inspection of carcasses can detect faecal and other contamination as a result of improper dressing process (EFSA, 2012). Furthermore, some detected conditions although do not have any direct public health implications, may indicate higher risk of chemical hazards in meat (residues of veterinary drugs).

Toxoplasma gondii is pointed out as a hazard that cannot be controlled by abattoir process hygiene or by official meat inspection. Therefore, it is certain that future meat inspection should be directed to the detection of this hazard. Furthermore, it is possible to inactivate intramuscular parasites through high or low temperature treatments (Nordic Council of Ministers, 2006). Therefore, temperature treatment (i.e. freezing) of meat in abattoirs could be "regular" risk management control measure for intramuscular parasites, in cases where it is estimated that the risks of these hazards are elevated - for instance, if FCI analysis indicate that birds originate from outdoor raising systems.

Table 3. Risk ranking of meat-borne hazards and strategies for their control

Hazard	Final risk category	Meat inspection or process hygiene as main risk management option
Campylobacter spp. (thermophilic)	high	abattoir process hygiene
Yersinia enterocolitica	low	abattoir process hygiene
Escherichia coli (human pathogenic)	low	abattoir process hygiene
Salmonella enterica	high	abattoir process hygiene
Listeria monocytogenes	low	control in later phases of the meat chain
Staphylococcus aureus	low	control in later phases of the meat chain
Clostridium difficile	low	control in later phases of the meat chain
Clostridium botulinum	low	control in later phases of the meat chain
Clostridium perfringens	low	control in later phases of the meat chain
Toxoplasma gondii	low	none
Zoonotic *Mycobacterium* spp.	negligible	meat inspection
Streptococcus spp. (haemolytic)	negligible	meat inspection
Erysipelothrix rhusiopathiae	negligible	meat inspection
Chlamydia psittaci	negligible	meat inspection
Pasteurella multocida	negligible	meat inspection
Highly pathogenic avian influenza virus	negligible	meat inspection
Newcastle disease virus	negligible	meat inspection
Cryptococcus neoformans	negligible	meat inspection
Cryptosporidium spp.	negligible	meat inspection
Aeromonas hydrophila	negligible	meat inspection

CONCLUSIONS

Analysis of the current two main risk management strategies in poultry abattoirs indicated that adequate hygiene of slaughter and carcass dressing has a higher potential to contribute to the overall biological safety of poultry carcasses than official meat inspection; this is primarily due to the fact that *Salmonella enterica* and thermophilic *Campylobacter* spp. are assessed as high risk hazards. For some hazards, data gaps were identified; further studies and zoonoses monitoring should be addressed to fill those gaps so to have more accurate inputs for risk assessment and selection of appropriate risk management measures.

ACKNOWLEDGEMENTS

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DEVELOPMENT OF NEW FUNCTIONAL COOKED SAUSAGES BY ADDITION OF ROSE PETAL EXTRACT

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ABSTRACT

An identification of the technological capabilities to develop new functional cooked sausages with ½ reduced content of nitrite, enriched with rose petal extract (*Rosa damascena Mill)* was studied. The experiments were conducted with samples containing 0.01, 0.03, and 0.05 g/kg sodium nitrite, and 0.1, 0.3, 0.5 g/kg rose petal extract. The control samples were produced with 0.05 or 0.10 g/kg of sodium nitrite. The samples were analyzed 1st day after manufacturing and on 6th day of storage at 0 - 4°C. It was found that the addition of 0.03% or 0.05% rose petal extract in the most significant extent contributes to preservation of the sensory properties of studied sausages with half nitrite content. The combination of 0.03 % or 0.05 % rose petal extract most preferably preserves the color characteristics of the sausages, but addition of 0.05% rose petal extract with reducing the nitrite content to 10% was not found so effective. The conclusion was made that the rose petal extract can be used as additive for development new functional meat products with halfway reduced amounts of nitrites. Future experiments are needed for optimization the sausage formulation.
Keywords: *Functional sausages, rose petal extract, color, reduced nitrite content*

INTRODUCTION

In wealthier societies, consumers attach increasing importance to all aspects that can contribute to improved quality of life (Jiménez-Colmenero *et al*, 2001). Meat plays a very important role in the diet by contributing quality protein, essential minerals and trace elements (Buckley *et al.*, 1995). In the last years red meat is associated as cancer-promoting food (Michaud *et al*, 2001) obesity and cardiovascular diseases (Toldrá and Reig, 2011). Due to increasing concerns for health, efforts have been made by food industries in many countries to develop new foods with tertiary functions.

Natural herbs as Rosemary extracts (Coronado *et al.* 2002), green tea extract, thymbra spicata oil (Bozkurt 2006), rutin (Balev *et al.* 2005), lycopene from tomato peel (Calvo *et al.* 2008) pumpkin powder, dried goji berry fruits and pumpkin polder (Serikaisai *et al.*, 2014) were added to sausages and salami as functional ingredients.

Rose petal extract, as by-product of rose oil (*Rosa damascena Mill*) processing is disposal problem for the food industry, but on the other hand is promising source of polyphenols (Kammerer *et al.*, 2005) with antioxidant (Wang *et al.*, 2006) and antibacterial activity (Özkan *et al.*, 2004). Moreover, strategies for the exploitation of polyphenol-rich extracts as functional (Larrosa *et al.*, 2002) or technological (Ivanov *et al.*, 2009) food additives have been proposed.

In the available literature did not have any reports of application of rose petal extracts in meat products.

Therefore purpose of this study was to identify the technological capabilities for developing new functional cooked sausages with ½ nitrites, by enrichment with rose petal extracts.

MATERIAL AND METHODS

Meat raw materials

The chilled to 0 – 4ºC beef topside (pH 6.60) and pork chest (pH 6.55) were used in this experiment. The beef and pork meat were supplied by the company Kartevi brother's Ltd, village Benkovski, district Plovdiv, Bulgaria.

Other ingredients and additives

The sodium chloride (salt), sodium tripolyphosphates (STPP), sodium nitrite (E250) were bought from the local market.

The rose petal extract (*Rosa damascena Mill*) was produced in the Department of Food Preservation and Refrigeration Technology, Technological Faculty, University of Food Technology, Plovdiv, Bulgaria.

Sample preparation

Sausages were manufactured according to the requirements of cooked meat product appropriate for EU (Table 1). The filling mass was prepared by cutter adding the phosphates, flake ice, NaNO2. The prepared filling mass was separated of seven equal portions and was filled in moisture and gases no-permeable five-layer polymer casings. The sausages were cooked to an internal temperature of 72°C and chilled in cold water. First half of the samples was immediacy studded (1d), and the second half was stored 6 day at 0 - 4ºC (6d). The samples were obtained according ISO 3100-1:1991.

Table 1. Formulation of different samples functional cooked sausages

Ingredients, g/kg	Samples						
	C	C½	RPE1	RPE2	RPE3	RPE4	RPE5
Beef topside, g/kg	500	500	500	500	500	500	500
Pork chest, g/kg	500	200	200	200	200	200	200
Sodium chloride, g/kg	20	20	20	20	20	20	20
Sodium nitrite, g/kg	0,10	0,05	0,01	0,01	0,05	0,05	0,03
Rose petal extract (%)	-	-	0,01	0,05	0,01	0,05	0,03
Sodium polyphosphates, g/kg	2	2	2	2	2	2	2
Flake ice, g/kg	200	200	200	200	200	200	200

pH value

pH value of the sausage samples was determined by pH-meter MS 2004, equipped by pH combination recorder S 450 CD (Sensorex pH Electrode Station, USA) (Young *et al*. 2004).

Sensory analysis

The sensory characteristics of the samples were determined after opening the packages. A panel consisting of five members with proven tasting abilities (Meilgaard *et al*. 1999) was used. The samples were scored using 1 to 5 scales.

Color properties establishment

Colorimeter Konica Minolta model CR-410 (Konica Minolta Holding, USA), purchased by Sending, Inc. (Tokyo, Japan) was used to evaluate the CIE L*, a*, b* color properties of sausages (Hunt *et al*. 2012) on 1st and 6th day of storage at 0 - 4ºC. On the 6th day of storage the changes of the color properties of sausage surface cross-sectional views were captured in dynamics during the 60 min of air exposure.

RESULTS AND DISCUSSION

pH value

At the first day (0 - 4ºC) the lowest pH ($p \leq 0.05$) was established in samples with 0.05 % and 0.03% rose petal extract addition ($RPE_2, RPE_4, RPE_5, RPE_3$) and in control sample with 100% nitrite content (Table 2). A comparison of pH values of samples on 6d of storage shows that in five of examined samples the changes was minimal – between 6.35-6.37.

Table 2. Changes in pH values of the studied sausages

	pH	
	1 d	**6 d**
C	$6,3\pm0,01^{a}$	$6,36\pm0,01^{b}$
C $_{1/2}$	$6,33\pm0,01^{a}$	$6,37\pm0,01^{b}$
RPE_1	$6,32\pm0,01^{a}$	$6,36\pm0,01^{b}$
RPE_2	$6,29\pm0,01^{a}$	$6,33\pm0,01^{b}$
RPE_3	$6,31\pm0,01^{a}$	$6,35\pm0,01^{b}$
RPE_4	$6,3\pm0,01^{a}$	$6,35\pm0,01^{b}$
RPE_5	$6,3\pm0,01^{a}$	$6,36\pm0,01^{b}$

[a, b,] *Means ± SD, in the same row with different superscripts are statistical different ($p \leq 0.05$)*

Sensory evaluations

After 6 d of storage (0-4°C) the sensory scores for flavour was classified as follows RPE_3 > RPE_5 > RPE_4 > C> RPE_2> RPE_1> C½ (Fig. 1).

The most attracted odour was found in samples RPE_5 followed by RPE_2, C and RPE_1, RPE_3 RPE_4 where the sensory scores for odour was not statistically different (p>0.05). The lowest score for odour was awarded to control sample with half reduced nitrite content without addition of rose petal extract.

The results obtained for the color of sausage surface cross-sectional view ranked the first and second place for samples RPE_5 and RPE_3. Again, the color of sausage surface cross-sectional view of the samples C½ was rated the lowest (Fig. 1).

Those results allow us to conclude that addition of 0.05% or 0.03% rose petal extract in the most significant extend contribute to the preservation of the bright red color, fresh smell and taste of the functional cooked sausages.

Changes of the color characteristics

The higher decrease in color brightness (L*) during studied period (six days of storage, 0-4°C) (Table 3) was found in control sample with half reduced nitrite content (p<0.05). Compared to samples C and $C_{1/2}$ on the six day of storage the L* value in samples with rose petal extract remain more stable.

This means that the addition of rose petal extract to 0.05% may kept the L* value of the cross-sectional surface view of the functional sausages produced with a half reduced sodium nitrite concentration.

On the 1d closest close to the control samples C was a* values of samples RPE_3 while on the 6d - a* values of samples RPE_4 (Table 3). Compared to the control samples C and C½, the a* value remained stable during the all storage period (six days, 0-4°C) in samples RPE_4 and RPE_5 with 0.05% and 0.03% rose petal extract addition. Those results confirming that the addition of 0.05% RPE in sausages with half nitrite content and 0.03% RPE in sausages with 1/3 reduced nitrite content relatively well stored red component of the color of the cross-sectional surface view of the studied functional sausages, compared to the other samples.

During the storage period different trends in yellow color component was found (Table 3). For five of samples (C½, RPE_1, RPE_2, RPE_3, RPE_4, RPE_5) the b* values decreases and a little increase only in control sample with 100% nitrite content was established (p>0.05).

Dynamics of the changes of color characteristics of the cross-sectional surface view of the sausages during of 60 min exposure on the air

The L* values of samples C was stabled and not statistically significantly (p > 0.05) different throughout the entire studied period of 60 minutes.
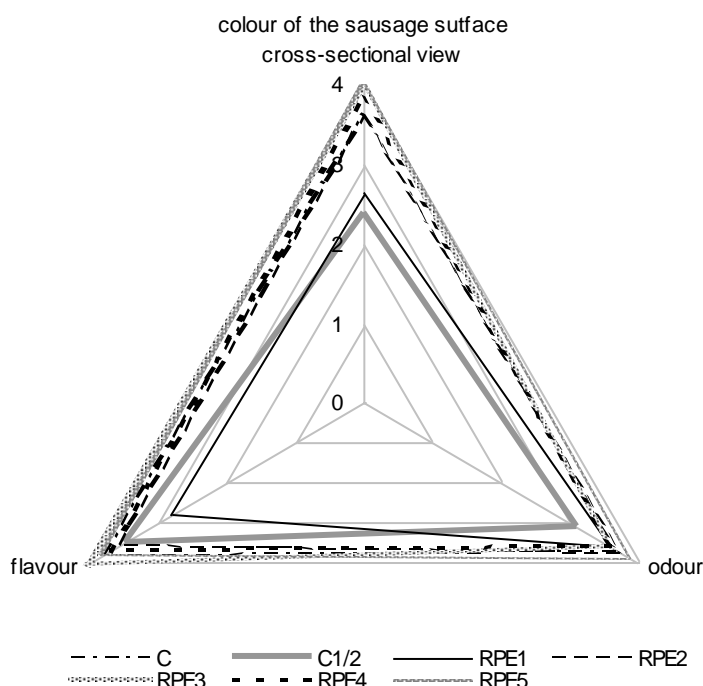
Figure 1. Sensory evaluations of the sausage color surface sectional view, flavour, and odor

Table 3. Changes of the color characteristics (L*, a*, b*) of the sausage cut surface

Color characteristics	Samples	Storage time at 0-4ºC	
		1 d	6 d
L*	C	60,41±0,12 [a]	59,69±0,18 [b]
	C½	61,35±0,22 [a]	59,08±0,23 [b]
	RPE$_1$	59,28±0,21 [a]	58,49±0,12 [a]
	RPE$_2$	58,67±0,18 [a]	58,65±0,18 [a]
	RPE$_3$	58,07±0,2 [a]	59,37±0,15 [b]
	RPE$_4$	59,23±0,16 [a]	58,79±0,12 [b]
	RPE$_5$	59,06±0,13 [a]	58,59±,17 [b]
a*	C	18,5±0,14 [a]	19,6±0,15 [b]
	C½	14,73±0,18 [a]	15,53±0,19 [b]
	RPE$_1$	13,65±0,13 [a]	13,37±0,16 [b]
	RPE$_2$	14,03±0,09 [a]	13,67±0,23 [b]
	RPE$_3$	15,03±0,22 [a]	13,06±0,18 [b]
	RPE$_4$	14,87±0,18 [a]	14,36±0,37 [a]
	RPE$_5$	14,51±0,27 [a]	13,95±0,28 [a]
b*	C	9,87±0,15 [a]	10,08±0,15 [a]
	C½	11,44±0,17 [a]	8,79±0,25 [b]
	RPE$_1$	8,93±0,12 [a]	8,18±0,16 [b]
	RPE$_2$	9,38±0,22 [a]	9,02±0,3 [a]
	RPE$_3$	8,45±0,3 [a]	7,15±0,28 [b]
	RPE$_4$	8,91±0,17 [a]	8,58±0,4 [a]
	RPE$_5$	8,77±0,4 [a]	8,73±0,32 [a]

[a, b,] *Means ± SD, in the same row with different superscripts are statistical different (p ≤ 0.05)*
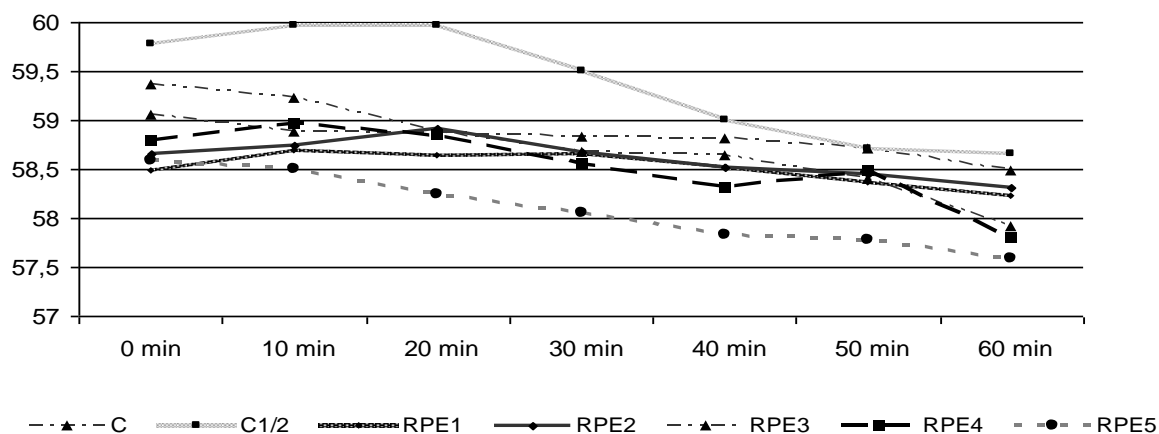
Figure 2. Dynamics of the changes in the color brightness (L* value) of sausage surface cross-sectional views during the 60 minutes air exposure after 6 days storage at 0 - 4⁰C
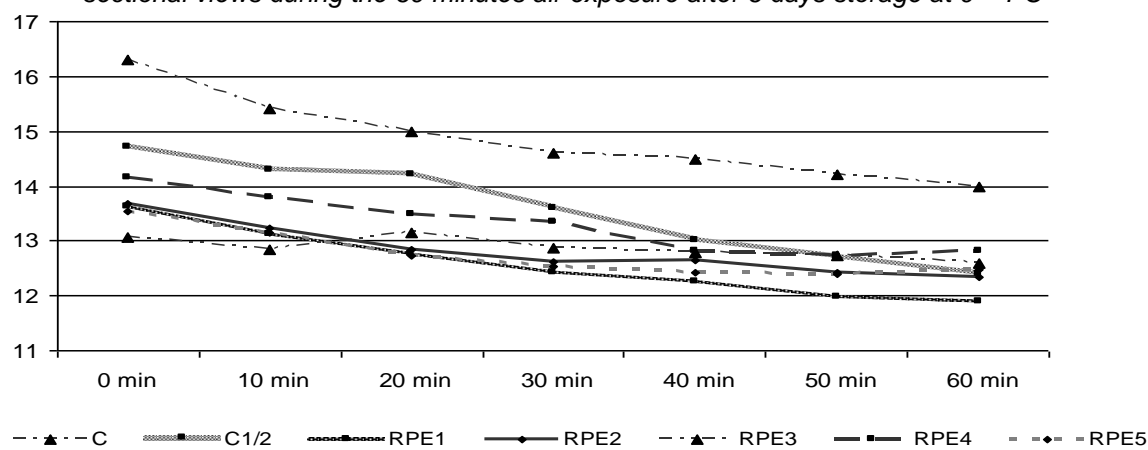
Figure 3. Dynamics of the changes in the red component of the color (a* value) of sausage surface sectional views during the 60 minutes air exposure after 6 days storage at 0 - 4⁰C
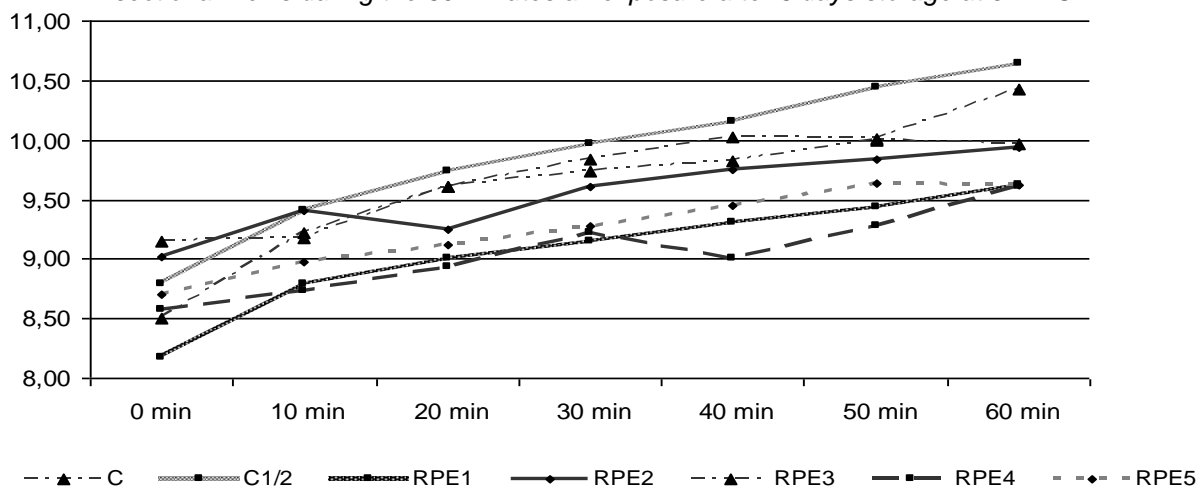
Figure 4. Dynamics of the changes in the yellow component of the color (b* value) of sausage surface sectional views during the 60 minutes air exposure after 6 days storage at 0 - 4⁰C

After 60 min of air exposure closest to control samples C was the color brightness (L*) of the samples RPE_4. The L* value decrease was observed for samples C½ and RPE_1- after 20 min, RPE_3 RPE_4 RPE_5 - after 30 min on air exposure (Fig. 2). For all studied period of 60 minutes a little decrease with only 0.96% was established in samples RPE_3.

The a* values of all examined samples decreased statistically significantly ($p < 0.05$) throughout the entire period of 60 minutes (Fig. 3). The decrease of a* values ($p < 0.05$) was observed for samples sample C, C½ and RPE_1 - after 10 min, for the samples RPE_2, RPE_4,

RPE$_5$ - after 20 min, while for rest sample RPE$_3$ - after 30 min of exposure on the air. After 60 min the minimal decrease was observed in a* value at samples RPE$_3$, and RPE$_5$.

The b* values of all examined samples were increased statistically significantly (p ≤ 0.05) throughout the entire period of 60 minutes (Fig. 4). After 60 min of air exposure the b*value in samples with rose petal extract addition were more stable compared to the control samples.

The results of the influence of the studied additives and their concentrations on the color characteristics indicate that rose petal extract had the positive effect and preserved the color characteristics of the sausages with half nitrite content.

CONCLUSIONS

The enrichment of cooked sausages with halfway reduced amounts of nitrites with rose petal extract (*Rosa Damascena Mill*) can be used as a technological capability to develop new functional meat products. The results indicate that the application of 0.03 or 0.05 % rose petal extract (*Rosa Damascena Mill*) can guarantee good sensory quality and preservation of color characteristic of new functional sausages. The addition of 0.05% rose petal extract with reducing the nitrite content to 10% was not found so effectively.

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PHOSPHORUS CONTENTS IN THE LONGISSIMUS DORSI AND SEMIMEMBRANOSUS MUSCLES FOR FIVE PUREBRED PIGS FROM VOJVODINA (NORTHERN SERBIA)

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ABSTRACT

The content of phosphorus was investigated in *M. longissimus dorsi* and *M. semimembranosus* for five purebred pigs (Large White – LW, n = 8; Landrace – L, n = 7; Duroc – D, n = 6; Hampshire – H, n = 7; Pietrain – P, n = 7), produced in Vojvodina, northern Serbia. Phosphorus was determined by standard spectrophotometric method. The difference in the phosphorus content among the five purebred pigs was not significant in the analysed longissimus dorsi (P=0.775) and semimembranosus (P=0.108) muscles. Also, muscle had no significant effect on the phosphorus content (P=0.162). The order of the purebred pigs regarding phosphorus content in the longissimus dorsi muscle samples (in mg/100g) was: H (202–251, on average 227) > L (211–239, on average 223) > LW (201–238, on average 222) > D (207–236, on average 219) > P (204–239, on average 217). The average phosphorus content in all investigated longissimus dorsi muscle samples was 222 mg/100g. The order of the purebred pigs regarding phosphorus content in the semimembranosus muscle samples (in mg/100g) was: L (232–243, on average 238) > LW (202–248, on average 227) > H (211–236, on average 226) > D (212–243, on average 225) > P (204–239, on average 217). The average phosphorus content in all investigated semimembranosus muscle samples was 227 mg/100g. The Vojvodian pig meat showed similar phosphorus content compared with the values found in other countries.
Keywords: *pigs, M. longissimus dorsi, M. semimembranosus, phosphorus*

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 macro-minerals 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.

Phosphorus is widely found in many food groups, largely as phosphate(s). Dietary sources that are rich in phosphorus include red meats (1600 mg/kg), dairy products (>900 mg/kg), fish (4000 mg/kg), poultry (2100 mg/kg) and bread and other cereal products (>900 mg/kg). A number of phosphate salts are used in foods and soft drinks as additives. Phosphorus is also used in food supplements (at levels up to a daily dose of 1100 mg/day) and licensed medicines, in the form of inorganic phosphate salts and sodium acid phosphate, respectively (EVM, 2003). Phosphorus requirements are conventionally set as equal to calcium in mass terms, i.e. 1 mg phosphorus: 1 mg calcium (COMA, 1991). According to FDA (2009), the Recommended Dietary Allowance (RDA) for phosphorus is 1000 mg for adults and children four or more years of age.

Phosphorus is abundant in the body with largest amounts found in bone. It is also found in all soft tissues including muscle, liver, heart and kidneys (EVM, 2003). Phosphorus is a constituent of all major classes of biochemical compounds. Structurally, phosphorus occurs as phospholipids, which are a major constituent of most biological membranes, and as nucleotides and nucleic acids. Phosphorus plays an important role in carbohydrate, fat and protein metabolism and is essential for optimum bone health. The energy that is required for most metabolic processes is derived from the phosphate bonds of adenosine triphosphate and other high energy phosphate compounds (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 aim of this study was to investigate the phosphorus levels of *M. longissimus dorsi* and *M. semimembranosus* of five pig purebreds used nowadays in Vojvodina for pork production, and to determine the possible effects of breed or muscle on phosphorus levels.

The overall objective is to produce high quality pork to ensure the competitiveness of Vojvodian pork in the international meat markets, and to update and improve regularly nutrient compositional data of meat.

MATERIAL AND METHODS

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 total phosphorous (P) content was determined by a colorimetric method after dry ashing mineralization of samples, according to ISO method (ISO 13730, 1996).

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 phosphorus in M. longissimus dorsi and M. Semimembranosus

Element	P
Certified concentration (mg/kg)	1080±110
Recovery (%)	98.1

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 (2012) was used for analysis.

RESULTS AND DISCUSSION

Average concentrations, standard deviations and ranges of phosphorus in the *M. longissiumus dorsi* and *M. semimembranosus* tissue samples from five different purebred pigs are presented in Table 2. The order of the purebred pigs according to average phosphorus content in the *M. longissiumus dorsi* samples in mg/100g was: H (202–251, on average 227) > L (211–239, on average 223) > LW (201–238, on average 222) > D (207–236, on average 219) > P (204–239, on average 217). Phosphorus levels found in the present study did not differ significantly ($P = 0.775$) among *M. longissiumus dorsi* for the different purebred pigs. The average phosphorus content in all investigated longissimus dorsi muscle samples was 222 mg/100g.

Table 2. Phosphorus levels (mg/100g wet weight) in the M. longissiumusdorsi and M. semimembranosus of various purebred pigs from Vojvodina

Muscle	Purebred	LW	L	D	H	P	*P* value[1]	All animals
LD	X	222	223	219	227	217	0.775	222
	Sd	14	10	10	19	15		14
	Min	201	211	207	202	204		201
	Max	238	239	236	251	239		251
SM	X	227	238	225	226	217	0.108	227
	Sd	17	5	13	9	12		13
	Min	202	232	212	211	204		202
	Max	248	243	243	236	239		248
	P value[2]	0.569	0.011	0.457	0.910	0.968		0.162

[1] *indicates significant of difference between purebred within raw*
[2] *indicates significant of difference between LD and SM within column*

The order of the purebred pigs according to average phosphorus content in the *M. semimembranosus* samples in mg/100g was: L (232–243, on average 238) > LW (202–248, on average 227) > H (211–236, on average 226) > D (212–243, on average 225) > P (204–239, on average 217). Phosphorus levels found in the present study did not differ significantly (P = 0.108) among *M. semimembranosus* for the different purebred pigs. The average phosphorus content in all investigated semimembranosus muscle samples was 227 mg/100g. Muscles had no significant effect on the phosphorus content (P = 0.162).

The average phosphorus contents in the *M. longissimus dorsi* and *M. semimembranosus* found in this study were at the same level compared to the data presented in the food composition tables of other countries (loin: 217 mg/100g; LD: 195 mg/100g and SM: 175 mg/100g, Denmark – National Food Institute, 2009; ham: 233 mg/100g, Italy – INRAN, 2007; loin: 210 mg/100g and ham: 210 mg/100g, Norway – The Norwegian Food Safety Authority, 2006; loin: 211 mg/100g and ham: 229 mg/100g, USA – Romans *et al.*, 1994, The US Department of Agriculture's, 2009). Additionally, the average phosphorus 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 Finland (loin: 180 mg/100g; ham: 160 mg/100g) (Finland – National Institute for Health and Welfare, 2009).

Table 3. Contribution of *M. longissimus dorsi* and *M. semimembranosus*to RDA* for phosphorus 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 (1000 mg/day)	Average	22.2	22.7
	Min	20.1	20.2
	Max	25.1	24.8

* RDA –Recommended Dietary Allowance
** Daily values based on a caloric intake of 2.000 calories (FDA, 2009)

According to obtained results (Table 3), the phosphorus content in 100 g of *M. longissimus dorsi* and *M. semimembranosus* of pigs produced in Vojvodina contributes minimally 20.1% (up to 25.1%) of the RDA value.

CONCLUSIONS

The results of the present study show that the levels of phosphorus in the *M. longissimus dorsi* and *M. semimembranosus* were not influenced by purebred or muscle. The Vojvodian pig meat showed similar P content compared with the values found in other countries.

ACKNOWLEDGEMENTS

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MEAT QUALITY AND EFFECT OF DRYING CONDITIONS ON COLOR, TEXTURAL AND SENSORY ATTRIBUTES OF PETROVSKÁ KLOBÁSA

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ABSTRACT

In this study the colour, textural and sensory attributes of *Petrovská klobása*, Serbian dry-fermented sausages with protected designation of origin at national level, dried in traditional and industrial conditions, were analysed at the end of drying process. *Petrovská klobása* sausages were made from meat and back fat of Landrace pigs (9-12 months old animals of live weight above 130 kg) with addition of spices (red hot paprika powder, raw garlic paste, caraway), salt and sugar. Sausages were produced in the household, manually stuffed in collagen casings (500 mm long and 55 mm in diameter) and divided in two batches: sample BT - drying process took place in the household, in naturally ventilated storeroom and sample BI - drying process took place in industrial chamber, under controlled conditions.

Quality of meat used for sausage processing was determined by criteria for pH, water holding capacity (WHC) and colour (CIE L^*) 24h *post mortem*. The colour measurements (CIE L^*, a^*, b^*, h, C^*, R, Bi) of sausages were carried out on the fresh cut slices. TPA test was performed for evaluation of texture attributes of sausages. Sensory attributes of sausages (colour, texture and juiciness) were evaluated by a panel of 10 trained panellist using scale from 0 to 5 (0 – atypical, 5 - optimal).

According to analysed parameters and quality criteria pork used for sausage production have red, firm and non-exudative (RFN) quality. At the end of drying process BT sausages had significantly ($P<0.05$) lower L^*, a^*, b^*, h, C^* and Bi values, and significantly higher R value. Values for textural attributes of sausages (hardness, springiness, cohesiveness and chewiness) were significantly ($P<0.05$) higher for sausages BT. Also, scores for sensory evaluation of colour and texture were significantly ($P<0.05$) higher for BT sausages, comparing to BI sausages.

Keywords: fermented sausage, meat quality, colour, texture, sensory quality

INTRODUCTION

Although many fermented sausages are commonly produced in industrial plants, there are still regions in Europe where these products are obtained through traditional technologies (Casaburi *et al.*, 2007). *Petrovská klobása* is traditional dry fermented sausage from the north of Serbia (City of Bački Petrovac in the Autonomous Province of Vojvodina) with protected designation of origin at national level. *Petrovská klobása* is usually manufactured traditionally, according to original recipe of the ancestors. As many other traditional dry fermented sausages it is produced without added starter culture (Petrović *et al.*,2007; Tasić *et al.*, 2012; Ikonić *et al.*, 2013).The required microorganisms originate from the meat itself or from the environment, and constitute a part of the so-called ''house-flora'' (Casaburi *et al.*, 2007). Sausages are only produced during winter, at low atmospheric temperatures, undergoing a more or less prolonged process of drying-ripening before consumption. *Petrovská klobása* is produced from pork meat and pork back fat, with addition of specific spices like red hot paprika powder, garlic and caraway, and with addition of salt and sugar. At the end of ripening *Petrovská klobása* is characterised by specific savoury taste, aromatic and spicy flavour, dark red colour and hard consistency (Petrović *et al.*,2007; Tasić *et al.*, 2012; Ikonić *et al.*, 2013). Meat and back fat used for sausage production are from Landrace pigs, animals from prolonged fattening, which are from 9 to 12 months old and more than 130 kg of live weight (Petrović *et al.,* 2007). The sensorial quality of dry sausages depends

on raw material characteristics and on technological parameters and is determined by the formation of end products, originating from the breakdown of proteins, lipids and carbohydrates (Spaziani *et al.*, 2009). The technological quality attributes of meat include its pH value, color, texture, water-holding capacity (WHC), and chemical composition (Rosenvold and Andersen, 2003; Olsson and Pickova, 2005). Fresh pork has been traditionally classified into three technological quality categories according to measurements of pH value, color, firmness (texture) and drip loss (exudation): PSE (pale, soft, exudative), RFN (reddish-pink, firm, non-exudative or normal pork) and DFD (dark, firm, dry). Beside these three traditional categories, during the last two decades many authors introduced two additional intermediate quality variations lying between them: RSE (reddish-pink, soft, exudative) and PFN (pale, firm, non-exudative) (Džinić, 2005; Tomović *et al.*, 2008; Faucitano *et al.*, 2010; Van de Perre *et al.*, 2010). Colour is probably the most influential characteristic of meat products, and its modifications with respect to the original meat product directly affect the quality and acceptability of the product. Off coloured food is likely to be rejected even though it may have good flavour and texture. The addition of paprika confers redness to sausages because of its high content of carotenoid pigments, it also contributes to characteristic flavour and taste, which differentiates such products from others (Bozkurt and Bayram, 2006; Gómez *et al.*, 2008). Beside colour, texture as a multi-parameter attribute, is one of the most important components of meat products quality. Many factors affect final texture of fermented sausages, including ingredients used, processing parameters, acidification method, drying/ripening conditions, as well as interactions among these factors over an extended period of time (Gonazales-Fernandez *et al.*, 2006; Barbut, 2007).

In order to meet market demands and produce larger quantities of *Petrovská klobása* with standard quality, and due to long processing time and dependence on natural climatic conditions, manufacturers are looking for some modifications in the traditional method like production of this fermented sausage in controlled, industrial conditions.

Thus, the objective of this study was to determine the raw meat quality, used for sausage production, and to determine instrumental and validate sensory characteristics of the colour and textural for *Petrovská klobása* dried in traditional manner (household) and in industrial chamber (controlled conditions) at the end of drying process.

MATERIAL AND METHODS

For sausages production lean pork and back fat were obtained from Landrace pigs. The animals were farmed in standard production system, with prolonged fattening period (9-12 months; live weight above 130 kg). Technological quality parameters were measured 24 hours *post mortem*. The pH value was measured using the portable pH meter (Consort T651, Turnhout, Belgium) equipped with an insertion glass combination electrode (Mettler Toledo Greifensee, Switzerland). According to pH_{24h} the potential (24h *post-mortem*) pork quality was classified as normal (pH<6.2) and DFD (pH≥6.2) (Džinić, 2005). Eight replicate measures of meat surface colour were performed on each sample, after 60 min of blooming at 3°C. The CIE *L** (lightness), CIE *a** (redness) and CIE *b** (yellowness) colour coordinates (CIE, 1976) were determined using MINOLTA Chroma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan) using D-65 lighting, a 2° standard observer angle and a 8-mm aperture in the measuring head. The colour quality was determined according to criteria for pork (pale colour: CIE *L**>50; reddish-pink colour: CIE *L**≤50; dark colour: CIE *L**≤43) (Džinić, 2005, Tomović *et al.,* 2008). Determination of the water-holding capacity (WHC) was assessed using a filter paper press method (Grau and Hamm, 1953).The WHC was expressed as percent of bounded water and WHC quality was determined according to criteria for pork (exudative meat: <0.50; non-exudative meat: >50; dry meat: >0.50) (Džinić, 2005).

Dry fermented sausages *Petrovská klobása* were manufactured in traditional way in rural household. All sausages were produced from a mixture of lean minced pork (80%) and pig fat (20%). The other added ingredients were: red hot paprika powder (2.5%), salt (1.8%), raw garlic paste (0.2%), caraway (0.2%) and sugar (0.1%). After grinding the meat and the fat to

a size of about 10 mm, spices were added and raw materials were manually mixed. The mixture was immediately manually stuffed in collagen casings, in units approximately 500 mm long and 55 mm in diameter. The sausages were stored in a cold room (0–4 °C) for 24 h, and after a resting day, were divided in two groups: BT – sausages were cold smoked in a traditional way and with specific kinds of wood during 10 days (with pauses), drying and ripening were under atmospheric conditions in naturally ventilated storerooms; BI – sausages were cold smoked, drying and ripening were under controlled (industrial chamber) conditions of temperature, relative humidity and air velocity. The production of sausages started in the end of November, when atmospheric temperatures were around 0°C or lower.

Moisture content, pH value, colour and texture instrumental measurements, and sensory analysis (colour and texture) were carried out at the end of drying process. Drying process is considered to be finished when moisture content is approximately 35%.

Moisture content was quantified according to the ISO recommended standard (ISO 1442:1997). pH value was determined using the portable pH meter, same as for pH measurements in meat.

Colour measurements were performed on the fresh cut of the sausage at room temperature using the same MINOLTA Chroma Meter and instruction as for measuring meat colour characteristics. Sausage colour characteristics were expressed by CIE *L*a*b** system (lightness - *L**, redness and greenness - *a**; yellowness and blueness - *b**). Hue angle (h), chroma (*C**), ratio of redness over yellowness (R) and browning index (Bi) were calculated using CIE *L*a*b**values (Bozkurt and Bayram, 2006; Ergunes and Tarhan, 2006):

$$h = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

$$C^* = \sqrt{a^{*2} + b^{*2}}$$

$$R = \frac{a^*}{b^*}$$

$$Bi = \frac{(100 - (x - 0.31))}{0.17} \qquad x = \frac{(a^* - 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)}$$

Texture profile analysis (TPA) was performed as described by Bourne (1978) with a universal testing machine Texture Analyser TA XP (Stable Micro System, Godalming, England). The samples (six cylinders) 2 cm high and 2.54 cm (1 inch) in diameter, taken from the centre of sausage, were equilibrated to room temperature and compressed twice to 50% of their original height at a constant speed of 1 mm/s. The following parameters from the force–time curves were determined: hardness, springiness, cohesiveness and chewiness. Sensory analysis (colour and texture) was conducted by a group of 10 experienced evaluators of different ages according to point system of analytical descriptive test using scale from 0 to 5 (5-optimal; 0-atypical product). All data are presented as mean values. Analysis of variance (Duncan test) was used to test the hypothesis about differences between obtained results. The software package STATISTICA 12.0 was used for analysis.

RESULTS AND DISCUSSION

Results of meat quality measurements are presented in Table 1. The measurement of pH values is the most direct way to obtained information about meat quality characteristics. Mean pH$_{24h}$ values was 5.50, what represents meat of normal quality since pH$_{24h}$ identifies pork quality as normal if pH<6.2. Further, mean WHC value was 82.07% representing, in accordance with meat quality criteria, meat of non-exudative or dry quality. Finally, mean *L** value was lower than 50 (46.40) representing red meat. Combining all the parameters and criteria for these parameters obtained results showed that meat used for *Petrovská klobása* production was of RFN quality – red, firm and non-exudative.

Table 1. Technological quality characteristics of meat (24 h post mortem) used for Petrovská klobása production

pH	WHC	*L**	*a**	b*
5.50 ± 0.06	82.07±2.52	46.40 ±0.26	12.41 ±1.51	5.53 ±1.07

Characteristics of *Petrovská klobása* at the end of drying process are presented in Table 2. Sausages BT were process under atmospherics conditions, which represented conditions of lower temperature and higher humidity, and had longer processing time. In other to archive demanded moisture content the drying process in industrial chamber lasted 45 and in the household (traditional way) 90 days. Differences in moisture content between samples were significant ($P< 0.05$), being lower for sausages BT. At the end of drying process pH values of sausages were 5.47 and 5.34 for the sausages of BT and BI group respectively, with significant ($P< 0.05$) difference between these values.

Results of colour measurements for CIE *L**, *a**, *b**, so as values calculated from *L**, *a** and *b** values like hue angle, chroma, ratio of redness over yellowness and browning index, of *Petrovská klobása* processed in different conditions, at the end of drying process are presented in Table 2.

Table 2. Characteristics of Petrovská klobása at the end of drying process

Characteristic	Sample		*P*
	BT	**BI**	
pH value	5.47 ± 0.02	5.34 ± 0.01	<0.05
Moisture content (%)	33.10 ± 0.10	37.54 ± 0.09	<0.05
L*	30.50 ± 1.76	34.21 ± 2.23	<0.05
a*	19.42 ± 2.03	24.34 ± 2.92	<0.05
b*	13.97± 2.61	20.58 ± 3.50	<0.05
h	35.53± 4.21	40.09 ± 2.43	<0.05
C*	23.97± 2.81	31.89 ± 4.36	<0.05
R	1.42 ± 0.21	1.19 ± 0.10	<0.05
Bi	104.25 ± 14.45	138.20 ± 30.45	<0.05
Hardness (g)	2225.89 ± 254.74	1494.02 ± 267.45	<0.05
Springiness	0.401 ± 0.015	0.358 ± 0.025	<0.05
Cohesiveness	0.565 ± 0.022	0.524 ± 0.013	<0.05
Chewiness (g)	503.55 ± 52.49	283.85 ± 74.68	<0.05
Sensory evaluation of colour	5.00 ± 0.0	4.25 ± 0.27	<0.05
Sensory evaluation of texture	5.00 ± 0	4.22 ± 0.19	<0.05

Processing method, i.e. different conditions of drying and ripening, significantly ($P< 0.05$) affected all colour characteristics of *Petrovská klobása.* BT sausages had significantly lower *L**, *a** and *b** values ($P< 0.05$) comparing to sausage BI. Lower *L** value represented formation of darker colour in the sausages due to the browning reaction (Bozkurt and Bayram, 2006). The difference in *L** values could also be correlated with the difference in moisture values. With the moisture loss the concentration of myoglobin in product increased, and on the other hand dehydrated muscle tissue absorbed a greater amount of light what resulted in a darker colour of the products, i.e. lower *L** value (Pérez-Alvarez *et al.*, 1999). In meat and meat products lightness (*L**) seems to be the most informative parameter for colour changes, but the importance of red (*a**) should not be ignored (Gimeno *et al.*, 2000). During production of dry fermented sausages *a** value increases as the result of formation of nitrosomyoglobin and moisture loss, and then again decreases by partial or total denaturation of nitrosomyoglobin because of the production of lactic acid (Bozkurt and Bayram, 2006). Also, it should be noted that probably the greatest influence on the share of the red colour in *Petrovská klobása* sausages had red hot paprika powder addition. During

processing red colour (*a**) of cut surface decreases as a result of oxidation of red hot paprika powder (Martinez *et al.,* 2007). Also, *b** value of analyzed sausages could probably be related to the presence of yellow carotenoids coming from red hot paprika powder (Gimeno *et al.,* 2000). Comparing the *L** and *a** values of both groups of sausages, with values reported for some other traditional dry fermented sausages *L** values were at the same level as for sucuk Bozkurt and Bayram, 2006), but lower than for *chorizo de Pamplona* (Gimeno *et al.,* 2000), while *a**values were higher than for sucuk (Bozkurt and Bayram, 2006), and on the same level as *for chorizo de Pamplona* (Gimeno *et al.,* 2000). Further, sausages BT were significantly (*P*< 0.05) lower in h, *C** and Bi value, but significantly (*P*< 0.05) higher in ratio of redness over yellowness (R value). Higher value of hue angle indicates the production of an orange-red colour of sausages. Chroma values is the indicator of color saturation and intensity, and as reported by Bozkurt and Bayram (2006) decreases during the ripening of sausages in parallel to the *b** values. Finally, colour of BT sausages was sensory evaluated with maximal score (5), what was significantly (*P*< 0.05) higher than score for sausages BI (Table 2).

Texture profiles of *Petrovská klobása* (hardness, springiness, cohesiveness and chewiness), produced in different conditions at the end of drying process are presented in Table 2.

Processing method, i.e. different conditions of drying and ripening, significantly (*P*< 0.05) affected all textural characteristics of *Petrovská klobása.* At the end of drying process hardness of sausage BT was significantly (*P*< 0.05) higher than for sausage BI. Hardness of sausages is partly the result of protein coagulation at low pH, and also partly the result of decreasing moisture content (Bozkurt and Bayram, 2006). Comparing the values of hardness, recorded for both groups of sausages, with values of hardness reported for some other traditional dry fermented sausages were lower than for sucuk (Bozkurt and Bayram, 2006) or chorizo de Pamplona (Gimeno *et al.*, 2000), but similar to those of Italian low-acid (Spaziani *et al.*, 2009) and slow fermented sausage analyzed by Olivares et al. (2010). Generally, the major changes in fermented sausage structure take place during fermentation when the pH declines and the myofibrillar proteins aggregate to form a gel (Spaziani *et al.*, 2009). Significant difference in hardness could be attributed to the differences in pH vales, and to difference in drying process that is to final moisture values. After fermentation, drying is a major factor affecting binding and rheological properties of fermented sausages (Gonzalez-Fernandez *et al.,* 2006; Spaziani *et al.*, 2009). Significant differences in chewiness values (*P*< 0.05) between samples could also be connected to difference in moisture content. Springiness, sometimes referred to as "elasticity" (Yang *et al.*, 2007), was also significantly affected by the production parameters, being higher (*P*< 0.05) for BT sausages. In addition to the springiness, cohesiveness, as a measure of the degree of difficulty in breaking down the internal structure of the sausage (Yang *et al.*, 2007), was also significantly affected by the production parameters and higher (*P*< 0.05) for BT sausage.

Finally, texture of sausages BT was sensory evaluated with maximal score (5), what was significantly (*P*< 0.05) higher than score for sausages BI (4.22) (Table 2).

CONCLUSIONS

The results obtained in this study showed that, as expected, meat of older animals revealed optimal quality for traditional dry sausage production, than drying process under controlled conditions of temperature, relative humidity and air velocity, lasted significantly less than in traditional manner, under atmospheric conditions.

Differences between sausages, processed in different conditions, in instrumental measurements of colour and texture were significant.

Sausages processed in traditional way had significantly higher scores for sensory evaluation of colour and texture.

ACKNOWLEDGEMENTS

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EVALUATION OF MASS TRANSFER KINETICS DURING OSMOTIC TREATMENT OF CELERY LEAVES

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ABSTRACT

In order to analyze mass transfer kinetics during osmotic treatment, celery leaves *(Apium graveolens)* was dehydrated in two different osmotic solutions (sugar beet molasses and the mixed solution of sodium chloride and sucrose) under atmospheric pressure, at three different temperatures (20°C, 35°C and 50°C). The main objective was to examine the influence of osmotic agents, temperature and dehydration time on the mass transfer phenomena during osmotic treatment. Response Surface Methodology (RSM) was used for optimization of osmotic treatment of celery leaves in respect of temperature, time and type of osmotic solution. The significance of used hypertonic solutions, temperature and immersion time on the various kinetics parameters: water loss (WL), solid gain (SG) and dehydration efficiency index (DEI), were tested during the process, using Analysis of variance (ANOVA). The results showed that the optimum process parameters for osmotic treatment of celery leaves were: osmotic time of 5 hours, sugar beet molasses as osmotic solution and temperature of 50°C.
Keywords: osmotic treatment, celery leaves, sugar beet molasses, mass transfer kinetics

INTRODUCTION

Celery is widely recognized as a healthy plant and spice and numerous research studies have proved that it contains many bioactive compounds. There is now a growing interest in the celery leaves for their potential healthy benefits, such as lowering cholesterol level, anti-inflammatory activity, antimicrobial activity and anticancer activity(Han and Row, 2011; Popović *et al.*, 2006). Average water content in celery leaves is high (more than 80%), and its dehydration provides improved physical, mechanical, chemical and microbiological stability (Ježek *et al.*, 2008). Many traditionally dehydration techniques and their combination are used to prevent a spoilage of food by reducing a water content, but these methods have negative effects on nutritive and sensorial properties on the final product (Nistor *et al.*, 2011, Ratti*, 2009). Compared to the other preservation treatments, osmotic treatment (OT), proved to be one of the most useful, primarily due to the low temperature and energy requirements, low waste material and good quality of final product. (Ramallo and Mascheroni, 2005). OT process represents partial removal of water from food (fruit, vegetable, meat), which is performed by immersing them in various hypertonic solution. (Sablani *et al.*, 2002; Koprivica *et al.*, 2010). Driving force for water removal is the concentration gradient between the surrounding hypertonic solution and the immersed plant material. The complex cellular structure of plant tissue acts as a semi-permeable membrane, which allows two main countercurrent flows: water outflow from the plant tissue into the osmotic solution whereas solute transfer from solution into the tissue (Lević *et al.*, 2007, Mišljenović *et al.,* 2009). The main aim of OT process is to provide maximum removal of water from the material (WL), with a simultaneous minimum adoption of dry matter from osmotic solution (SG), in order to obtain a better quality of treated product. Mass transfer mechanisms during OT are affected by many factors such as composition and concentration of osmotic agents, immersion time of the product in the solution, operating temperature, solution to sample ratio, nature and thickness of food material and pre-treatment (Ćurčić *et al.*, 2013; Koprivica *et al.*, 2010).

Considerable influence on the kinetics of WL and SG has the type of osmotic agent. Concentrated sucrose solution, sodium chloride solutions and their combinations are usually used as hypertonic solution (Mišljenović *et al.,* 2012).

Recent research has shown that use of sugar beet molasses as a hypertonic solution improves OT processes (Lević *et al.*, 2007). High content of dry matter (around 80%) provide high osmotic pressure in the molasses and maintains a high transfer potential favorable to WL during OT and thus enhances the efficiency of this process. On the other hand, specific chemical composition of molasses enriches chemical and nutritional composition of dehydrated products.The application of sugar beet molasses as osmotic agent has many advantages: it is sensory acceptable, always accessible in large quantities and cheap raw material and could be used as a replacement for sucrose (Filipović *et al,* 2012; Ćurčić *et al.*, 2013).

The objective in this study was to investigate the influence of temperature, immersion time and the type of osmotic solution on the efficiency of OT process of celery leaves. To assess the quality of these products their water loss (WL), solid gain (SG) and dehydration efficiency index (DEI) have been determined. Experimental results have been subjected to analysis of variance (ANOVA) to show relations between applied assays. In order to enable more comprehensive comparison between investigated samples, standard score (SS) has been introduced.

MATERIAL AND METHODS

Celery leaves (*Apium graveolens*) for the experiment were purchased on the local market, shortly before their use. Prior to the treatment, celery leaves were cut into small squares of dimension approximately 1x1 cm. Initial moisture content of celery leaves was 80.92%. As hypertonic solutions two different solutions were used. The first one, concentrated sugar beet molasses, with initial dry matter content of 80.00%, was obtained from the sugar factory Pećinci, Serbia. The second osmotic medium was ternary solution, prepared by mixing commercial sugar (in the quantity of 1.200 g/kg water) and NaCl (in the quantity of 350 g/kg water) with distilled water. The material to solution ratio of 1:20 (w/w) was used during all experiments. After measuring the initial mass, the samples of celery leaves were submerged in laboratory jars and the immersion lasted for 1, 3 and 5 hours, under atmospheric pressure. The OT process was performed at the three different temperatures, 20°C, 35°C and 50°C. Samples were withdrawn from the osmotic solution at determined intervals of time, then lightly washed with water and gently absorbed with paper towels to remove excessive water from the surface. The next step was to measure the mass of the dehydrated samples. After that, dry matter content of all samples was determined by drying in a heat chamber at 105°C for 24h (Instrumentaria Sutjeska, Croatia) until constant weight. All analytical measurements were carried out in accordance to AOAC (2000). In order to follow the mass transfer kinetics of the OT, three key process variables were measured: moisture content, change in weight and change in the soluble solids. Based on the experimental data, water loss (WL), and solid gain (SG), were calculated, as described by Mišljenović *et al.*, 2009.

Important process parameters as a function of different type of osmotic solution and dehydration time were analyzed using the ANOVA. During ANOVA calculation, the independent variables were: immersion time (X_1) - 1, 3 and 5h; temperature (X_2) - 20°C, 35°C and 50°C and the type of osmotic solution (X_3) - sugar beet molasses (1) and ternary solution (2), and the dependent variables were the responses: WL (Y_1) and SG (Y_2). Two mathematical models of the following form were developed to relate two responses (Y) to two process variables (X):

$$Y_k = \beta_{k0} + \sum_{i=1}^{2} \beta_{ki} X_i + \sum_{i=1}^{2} \beta_{kii} X_i^2 + \beta_{k12} X_1 X_2, \ k\text{=1-2,} \qquad (1)$$

where: β_{k0}, β_{ki}, β_{kii}, β_{k12} are constant regression coefficients, k-index.

Min-max normalization is a technique which is commonly applied for comparison of various characteristics of complex samples determined using multiple assays, where samples are classified on the basis of the ratio of raw data and extreme values of the measurement used. Considering that the scale of the data from various parameters concerning mass transfer (WL and SG) are different, the data in each data set should be transformed into normalized scores, in accordance with the following equations:

$$\overline{x}_i = 1 - \frac{\max_i x_i - x_i}{\max_i x_i - \min_i x_i}, \quad \forall i \ , \tag{2}$$

in case of "the higher, the better" criteria, used for WL score calculation, or

$$\overline{x}_i = \frac{\max_i x_i - x_i}{\max_i x_i - \min_i x_i}, \quad \forall i \ , \tag{3}$$

in case of "the lower, the better" criteria, used for SG score calculation.
Obtained data have been subjected to analysis of variance (ANOVA) for the comparison of means, and significant differences are calculated according to post-hoc Tukey's HSD ("honestly significant differences") test at p<0.05 significant level, 95% confidence limit. All statistical analyses of the collected results have been performed using StatSoft Statistica 10.0® software.

RESULTS AND DISCUSSION

Table 1 provides an overview on the average values and standard deviations of *WL* and *SG* parameters, as a function of different type of osmotic solution, temperature of process and dehydration time. Dehydration efficiency index-DEI (WL/SG ratio) were calculated and shown in the table 1. This ratio is considered to best determine optimal condition for the OT. High DEI ratios point to intensive water removal from the samples accompanied with minimal solid uptake. Standard score (SS) analysis was also calculated using min-max normalization, and the SS of samples are written in table 1.

Table 1. Experimental results for celery leaves during osmotic treatment

Time	Temperature	Sugar beet molasses				Ternary solution			
		WL	SG	DEI	SS	WL	SG	DEI	SS
1	20	0.28±0.02[a]	0.19±0.06[a]	1.48	0.20	0.32±0.07[a]	0.20±0.07[abc]	1.62	0.20
1	35	0.31±0.05[a]	0.11±0.04[ab]	2.82	0.54	0.37±0.04[ab]	0.13±0.04[ab]	2.83	0.51
1	50	0.38±0.03[ab]	0.15±0.02[abc]	2.59	0.46	0.41±0.01[abc]	0.14±0.00[ab]	2.95	0.52
3	20	0.34±0.12[a]	0.13±0.02[a]	2.65	0.49	0.40±0.05[ab]	0.18±0.02[abc]	2.20	0.34
3	35	0.55±0.04[cde]	0.16±0.01[abc]	3.53	0.59	0.57±0.04[def]	0.17±0.03[abc]	3.35	0.55
3	50	0.74±0.01[g]	0.18±0.02[abc]	4.19	0.69	0.66±0.02[efg]	0.20±0.02[abc]	3.28	0.51
5	20	0.49±0.08[bcd]	0.18±0.01[abc]	2.69	0.42	0.50±0.02[bcd]	0.24±0.02[c]	2.11	0.22
5	35	0.72±0.01[fg]	0.19±0.02[abc]	3.80	0.62	0.71±0.06[fg]	0.20±0.01[abc]	**3.51**	**0.56**
5	50	0.79±0.02[g]	0.15±0.02[abc]	**5.30**	**0.85**	0.69±0.03[efg]	0.23±0.01[bc]	3.05	0.44
Polarity		+	-			+	-		

The results are presented as mean±SD; Different letter within the same column indicate significant differences (p <0.05), according to Tukey's test. Polarity: '+' = the higher the better criteria, '−' = the lower the better criteria

Because of the great difference in osmotic pressure between hypertonic solution and the celery leaves tissue, loss of water is fast at the beginning of the process. But, increasing the dehydration time resulted in greater removal of water from the all samples, regardless of the type of solution. The highest value of WL was achieved at temperature of 50°C, after 5 hours of OT in sugar beet molasses (0.79 g/g i.s.w.). Table 1 shows that *SG* also had a tendency to increase with increasing the immersion time for most of the cases. Greater increase in values of SG was noticed in the samples which were treated in ternary solution, as compared to those treated with the molasses (which can be observed by overally higher DEI and SS values for molasses). It is important to keep solid uptake as low as possible during OT, and that is the reason why the most acceptable values for SG were achieved by using molasses as osmotic solution (higher DEI and SS values). Based on the data in the Table 1, it was observed that temperature is also an important factor, which considerable affects on the values of WL, from all samples. But, in the case of the SG, only linear terms of time and type of osmotic solution have a significant influence on the SG calculation. Optimum OT conditions which define maximum WL with lesser SG were determined using DEI and SS (Table 1). The maximum value of DEI that indicates the most efficient dehydration process was 5.30, achieved by immersion of celery leaves for 5 hours, at temperature of 50°C, in sugar beet molasses. Maximum SS (0.85) was observed when using sugar beet molasses solution, after 5h and at temperature of 50°C. The most appropriate process conditions for samples treated in ternary solution were: temperature of 35°C and immersion time of 5 hours (DEI = 3.51; SS=0.56).

Table 2. ANOVA table (sum of squares for each assay)

Factor	dF	WL	SG
Solution	1	0.000	0.004[*]
Time	1	0.279[*]	0.006[*]
Time^2	1	0.010[**]	0.000
Temp.	1	0.151[*]	0.000
Temp.^{2}	1	0.006	0.001
Solution x Time	1	0.004	0.001
Solution x Temp.	1	0.005	0.000
Time x Temp.	1	0.011[**]	0.000
Error	9	0.027	0.006
r^2		0.945	0.685

[*]*Significant at p<0.05 level,* [**]*Significant at p<0.10 level, 95% confidence limit, error terms have been found statistically insignificant*

ANOVA analysis revealed that the observed variables contributed substantially in all of the cases to generate a significant second order polynomial (SOP) model. Calculation of WL was mostly affected by linear terms of treatment time and processing temperature (statistically significant at *p*<0.05 level, 95% confidence level).. Interchange term of these two process variables was also influential for WL calculation, as well as quadratic term of immersion time (both statistically significant at p<0.10 level). *SG* calculation was more influenced by immersion time and the type of used solution, statistically significant at *p*<0.05 level. Also shown in Table 2 is the residual variance where the lack of fit variation represents other contributions except for the second order terms. All SOP models had insignificant lack of fit tests, which means that all the models represented the data satisfactorily. A high *r^2* is indicative that the variation was accounted and that the data fitted satisfactorily to the proposed model. The *r^2* values for *WL* (0.945) and *SG* (0.685), were found satisfactory and showed the good fitting of the model to experimental results.

In Figure 1, it is shown how the WL (a) and SG (b) vary depending on the temperature, time of dehydration, and type of used osmotic solution.
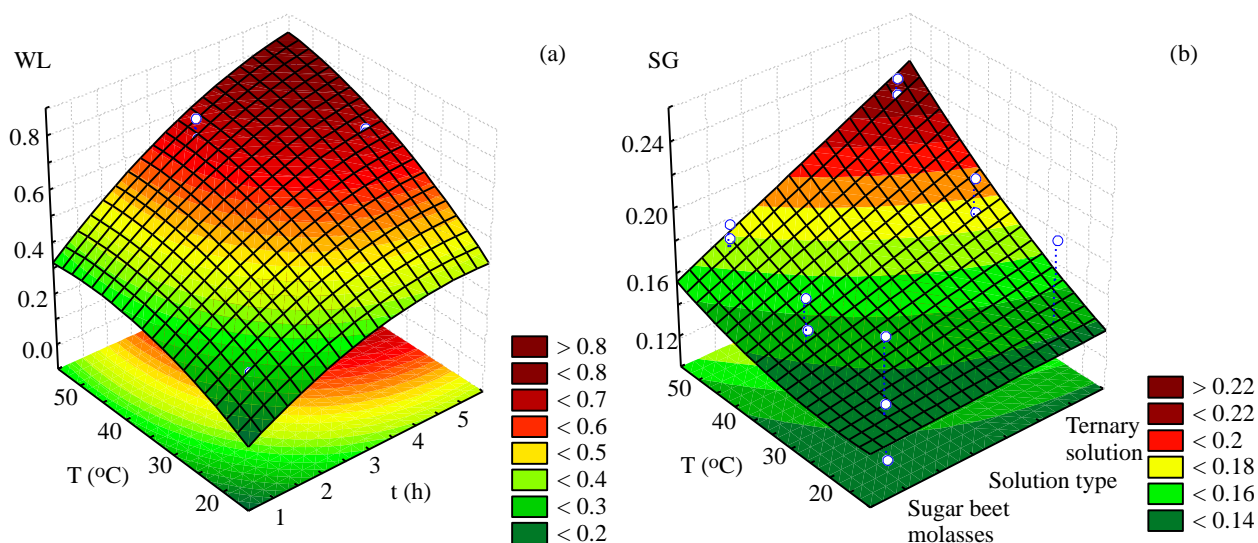
Figure 1. WL and SG for celery leaves during osmotic treatment

CONCLUSION

Efficiency of osmotic dehydration is directly dependent on temperature and duration of process, which both have great influence on complex mass transfer mechanisms. On the basis of presented results it can be concluded that both solutions are satisfying osmotic mediums, considering the favorable loss of water during both processes. According to results, optimal solution for drying celery leaves was sugar beet molasses, optimum temperature of process was 50^0C, while the optimum immersion time was 5 h. The predicted responses for the optimum dehydration conditions in sugar beet molasses were: *WL* about of 0.79 and *SG* about 0.15 g/g i.s.w. (*DEI*=5.30, *SS*=0.85). Beside the fact that sugar beet molasses proved to be more effective osmotic solution, its use is also environmental and economically reasonable, because molasses is side product of sugar industry.

ACKNOWLEDGEMENTS

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MINERAL CONTENT AND MICROBIOLOGICAL PROFILE AFTER OSMOTIC TREATMENT OF NETTLE LEAVES

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ABSTRACT

The aim of this study was to investigate the effects of osmotic treatment performed in two different solutions, on mineral content and microbiological profile of nettle leaves. Drying process was conducted using low temperature regime to preserve original thermo-sensitive ingredients. Nettle leaves were dehydrated in order to lower the water activity and microbiological load.

Nettle leaves were dehydrated in sugar beet molasses (concentrated at 80%) and aqueous solution of sodium chloride and sucrose (sucrose in the quantity of 1.200 g/kg water, NaCl in the quantity of 350 g/kg water and distilled water - ASSS), at temperatures of 20°C, for 1 hour. Mineral content (Mg, Fe, Zn, Ca and K) was determined in fresh and dehydrated leaves.

The measured Mg content (expressed in relation to the dry mass) in fresh and osmotically treated leaves (in ASSS and molasses solution) were: 2.94%, 1.56% and 3.23%, respectively. Zn was found with the highest content: 23.20%, 15.01% and 27.32% (in fresh leaves, and treated in ASSS and molasses solution, respectively). Fe content was found with the lowest content in fresh leaves, and treated in ASSS and molasses solution: 1.42%, 0.98% and 1.53%. Ca was found with content of: 3.38%, 1.92% and 4.23%, while K content was: 5.83%, 3.02% and 7.51% (in fresh leaves, and treated in ASSS and molasses solution, respectively). The content of proteins in fresh leaves was 5.61% (expressed in relation to the dry mass).

Also, the change of the microbiological profile between the fresh and dehydrated nettle leaves was examined. It was noticed that the process of osmotic treatment does improve the initial microbiological profile of the nettle leaves due to increasing dry matter content and lowering aw value. Better results of the reduction of the observed microorganisms in nettle leaves were obtained in the process of osmotic treatment in molasses.

Keywords: *Osmotic treatment, Nettle leaves, Sugar beet molasses, Microbiological profile*

INTRODUCTION

Nettle (Urtica dioica L.), naturally found in pathway, field, and wildwood. Nettles are grown in mild climate areas, bottom of barriers, ruins and grassy places, between cultivated plants, street, and water runnels.(Semih Otles *et al*., 2012)

The presence of valuable biologically important compounds such as proteins, vitamins, phenolic components, macro and microelements, tannins, flavonoids, sterols, fatty acids, carotenoids and chlorophylls (Kukrić, *et al*., 2012, Rafajlovska *et al*., 2013), contributes to the utilization of stinging nettle in different ways. Stinging nettle is of high value in the folk medicine as well as in scientific medicine (Kukrića *et al*., 2012; Stanojević *et al*., 2013).

Traditionally, in herbal medicine stinging nettle is used as a diuretic agent and for the treatment of rheumatism and arthritis (El Haouari *et al*.,2006).

Osmotic treatment (OT) is a water removal process, based on soaking food (fruit, vegetable, meat and fish) in a hypertonic solution. In comparison to other drying treatments main advantages of OT process are water is removed in liquid form, at mild temperatures and osmotic solution can be reused (Koprivica *et al*.,2008; Ćurčić *et al.,* 2012)

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 (Mišljenović *et al.,* 2009; Koprivica *et al.*, 2010).

Recent research has shown that use of sugar beet molasses as a hypertonic solution improves OT processes . Sugar beet molasses proved to be excellent as the OT medium due to the high dry matter (80%) and specific nutrient content. Using sugar beet molasses as hypertonic solution is an important part of the material enrichment in minerals and vitamins, which penetrate from molasses into the plant tissue (Filipović *et al.,* 2012, Lević *et al.,* 2008)

The objective of this research was to examine the influence of two different osmotic solutions on the minerals content and microbiological profile of the nettle leaves after the process of OT as a confirmation of the process safety.

MATERIAL AND METHODS

The stinging nettle leaves were collected in the first week of October in 2013, near 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. Nettle leaves were cut into squares of dimensions approximately 1x1cm. Aqueous osmotic solution of sodium chloride and sucrose (ASSS) 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 (SBMS) from the sugar factory in Pećinci, Serbia. The material to solution ratio of 1:20 was used in all experiments. The experiments were performed under atmospheric pressure at the temperature of the process of $20^{o}C$. The process was performed in laboratory jars.

Samples of nettle leaves were dipped into ASSS and SBMS, and the immersion lasted for 1 hours. On every 15 minutes leaves samples in osmotic solutions were agitated to provide better homogenization of the osmotic solutions. After OT, nettle leaves samples were washed with sterilised water and gently blotted with paper towels to remove excessive water from the surface.

The dry material content of samples was determined by drying at 105 ^{o}C in a heat chamber until constant mass was achived (Instrumentaria Sutjeska, Croatia), the proteins content was determined from the nitrogen content by Kjeldahl method (AOAC, 978.04) using factor 6.25, and calculated as N x 6.25 (AOAC, 1995). Mineral content (Mg, Fe, Zn, Ca and K) was determined by examining:

-Content of K, Mg and Ca (was done accordant to the SRPS EN 1134:2005, by AAS method),

-Content of Fe and Zn (was determined according to the BS EN 15763:2009).

Determination of the Enterobacteriaceae was done by the SRPS ISO 21528-2.

RESULTS AND DISCUSSION

In the Table 1, data of the average dry material, a_w, microbiological profile, mineral and protein content in nettle leaves values and standard deviation of the fresh and OT nettle leaves in ASSS and SBMS at the $20^{o}C$ are shown. Fresh sample of nettle leaves in initial state had a_w of 0.935 ± 0.01 which is close to the optimum growth level of most microorganisms. In all samples after the process of OT lowered a_w values were observed which should mean the reduction of microbial load. The sample of nettle leaves treated in ASSS as osmotic solution showed lower a_w value (0.855 ± 0.03) in comparison to the nettle leaves treated in SBMS at the same temperature (0.882 ± 0.031). This is probably due to the higher values of solid gain in processed nettle leaves, which leads to decreasing in a_w values (Vereš, 1991).

Table 1. Dry mass, a_w, microbiological profile, mineral and protein content, in nettle leaves

	Fresh	**ASSS**	**SBMS**
DM	8.5±0.81	80.32±1.27	82.5±3.01
a_w	0.935±0.01	0.855±0.03	0.882±0.031
Enterobacteriaceae	<10	<10	<10
Mg	2.94±0.05	1.56±0.19	3.23±0.20
Zn	23.20±1.03	15.01±0.56	27.32±0.93
Fe	1.42±0.11	0.98±0.02	1.53±0.09
Ca	3.38±0.27	1.92±0.12	4.23±0.21
K	5.83±0.41	3.02±0.09	7.51±0.43
Protein	5.61±0.38	5.01±1.00	4.89±0.54

Considering that most nettle leaves spoiling bacteria do not grow below a_w = 0.91(Vereš, 1991), achieved a_w values of the nettle leaves 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 OT ensure a_w values which are within a specified range for nettle leaves quality and safety.

The results of the microbiological analysis of the fresh and dehydrated nettle leaves are presented in Table 1. Pathogenic bacteria such as *Salmonella spp.* which are frequent contaminants of food, 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 nettle leaves before and after OT in both osmotic solutions and these results are in accordance with the hygiene production criteria of the Serbian National Regulation (72/2010).

Number of *Enterobacteriaceae* in the fresh nettle leaves was <10 CFU/g (Table 1). After the OT number of Enterobacteriaceae in the dehydrated nettle leaves was <10 CFU/g in samples treated in both osmotic solutions.

Although, Serbian National Regulation (72/2010) doesn't determine reference values for the total number of *Enterobacteriaceae* for the nettle leaves 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 OT is hygienically safe.

In Table 1, the content of macro (K, Mg and Ca) and microelements (Zn and Fe) determined in the stinging nettle leaves is given. The stinging nettle leaves contained higher amount of K then Ca and Mg.

Mineral content (Mg, Fe, Zn, Ca and K) was determined in fresh and OT leaves. The measured Mg content (expressed in relation to the dry mass) in fresh and OT leaves (in ASSS and SBMS) were: 2.94±0.05%, 1.56±0.19% and 3.23±0.20%, respectively. Zn was found with the highest content: 23.2%, 15.01±0.56% and 27.32±0.93% 0±1.03 (in fresh leaves, and treated in ASSS and SBMS, respectively). Fe content was found with the lowest content in fresh leaves, and treated in ASSS and SBMS: 1.42±0.11%, 0.98±0.02% and 1.53±0.09%. Ca was found with content of: 3.38±0.27%, 1.92±0.12% and 4.23±0.21%, while K content was: 5.83±0.41%, 3.02±0.09% and 7.51±0.43% (in fresh leaves, and treated in ASSS and SBMS, respectively). The content of proteins in fresh leaves was 5.61±0.38% (expressed in relation to the dry mass).

CONCLUSIONS

The results of this study show that the stinging nettle can be considered as a source of valuable components. High content of proteins and minerals determined in leaves is of great importance for the introduction of the stinging nettle in nourishment, as well as from medicinal and phytotherapeutic point of view. Considering that the data given in the literature is in regard to the components in leaves, the determined values for the quantities of proteins and minerals in the stem of the stinging nettle are of significance, with indications of new directions for its utilization.

Achieved a_w values of OT nettle leaves indicate that growth of microorganisms in that environment would be inhibited. On the basis of measured a_w values can be assumed that OT of nettle leaves contribute to reduction of microbial load, providing products extended sustainability. The results of the microbiological analysis of the fresh and dehydrated material confirmed the validity of this assumption.

OT of the fresh nettle leaves in both osmotic solutions at the temperature of 20°C 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, OT represents energy efficient process, which provides safe products.

The microbiological profile of the OT nettle leaves has shown that the treatment is hygienically safe and semi products are microbiologically stable for further technological production.

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ZINC CONTENT IN DIFFERENT TYPES OF MEAT FROM THE SERBIAN MARKET

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ABSTRACT

Zinc is an essential element and vital micronutrient necessary for more than 300 biochemical reactions in the body. Insufficient intake of zinc leads to several disorders, but an excessive zinc intake can cause adverse acute effects. The Recommended Dietary Allowances (RDA) for Zn for adults over 19 years old is 11 mg/day for men and 8 mg/day for women.

The purpose of this study was to analyze and evaluate the levels of zinc in the beef, pork, turkey and chicken meat from the Serbian market. A total of 140 samples (29 samples of beef, 52 of pork, 29 of chicken and 30 of turkey) were collected from January 2014 to May 2014. The Zn analysis was performed using inductively-coupled plasma-mass spectrometry (ICP-MS) measuring ^{66}Zn isotope. In order to establish the differences between the zinc content in four types of meat, the obtained data were tested using one-way ANOVA and Tukey's test (95% confidence intervals).

The lowest and the highest obtained Zn levels were 3.93 mg kg^{-1} in chicken and 59.50 mg kg^{-1} in beef. The mean values for Zn content were 42.56±4.55 mg kg^{-1}, 30.51±5.88 mg kg^{-1}, 10.66±2.06 mg kg^{-1} and 9.94±1.00 mg kg^{-1} in beef, pork, turkey and chicken samples of meat, respectively. Statistical analysis of the data showed significant differences between the zinc content in all types of meat except between the chicken and turkey meat.

According to the data of the World Health Organization (WHO) the daily intake of meat for adult Serbian population is 114.1 g of mammalian and 55.6 g of poultry species. Taking into account the results of this study, RDA for Zn, as well as WHO data for meat daily intake, it can be concluded that meat intake (poultry and mammalian meat) provides 43.1% of Zn RDA for men and 59.3% of Zn RDA for women in Serbian population.

Keywords: zinc, beef, pork, chicken, turkey

INTRODUCTION

Meat is considered a highly nutritious food, previously considered essential to optimal human growth and development (Higgs, 2000). Although some investigations indicate that consuming of meat has been associated with various cancers (Bingham, 1999; Wie *et al.*, 2014), cardiovascular and metabolic diseases (Wu *et al.*, 2014), meat consumption has been important in human species evolution, especially the brain and intellectual development (Broadhurst *et al.*, 2002). Meat and meat products are important sources of a wide range of micronutrients which are vital in human health. It is particularly rich in high biological value protein, as well as micronutrients like iron, selenium, zinc and vitamin B12 (Pereira and Vicente, 2013).

Among all the vitamins and minerals, zinc shows the strongest effect on our all-important immune system (Pae *et al.*, 2012; Roohani *et al.*, 2013). Zinc is known as an essential trace element necessary for more than 300 metalloenzymes and fulfills many biochemical functions in human metabolism. The best sources of zinc are oysters (25 mg/100g), meat, especially red meat (5.2 mg/100g), nuts (3 mg/100g) and poultry (1.5 mg/100g), (www.zinc.org). Bioavailability of zinc is enhanced when consumed with animal protein, and is reduced by inhibitors such as phytate and oxalate. This explains the results from a few studies in which were shown that vegetarians had lower plasma zinc (Freeland-Graves, 1988). Thus, meat is important part of diet associated with good health and prosperity.

The aim of this study was to analyze and evaluate the levels of zinc in four types of meat, which were taken from Serbian market and ordinarily consumed by the population of Serbia.

On the other hand, data from this study were used in order to assessed daily intake of zinc by meat.

MATERIAL AND METHODS

Sample collection
A total, 140 meat samples were collected in Serbian markets from January 2014 to May 2014 (29 beef, 52 pork, 29 chicken and 30 turkey meat). After collection, samples were labelled and stored in polyethylene bags and frozen at -18°C prior to analysis.

Sample preparation
Meat samples were partially thawed at +4 °C 1 day before analysis. Approximately 0.3 g of samples were weighted into Teflon vessels. Samples were mineralized by adding 5ml nitric acid (p.a. SIGMA) and 1.5ml hydrogen peroxide (30%, p.a., MERCK). Microwave assisted digestion was performed in Milestone, Start D, Microwave Digestion System (Via Fatebenefratelli, 1/5-24010 Sorisole (BG), Italy). The microwave program consisted of three steps as follows: 5 min from room temperature to 180°C, 10 min hold 180°C, 20 min vent. After cooling at room temperature, the digested sample solutions were quantitatively transferred into disposable flasks and diluted to 100ml with deionized water (ELGA).
The analysis was performed by inductively-coupled plasma mass spectrometry (ICP-MS). Measurements were performed using the instrument "iCap Q" (Thermo Scientific, Bremen, Germany), equipped with collision cell and operating in kinetic energy discrimination (KED) mode. The ^{66}Zn isotope was measured:
Torch position, ion optics and detector settings were adjusted daily using tuning solution (Thermo Scientific Tune B) in order to optimize measurements and minimize possible interferences. For the qualitative analysis of the samples, five-point calibration curve (including zero) was constructed for the ^{66}Zn isotope in the concentration range of 0.1 – 2.0 mg/L. Additional line of the peristaltic pump was used for on-line introduction of multi-element internal standard (^{45}Sc – 10 ng/mL; ^{71}Ga – 2 ng/mL). Concentrations of each measured sample were corrected for response factors of both higher and lower mass internal standard using interpolation method.
The quality of the analytical process was controlled by the analysis of the standard reference material (NIST SRM 1577c, Gaithersburg, USA). Measured concentrations were within the range of the certified values for all isotopes.

Statistical Analysis
For data analysis a one-way analysis of variance (ANOVA) and Student's paired t-test, which was performed using Microsoft Office Excel 2007, was used for the comparison of the mean content of Zn in different types of meat.

RESULTS AND DISCUSSION

Contents of Zn (mg/kg) in four types of meat are expressed as mean value ± standard deviation (SD) and are graphically presented in Figure 1. Results for ANOVA and Student's t-test of the data are showed in Figure 1, as well. Statistical analysis of the data showed significant differences between the zinc content in all types of meat except between Zn content in chicken and turkey meat ($p < 0.05$).
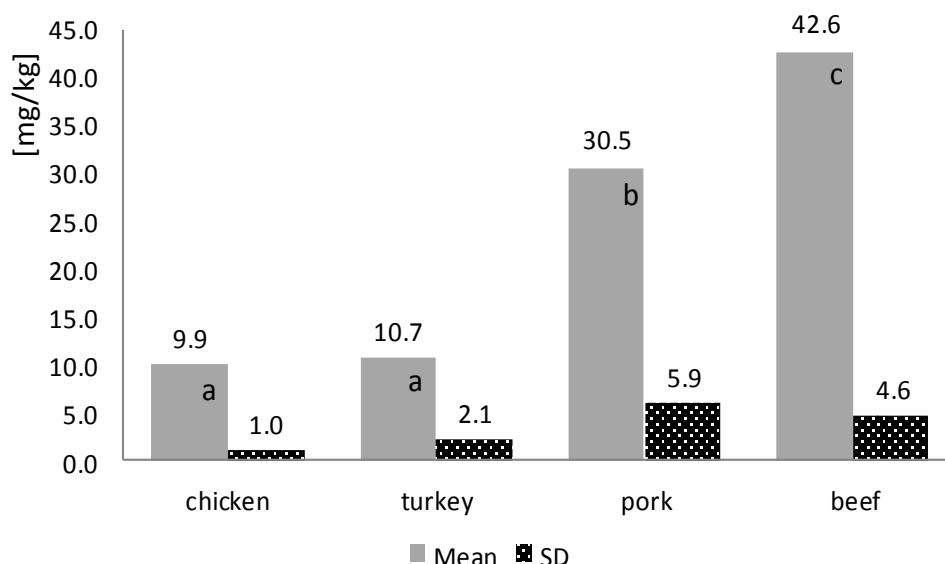
Figure1. The mean values for Zn content [mg/kg] in different types of meat (a, b, c – values expressed as columns followed by different letters are differ significantly (p<0.05))

The World Health Organization (WHO) through Global Environment Monitoring System - Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets 2012 (www.who.int/foodsafety/chem/gems/en) gives data for the daily intake of 383 different food items of 183 countries. According to the data of WHO, the daily intake of meat for adult Serbian population is 114.1g and 55.6g of mammalian and poultry species, respectively. The Recommended Dietary Allowances (RDA) for Zn, recommended by Food and Nutrition Board of the Institute of Medicine (Institute of Medicine, 2001) is 11 mg/day and 8 mg/day for man and woman up to 19 years old, respectively. Taking into account the obtained data of this study, RDA for Zn, as well as WHO data for meat daily intake Figure 2 shows percent of recommended daily intake of Zn by poultry and mammalian meat for man and woman in Serbian population.

a) man
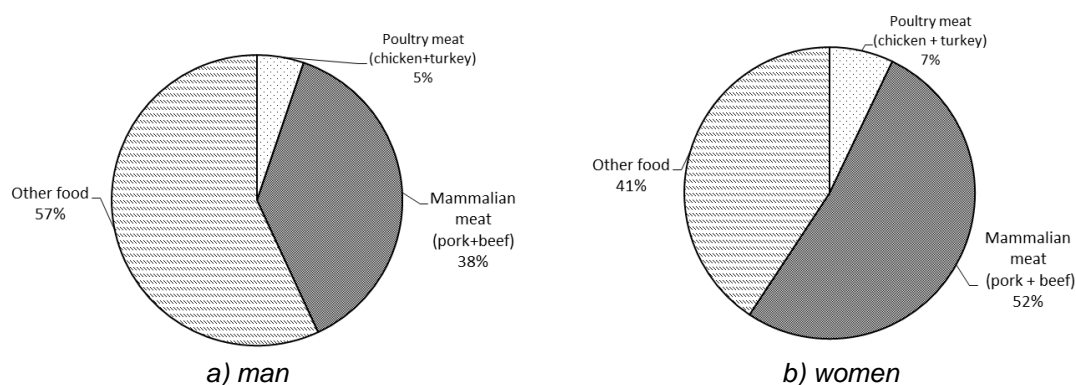
b) women

Figure 2. Percent of recommended daily intake of Zn by consuming poultry and mammalian meat for man and woman in Serbian population

As it is showed in Figure 2 poultry meat (chiken and turkey) provides 5% and 7% of Zn RDA for men and women, respectively, while mammalian meat (pork and beef) provides 38% and 52% of Zn RDA for men and women, respectively, in Serbian population.

CONCLUSIONS

The mean contents of Zn in analyzed samples (beef, pork, turkey and chicken meat) were in the range 9.9-42.6 mg/kg; the highest Zn content was determined in one beef sample (59.5 mg/kg). According to the results of this study, taking into account the RDA data for Zn, as well as WHO data for meat daily intake, it can be concluded that meat intake (poultry and mammalian meat) provides 43.1% of Zn RDA for men and 59.3% of Zn RDA for women in Serbian population.

ACKNOWLEDGEMENTS

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OCCURRENCE OF LIPID OXIDATION PRODUCTS AND HETEROCYCLIC AMINES IN GRILLED *n*-3–ENRICHED CHICKEN PATTIES STORED UNDER OXYGEN RICH ATMOSPHERE

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ABSTRACT

The oxidation processes on lipids (instrumentally measured colour values, TBARs, cholesterol oxides – COPs) and formation of heterocyclic amines (HAs) were studied in chicken patties enriched in *n*-3 fatty acids (whole flaxseed addition in the chicken feed), after 8 days of storage at 4 °C under different aerobic conditions (MAP−high CO_2, wrapping (air permeable), MAP−high O_2, and MAP−low O_2). HA precursors – creatine and creatinine on raw patties before grilling, as well as COPs (7α-hydroxycholesterol, 7β-hydroxycholesterol, 20α-hydroxycholesterol, 22-hydroxycholesterol, 25-hydroxycholesterol) and HAs (quinoxalines (MeIQx, 7,8DiMeQx, IQx) and PhIP) on homogenate of the upper and the lower surface slices of the grilled (two-plate grill, at 220 °C for 5 min) patties were determined by LC-MS; TBARs were determined on raw samples spectrofotometrically. For the raw chicken patties, *n*-3 enrichment increased the colour L* values and TBARs, while after the heat treatment there were non-significant and slightly higher content of COPs. Generally, higher contents of individual and total HAs were seen for the *n*-3–enriched groups (not significant), compared to the control patties (mixed cereal diet that contained no flaxseed). In comparison with the low O_2 (<0.5%) package-atmosphere condition, O_2 enrichment (80%) increased the instrumentally measured colour values, TBARs, as well as total and individual COPs. On the contrary we found that content of total HAs was significantly lower in patties stored in packages with the highest O_2 (MAP−high O_2 and wrapping) than with the lowest O_2 concentration (MAP−low O_2, MAP−high CO_2).
Keywords: chicken patties, n-3 fatty acids, cholesterol oxides, TBARs, heterocyclic amines

INTRODUCTION

Poultry is one of the most important protein-rich muscle food sources available today, and production and consumption are increasing rapidly. It has been shown that heterocyclic amines (HAs) are formed at ppb levels during the cooking of muscle foods and play a role in the etiology of human cancers (Sugimura *et al.*, 2004). Therefore, to assess the human intake of HAs, it is important to collect data on the content of HAs in chicken meat prepared in various ways. As well as cooking conditions, there are other factors that can influence the formation of HAs in cooked meat, such as the amounts of their different precursors (creatine/creatinine, free amino acids, and reducing sugars) and the presence of other compounds with enhancing or inhibiting effects (Skog and Solyakov, 2002). To our knowledge *n*-3 polyunsaturated fatty acids (PUFAs) enriched chicken meat, which is more susceptible to oxidation in O_2−enriched package-atmosphere conditions, have not been used in studies on HAs.

It is well known that *n*-3 PUFAs consumption reduces the risk of cardiovascular disease, inhibits the growth of malignant mammary and prostate gland tumours, delays the loss of immunological functions, and is required for normal brain development in the foetus (Azcona *et al.*, 2008; Siriwardhana *et al.*, 2012). However, the quality of meat enriched in *n*-3 PUFAs might be adversely influenced by the supplementation with high levels of flaxseed, fish meat, or other sea products in the animal diet (Azcona *et al.*, 2008). Indeed, undesirable changes in several quality parameters can occur, including loss of water-holding capacity, and changes to the texture and flavour (Morrissey *et al.*, 1998). However, oxidation is a major cause of deterioration of fat and fat-containing muscle foods, and especially of *n*-3–enriched meat. Some studies have reported that the supplementation of a high level of flaxseed in the

diet of broiler chickens increases the degree of unsaturation of the lipids in their muscle membranes, which can induce greater oxidation intensity in the muscle (Morrissey *et al.,* 1998; Betti *et al.*, 2009). Lipid oxidation can affect the cholesterol stability, and thus produce cholesterol oxidation products (COPs). In particular, meat processing techniques and meat storage conditions can disrupt the muscle-cell membranes and facilitate interactions of fatty acids (FA) with cystosolic pro-oxidants, thereby further accelerating lipid and cholesterol oxidation (Boselli *et al.*, 2005).

Consequently, there is the need to determine the formation of lipid oxidation products and HAs in *n*-3 enriched chicken patties (from meat from chickens that had been fed ground whole flaxseed as an addition to their mixed cereal diet) after 8-day storing at 4 °C under different aerobic conditions used in the meat industry.

MATERIAL AND METHODS

The chicken patties were prepared by a local company (Pivka Perutninarstvo, d.d), using chicken meat that originated from chickens that followed two different diets. The first group of patties was made from meat from chickens that had been fed on a mixed cereal diet that contained no flaxseed (control). The second group of patties was made from meat from chickens that had been fed ground whole flaxseed as an addition to their mixed cereal diet (enriched with *n*-3 PUFAs). Therefore, the chicken meat enriched with *n*-3 PUFAs was obtained from the chicken named 'Pivka chicken with Omega 3', with a certificate defining their national level of higher quality (33203-35/2008/10) due to the favourable ratio of *n*-6/*n*-3 PUFAs (4.5:1).

On day 42, the control and '*n*-3–enriched' chickens were commercially processed after a 12-h feed-withdrawal period. The carcasses were then mechanically eviscerated and cut up after reaching an internal carcass temperature of 4 °C. The chicken breast and thigh meat were collected, minced (mincer hole diameter, 6 mm), spiced and mixed. The additives and spices used were: natural spices (e.g., garlic), bamboo fibre, dextrose, sodium acetate, sodium glutamate, ascorbic acid, sodium diphosphate, and carmine. The chicken patties weighed *ca.* 120 g each, and they were formed into an oval shape (*ca.* 140 mm × 100 mm × 10 mm). Four patties were placed onto each polypropylene tray (G018110, Marcato Sp. Z.o.o., Poland), ready for the packaging.

A total of 48 trays of four chicken patties were prepared, as the control (24 trays) and *n*-3–enriched (24 trays) chicken-meat groups. Each of these two groups was divided into four subgroups of 6 trays, each containing four chicken patties, for the different package-atmosphere conditions investigated. The full experimental procedure is summarised in Figure 1.

Two types of packaging films were used: multi-layer polyvinyl chloride (PVC) film (Zenium M; Linpac Packaging, Pointivy, France) and very high barrier sealable lidding film for food trays, of polypropylene and polyethylene (PP/PE) (Lin Top PP HB A 50; Linpac Plastic Pointivy, France). The four subgroups were defined according to the package-atmosphere conditions (wrapping and modified atmosphere), as follows (Figure 1): (i) the first subgroup were trays wrapped with the multi-layer PVC that is permeable to the air, as 21% O_2, < 1% CO_2 and 78% N_2 (WF−lCO_2); (ii) the high-CO_2 MAP subgroup were trays sealed with the high-barrier PP/PE and filled with modified atmosphere of 20% O_2, 30% CO_2 and 50% N_2 (MAP−hCO_2); (iii) the high-O_2 MAP subgroup were trays sealed with the high-barrier PP/PE and filled with modified atmosphere of 80% O_2 and 20% CO_2 (MAP−hO_2), and (iv) the low-O_2 MAP subgroup were trays sealed with the high-barrier PP/PE and filled with modified atmosphere of < 1% O_2, 25% CO_2 and 74% N_2 (MAP−lO_2). The initial gas compositions were measured for parallel packaged samples, which were then excluded due to the penetration of the packaging (data not presented). The packaged patties were stored in a refrigerator at 4 (±1) °C for eight days. Following this storage, the gas composition within the packaging was measured again (data not presented), accompanied by the instrumental analysis for patties colour. The thermal treatment (grilling) then followed. The patties were thermally treated under a two-plate grill (Silex, Kitchen Genius 610.95®, Germany) at 220 °C for 5 min. The

final internal temperature (Ti) was 85 (±5) °C. The Ti was monitored with a spear-pointed temperature probe (Testo 177-T4, coupled with a stainless-steel class 1 probe) that was inserted into the mid-point of patties.

Chicken broilers fed with conventional feed (Control)		Chicken broilers fed with conventional feed enriched in *n*-3 PUFAs (*n*-3 enriched)
	Breasts and thighs	
	Minced (d = 0.006 m)	
	Spiced (spices and additives)	
	Mixed	
	Patties formed	
	Packaged	
(Control) WF−lCO$_2$, MAP−hCO$_2$, MAP−hO$_2$, MAP−lO$_2$		(*n*-3 enriched) WF−lCO$_2$, MAP−hCO$_2$, MAP−hO$_2$, MAP−lO$_2$
	Gas composition measurement on packaging	
	Storage (8 days, at 4 ±1 °C)	
Colour measurement	Gas composition measurement	TBARs, HAs precursors
	Thermal treatment (grilled) (220 °C, 5 min)	
	Determination of cholesterol, COP, and HA content on average sample from each of three series	

Figure 1. Description of the procedures carried out in the present study for the chicken patty production, treatments and sampling (three production repeats were carried out).

The colour, TBARs, and content of HA precursors were assessed for the patties before the thermal treatment, and the other traits, the contents of HAs, cholesterol, and COPs were determined following the thermal treatment.

All of the chemical analyses were carried out in duplicate; the instrumental colour evaluations were measured in triplicate. For the determination of contents of HAs, cholesterol and COPs the means from each of the subgroups (means from 6 trays: 2 parallels × 3 production repeats = 6 determinations) were used.

A CR 200b colorimeter (Minolta; Illuminant C, 0° viewing angle) was used to determine the CIE L* (lightness), a* (±, red to green), and b* (±, yellow to blue) values on the surface of the raw chicken patties. The TBARs were determined using a modified extraction method described by Witte *et al.* (1970). The TBARs are calculated as mg malondialdehyde per kg meat sample. The cholesterol and the cholesterol oxides (COPs) contents were determined according to a modified method from Ubhayasekera *et al.* (2004), followed by LC-MS/MS analysis. The HA content was determined by the method described by Messner and Murkovic (2004) and Sentellas *et al.* (2004), with minor modifications.

The experiment for the evaluation of physico-chemical parameters of chicken patties was carried out using a 2 × 5 × 3 mixed factorial design (2 types of chicken diet (control and dietary flaxseed enrichment), 4 types of package-atmosphere condition (i.e., WF−lCO$_2$, MAP−hCO$_2$, MAP−hO$_2$, and MAP−lO$_2$), and 3 production repetitions) (SAS). Least square means for the experimental groups were obtained using the LSM procedure, and they were compared at a 5% probability level. The relationships between the parameters were assessed according to the Pearson correlation coefficients, using the CORR procedure.

RESULTS AND DISCUSSION

Packaging and storage at higher concentration of O_2 (MAP−hO_2 and WF−lCO_2) significantly changed the colour (Table 1), where it became lighter, compared to MAP−hCO_2 and MAP−lO_2; colour became paler in *n*-3 enriched ($\Box L^*$, 3.00) or more greyish and duller in control ($\Box L^*$, -0.30). The duller colour due to the low concentration of O_2 was expected, as low or no O_2 usually causes the pigments to be in the deoxymyoglobin state (Belcher, 2006). The evolution of TBARs formation in the raw patties decreased with the lowering of the concentration of O_2 in the packages (Table 1). The TBARs are significantly lower in the packages wrapped with the air-permeable film (WF−lCO_2) or filled with 20% O_2 and 30% CO_2 (MAP−hCO_2) than in packages with the highest O_2 concentration (MAP−hO_2); furthermore, the lowest TBAR levels are seen in the packages with O_2 at < 0.5% (MAP−lO_2). The *n*-3 enrichment of the chicken breast and thigh meat greatly affected the oxidative stability of the grilled patties, such that with this flaxseed supplementation, the highest TBARs levels occurred with *n*-3 enrichment, as compared to the control (69%). A possible explanation for the extensive lipid oxidation in *n*-3–enriched meat might also be the formation of reactive O_2 species, which are produced as a consequence of *n*-3 PUFA deposition (Morrissey et al., 1998). This is linked to transition metals such as iron, activation of adenosine monophosphate-activated protein kinase, increased release of calcium from the sarcoplasmic reticulum, and a high PUFA content in the phospholipid membranes (Betti *et al.*, 2009).

At closer (Figure 1), higher contents of COPs were seen for the *n*-3–enriched groups, compared to the control group, with the exception of the O_2-rich atmosphere. This result would be as expected, due to the extensive cholesterol oxidation in the chicken patties with lower oxidative stability. Over the 8 days of storage, in comparison with the very low O_2 atmosphere the total COPs increased by just under 2-fold in MAP−hCO_2, although these differences did not reach significance. For packaging in an O_2-rich atmosphere in comparison with a low-O_2 atmosphere the total COPs increased by more than 16-fold. Cayuela *et al.* (2004) reported that an O_2-rich atmosphere (O_2:CO_2, 70:30) resulted in a noticeable rise in the rate of cholesterol oxidation in pork loin chops stored at 4 °C for 13 days: from 0.2 mg kg^{-1} to 0.4 mg kg^{-1} meat. The trend (Pearson correlation coefficient = 0.70; $P < 0.001$) found for TBARs and COPs formation in the present study supports the catalysing influence of peroxyl radicals coming from neighbouring phospholipid polyunsaturated fatty acids on the onset of cholesterol oxidation (Paniangvait *et al.*, 1995).

Generally, higher contents of individual and total HAs were seen for the *n*-3–enriched groups (not significant, exception of MeIQx), compared to the control (Table 1). To our knowledge no data on HAs was acquired on *n*-3–enriched meats. We found that content of total HAs was significantly lower in patties stored in packages with the highest O_2 (MAP−high O_2 and wrapping) than with the lowest O_2 concentration (MAP−low O_2, MAP−high CO_2). However, this phenomenon holds mainly on account of significantly higher content of MeIQx.

If we look in more details the relationship between the parameters of lipid oxidation and HAs generated (Figure 2), we can conclude that accelerated lipid oxidation in both, *n*-3 enriched and control patties stored under rich O_2-conditions limits, perhaps even inhibits the formation of HAs. Plausible mechanism of HAs inhibition is based on reactions of bioactive compounds originating from cholesterol and lipid oxidation reactions with free radicals formed during thermal treatment and consequently on their inactivation. Lipid oxidation products can act as inhibitors along the different pathways of the reaction, perhaps by scavenging reactive molecules. On the contrary, in low-O_2 (<0.5%) atmosphere formation of COPs and TBARs is very limited and the noticeable formation of the HAs is noted, regardless diet *n*-3 enrichment. Additionally, the formation and amounts of the HAs are dependent on physical and chemical parameters, in terms of the levels of HA precursors (Skog *et al.,* 1998). Data about creatine and creatinine content in chicken patties showed that there are no differences between groups with different content of *n*-3 or concentration of O_2 in the packages.

Table 1. Effects of ground whole flaxseed addition in chicken feed (n-3 enriched) and package-atmosphere conditions on colour values, TBARs, content of cholesterol, cholesterol oxides, precursors of heterocyclic amines, quinoxalines, and PhIP of chicken patties, stored 8 days at 4 (±1) °C

Parameter, mean ± sd	Package-atmosphere conditions					Diet		
	$MAP-hCO_2$	$MAP-hO_2$	$MAP-lO_2$	$WF-lCO_2$	s.	Control	*n*-3	s.
Colour values								
L^* value	50.56 ± 2.46^b	51.78 ± 2.35^a	50.95 ± 3.27^b	51.26 ± 2.76^{ab}	***	49.90 ± 2.50^b	52.35 ± 2.45^a	***
a^* value	14.39 ± 2.00^b	14.76 ± 1.87^b	13.47 ± 1.55^c	15.91 ± 1.96^a	***	14.56 ± 2.11	14.65 ± 1.96	Ns
b^* value	15.38 ± 0.90^b	15.46 ± 1.03^b	13.95 ± 1.48^c	16.40 ± 1.25^a	***	15.15 ± 1.42	15.40 ± 1.51	Ns
Lipid oxidation parameter, mg malondialdehyde kg^{-1}								
TBARs	0.18 ± 0.06^b	0.54 ± 0.46^a	0.13 ± 0.02^c	0.21 ± 0.06^b	***	0.22 ± 0.15^b	0.32 ± 0.36^a	***
Cholesterol oxides (COPs) and cholesterol, mg kg^{-1}								
7α-HC	1.05 ± 0.26^c	14.16 ± 2.82^a	0.41 ± 0.29^c	2.43 ± 0.72^b	***	4.03 ± 5.76	4.48 ± 5.82	Ns
7β-HC	2.11 ± 0.55^c	28.35 ± 5.67^a	0.84 ± 0.57^c	4.79 ± 1.44^b	***	8.06 ± 11.58	8.94 ± 11.64	Ns
20α-HC	2.07 ± 0.50^c	28.29 ± 5.61^a	0.88 ± 0.56^c	4.55 ± 1.53^b	***	7.78 ± 11.28	8.91 ± 11.64	Ns
22-HC	1.01 ± 0.24^c	14.52 ± 2.96^a	0.47 ± 0.30^c	2.31 ± 0.75^b	***	4.54 ± 6.27	4.47 ± 5.50	Ns
25-HC	1.03 ± 0.34^c	14.59 ± 2.92^a	0.38 ± 0.30^c	2.32 ± 0.77^b	***	4.53 ± 6.33	4.46 ± 5.83	Ns
Total COPs	7.27 ± 1.85^c	99.16 ± 19.72^a	2.98 ± 1.95^c	16.76 ± 5.11^b	***	28.17 ± 40.43	31.27 ± 40.73	Ns
Cholesterol	571.8 ± 3.8^{ab}	495.6 ± 18.4^c	578.1 ± 4.5^a	565.2 ± 8.8^b	***	553.3 ± 36.2	552.1 ± 37.9	Ns
Oxidation, %	1.3 ± 0.3^c	16.7 ± 3.2^a	0.5 ± 0.3^c	2.9 ± 0.9^b	***	4.75 ± 6.77	5.28 ± 6.83	Ns
HA precursors[†], mMol kg^{-1}								
Creatine	6.30 ± 0.56	6.65 ± 0.33	6.50 ± 0.54	6.67 ± 0.58	Ns	6.44 ± 0.53	6.59 ± 0.45	Ns
Creatinine	0.56 ± 0.23	0.55 ± 0.25	0.53 ± 0.22	0.56 ± 0.26	Ns	0.54 ± 0.22	0.55 ± 0.23	Ns
Total creatine	6.85 ± 0.65	7.19 ± 0.36	7.02 ± 0.54	7.23 ± 0.63	Ns	6.97 ± 0.58	7.14 ± 0.48	Ns
Heterocyclic amines, g kg^{-1}								
MeIQx,	2.58 ± 2.55^a	1.45 ± 1.32^b	2.69 ± 2.09^a	1.12 ± 1.03^b	***	1.64 ± 1.49^b	2.36 ± 2.32^a	***
PhIP	5.11 ± 5.27^a	3.11 ± 4.30^b	5.73 ± 7.60^a	2.75 ± 3.61^b	***	4.07 ± 5.97	4.55 ± 5.37	Ns
7,8-DiMeIQx	0.32 ± 0.21^a	0.17 ± 0.14^b	0.33 ± 0.26^a	0.09 ± 0.11^b	***	0.21 ± 0.20	0.27 ± 0.23	Ns
IQx	2.03 ± 1.78^a	1.56 ± 1.52^b	1.93 ± 1.56^{ab}	1.71 ± 1.99^{ab}	*	1.86 ± 1.77	1.74 ± 1.62	Ns
Total HCA	8.56 ± 4.99^a	6.06 ± 6.02^b	9.88 ± 7.54^a	5.65 ± 6.57^b	***	7.48 ± 7.27	7.98 ± 5.73	Ns

[†] *Measurements on raw patties. HC, hydroxycholesterol. Levels of significance: Ns P > 0.05, *** P ≤ 0.001; * P ≤ 0.05. Means with a different superscript within effect (a,b,c,d) differ significantly (P ≤ 0.05).*
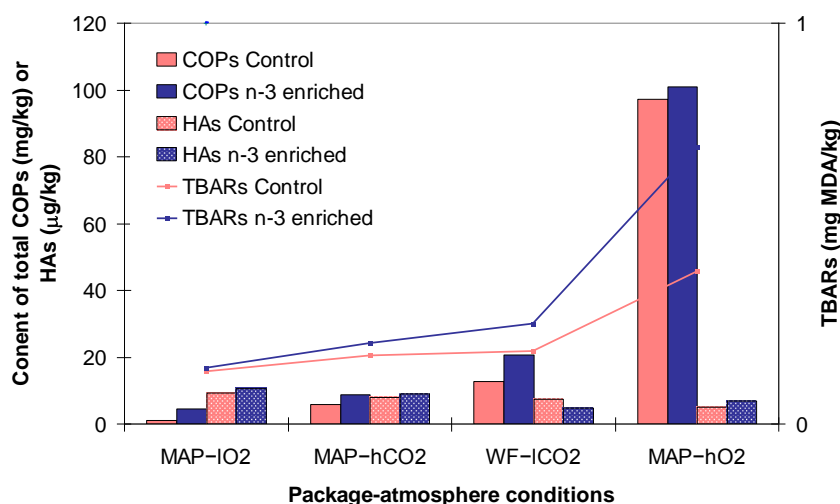
Figure 2. Effect of diet (n-3 enrichment) on content of cholesterol oxides (COPs) and heterocyclic amines (HAs), and TBARs of chicken patties, stored 8 days at 4 (±1) °C, with respect to package-atmosphere condition ($MAP-lO_2$ - <1% O_2, $MAP-hCO_2$ – 20% O_2, $WF-lCO_2$ – 21% O_2, and $MAP-hO_2$ – 87% O_2)

CONCLUSIONS

It is fascinating that on grilled poultry patties packed in low-O_2 (<0.5%) atmosphere after 8-day storage significantly higher amount of HAs compared to these stored in aerobic conditions was formed, which is very interesting for further research.

ACKNOWLEDGEMENTS

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CHEMICAL AND MICROBIOLOGICAL PROPERTIES OF BEVERAGES MADE FROM CAMEL, GOAT AND BOVINE WHEY USING KEFIR STARTER CULTURE

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ABSTRACT

Kefir is a traditional fermented milk beverage with a sharp acidic and slight yeasty flavor from the Caucasus Mountains in central Asia. In this study, kefir starter culture was used for production of beverage. Some chemical parameters (carbohydrate, dry matter, fat, and pH) and microbiological changes (lactic acid bacteria and yeasts) were determined during 24h of fermentation and then after 2, 7, 14, 21, and 28 days of storage at 4 °C. Three different substrates (camel, bovine and goat whey) were used as fermentation substrate for kefir starter culture. The incubation time (24h) and temperature (25 °C), inoculation rate (5%) and mixing rate (90 rpm) were the same for all the beverages. The changes in beverage properties, manufactured by three substrates, were more or less similar. During 0-4 weeks of storage, level of lactic acid bacteria decreased although population of yeast increased. During the fermentation, carbohydrate content (w/w) and pH decreased continuously, but dry matter and fat content of the beverages were not significantly different from dry matter and fat content of the substrates. At the end of 4 weeks of storage, carbohydrate content (w/w), and pH changed significantly in three substrates. Moreover, the results confirmed that changes in the properties of camel whey during fermentation were more pronounced than changes in goat and bovine whey substrate.

Keywords: camel whey, kefir starter culture, goat whey, 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). The appearance of kefir grains varies in size from approximately 3 to 30 mm and they comprise of the lactic acid bacteria (Lactobacillus, Lactococcus, Leuconostoc), yeasts (Kluyveromyces, Candida, Saccharomyces, Pichia) and acetic acid bacteria (Farnworth, 1999; Paraskevopoulou *et al.*, 2003). A prickling sensation on the tongue is typical when consuming kefir, due to CO_2 produced by the yeast microflora (Farnworth, 2005; Irigoyen *et al.,* 2005).

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).

Five major proteins in bovin whey comprise immunoglobulins (Igs), mainly immunoglobulin G (IgG), bovine serum albumin (BSA), α-lactalbumin (a-la), β-lactoglobulin (b-lg) and proteose peptones (Whitney, 1977; Eigel *et al.*, 1984). Serum proteins that were identified in camel milk include a-la, serum albumin, lactophorin A, lactoferrin, lac-toperoxidase and Igs.

In previous study 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. In the scientific literature, there are many reports on the

microbiological characteristics of Kefir grains and on changes occurring during storage of kefir beverage made from milk (Duitschaever *et al.*, 1988; Farnworth, 2005; Güzel-Seydim *et al.*, 2005; Irigoyen *et al.*, 2005; Kuo and Lin, 1999; Simova *et al.*, 2002). However, information on the microbiological and biochemical changes that occur during the storage of beverage inoculated with kefir starter culture is scarce. Lactic acid bacteria, yeast and acetic acid bacteria were used for production of beverage and the products were examined and analyzed for fat, carbohydrate, dry matter and pH. The aim of this study was to evaluate microbial and chemical changes during the fermentation of beverages made from camel, goat and bovine whey 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% N2, 10% H2 at 30 ºC, while *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, camel, goat and bovine whey was provided by Kalber S.A. which is a milk processing factory for cheese and other dairy products in the area of Arak, 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 (B1): The pasteurized camel whey was inoculated with combined kefir grain bacteria with yeast: 3% (v/v) bacterial strain and 2% (v/v) yeast kefir grains.

Beverage 2 (B2): The pasteurized goat whey was inoculated with combined kefir grain bacteria with yeast: 3% (v/v) bacterial strain and 2% (v/v) yeast kefir grains.

Beverage 3 (B3): The sterilized bovine whey was inoculated with combined kefir grain bacteria with yeast: 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. Carbohydrate content 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} (Wang *et al.*, 2004).

Microbiological analysis

The development in microflora was investigated by making serial dilutions of kefir in 0.1% peptone water (LP0040, Oxoid Ltd, Basingstoke, England) (Mian *et al.*, 1997). Dilutions of sample for counts of lactic acid bacteria were plated on MRS agar and Azid agar (Merck Co.), respectively. Both cultures were incubated for 3 days at 30 ºC. Malt extract agar (Merck Co.) was used for counts of yeasts. Samples were incubated for 3 days at 25 ºC (Atlas, 2006).

Statistical analysis

All results presented in this study are the mean of three independent experiments with three replicates for 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 the present study, by using the native microbial strains isolated from kefir grains starter culture, the following results were obtained.

Lactic acid bacteria levels in the beverage samples were 8 log10 cfu mL^{-1}. This finding is consistent with results obtained for Spanish, Turkish, South African, Scottish and Polish kefir (García-Fontán *et al.*, 2006; Güzel-Seydim *et al.*, 2005; Irigoyen *et al.*, 2005; Loretan *et al.*, 2003, Wszolek *et al.*, 2001). The significant ($P < 0.01$) decrease in camel whey substrate was found in both amount by 2 and 3 log10 units, respectively after 4 weeks. A significant ($P < 0.01$) decrease from 8 log10 cfu mL^{-1} to 5 log10 cfu mL^{-1} in level of lactic acid bacteria occurred in goat and bovine whey substrate, after 4 weeks of storage (Fig. 1.A).

Yeast levels in the beverage samples were approximately, 3.3 log10 cfu mL^{-1}. Although the amounts listed in CODEX STAN 243 (2003) for fermented milks were 10^4 cfu g^{-1} as minimum yeast number for kefir , the yeast level reported for household kefir from South Africa was 8 log10 cfu mL^{-1} (Loretan *et al.*, 2003). In camel whey substrate, after 3 weeks of storage, a significant ($P < 0.01$) increase in yeast level (4 log10 cfu mL^{-1}) occurred followed by further significant ($P < 0.01$) increase to 4.2 log10 cfu mL^{-1}, after 4 weeks of storage (Figure 1.B). During the 4 weeks storage period, yeast level in goat and bovine whey substrate increased significantly (Figure 1.B).

The carbohydrate content decreased significantly ($P < 0.05$) during the first 24 h, followed by slower decrease until the 48 h of storage. Carbohydrates were consumed during the first 24 h fermentation period, resulted in carbohydrates level decreased by 20–25% with respect to the initial carbohydrates level present in whey. In bovine whey substrate, the final carbohydrate content was within the range of the values observed by other authors for kefir produced from milk obtained from different species (Muir and Hunter, 1992; Muir *et al.*, 1999). In goat and camel whey, carbohydrate level decreased slowly (Figure 1.C). Alm (1982) did not detect galactose in the kefir samples, which was confirmed in this study. Namely, the galactose formed by hydrolysis of the lactose is employed by the kefir microflora to form the polymer kefiran, which is characteristic of kefir. The role of kefiran is to make the new granules formed during the fermentation process.

Dry matter in the three beverages did not significantly change after 48 h of fermentation (Figure 1.D). 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 it was the case for the fat content, fermentation did not affect the dry matter content of the source substrate used. Ottogalli *et al.* (1973) found that the dry matter in recently manufactured kefir differed according to the geographic origin of the granules, with variations in dry matter between 9.4% and 11.1% in the milk substrate. The percentage of kefir grains inoculated did not significantly influence the dry matter content in the samples.
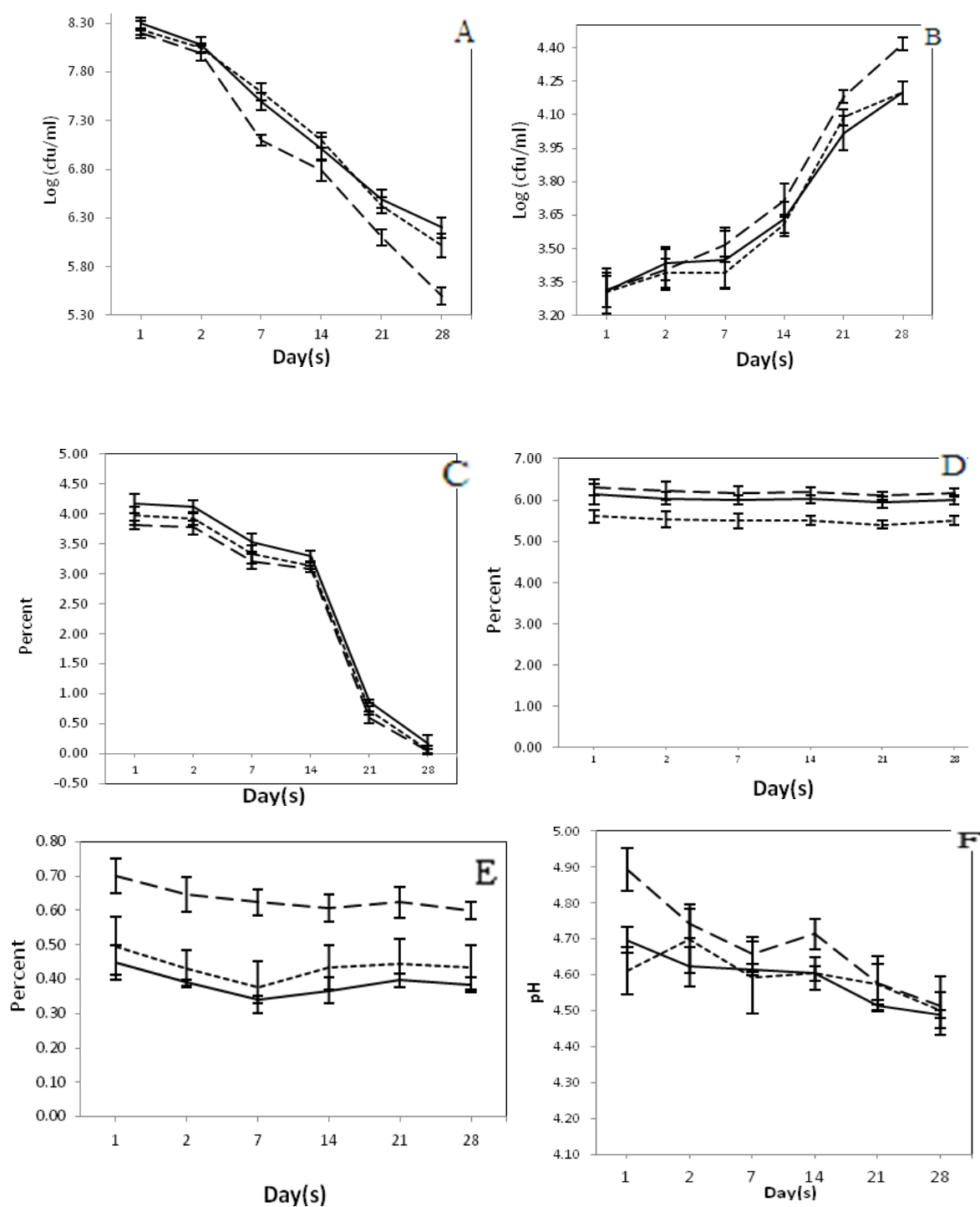
Figure 1. Changes in the physicochemical and microbiological characteristics of beverage samples made from camel whey (▬), goat whey (▬ ▬) and bovine whey (……) using kefir starter culture, during storage. (A) Lactic acid bacteria; (B) yeast; (C) carbohydrate; (D) dry matter; (E) fat; (F) pH

The fat content of three beverages did not differ significantly ($p > 0.05$) from the fat content of the substrates (milk, whey and soymilk) (Figure 1.E). 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 beverage.

There was a sharp decrease of around 2 pH units during fermentation in all three beverages. As already related above, the lactic acid bacterial population declined with time, which is why the kefir did not become more acidic (Figure 1.F). 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 of added kefir grain inoculate did significantly ($p < 0.05$) affect the pH values as reported by Irigoyen *et al.* (2003), who recorded significant differences during kefir manufacture influenced by the percentage of added kefir grain inoculate.

CONCLUSIONS

This study evaluated chemical and microbiological changes during the fermentation and cold storage of beverages made from camel, goat and bovine whey 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. The changes in beverage properties manufactured by three substrates were more or less similar. During 0-4 weeks of storage, level of lactic acid bacteria decreased although population of yeast increased.

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FRUIT WINES AS A SOURCE OF SOME OLIGOELEMENTS

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ABSTRACT

Fruit wines in Serbia have a reputation as a source of good health, immunity and blood count. They contain a low percentage of alcohol and are consumed as aperitifs. They are considered to contain haemoglobin precursors as well as oligoelements which are necessary for good health. Inductively coupled plasma-atomic emission spectrometry (ICP-AES) provides a rapid and precise means of monitoring elements simultaneously for minor- and trace- levels. The ICP-AES technique is widely regarded as the most versatile analytical technique in the chemistry laboratory.

The aim was determination of the oligoelements (iron, zinc, copper, chrome, manganese, cobalt) content in fruit wines, as well as in domestic red grape wines, using ICP-AES spectrometry, and to compare results obtained. We analysed 4 types of fruit wines (blackberry, raspberry, cherry, apple) and red wine of various producers, a total of 30 samples. Samples were prepared by microwave sample digestion. Domestic fruit wines are a good source of oligoelements, as well as some grape wines from the market. The advantage can be given to fruit wines because of the lower ethanol content, making them suitable for the general population.

***Keywords**: fruit wines, oligoelements, ICP-AES*

INTRODUCTION

The expansion of nutritional labeling programs and increased costumer interest in nutrition have created new demands on the food industry to specify the quantity of individual carbohydrate fractions in fresh and processed foods, rich in minerals (John Howlett, 2008). In spite of a rich offer of industrially produced wines, there is also a variety of traditionally made fruit wines. These are made from fresh squeezed fruit, without thermal treatment.

Minerals have many important physiologic functions. They assist in fluid regulation and energy production, are essential to the health of our bones and blood, and help rid the body of harmful by-products of metabolism. Minerals are classified according to the amounts we need in our diet and according to how much of the mineral is found in the body – oligoelements (trace minerals) and major minerals (Janice *et al.,* 2011).

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) provides a rapid and precise means of monitoring elements simultaneously for minor- and trace- levels. The ICP-AES technique is widely regarded as the most versatile analytical technique in the chemistry laboratory (Bingol *et al.,* 2010).

MATERIAL AND METHODS

Four types of fruit wines were analyzed (blackberry, raspberry, cherry, apple) and red grape wines of various producers, a total of 30 samples. Samples were prepared in two ways: by dilution with water to obtain 1% (v/v) ethanol, or microwave sample digestion:

5 g of the sample and 5 mL HNO3 65% were added. A microwave furnace was used for the digestion and dissolution of the experimental samples. In the method, the samples were dissolved at 180 ^{o}C and 400 psi pressure in apparatus. After further 20 min processing, the samples were put into 25 mL polyethylene flasks which were made up with deionized water.

The metals were analyzed by ICP-AES (ICAP Series 600 Thermo Fisher scientific). The calibration curves were constructed using a series of dilutions containing different levels of metals (0,005 mg/L to 2 mg/L). The reading was made at the emission wavelengths for zinc,

manganese, iron, copper, chrome and cobalt of 202.548, 257.610, 238.204, 324.754, 267.716 and 238.616 nm, respectively. The results were evaluated according to iTEVA iCAP Software ICP Spectrometer, and for the comparison of the metal values One-Sample *t*-test was used. The method was validated using certified reference material „Environment Canada TM-25.3" (Table 1).

RESULTS AND DISCUSSION

Zinc, manganese, copper, chrome and cobalt contents did not vary significantly in commercial wines, while iron contents did, depending on type of wine. In fruit wines, contents of manganese, iron and chrome varied significantly, depending on the origin of wine, while zinc, copper and cobalt contents showed less variation (Table 2). Certain oligoelement values are in accordance with their contents in corresponding fruit (Piotz and Jerone, 2007). There is statistically significant difference in terms of higher zinc and copper contents in fruit wines, compared to other wines. In terms of average manganese, chrome and cobalt contents, there is no statistically significant difference. Regarding iron content, average values differ, but there are significant differences between certain types of fruit wines, resulting in no statistical significance (Table 3).

Table 1. Method validation using certified reference material „Environment Canada TM-25.3"

CRM *	Mn mg/L	Fe mg/L	Cu mg/L	Cr mg/L	Co mg/L
Determined	26.8	27.9	25.1	23.7	30.3
Certificated	25.4+2.54	29.6+4.76	27.6+2.84	24.4+2.13	27.9+2.56

*Environment Canada TM-25.3 lot 0809

Table 2. Content of selected elements in grape wines and fruit wines originating from Serbia

Wines	Zn mg/L	Mn mg/L	Fe mg/L	Cu mg/L	Cr mg/L	Co mg/L
Chardonnay	1.371	0.928	1.430	0.804	0.104	0.007
	1.227	0.778	1.445	0.424	0.084	0.005
	1.121	0.754	1.767	0.742	0.094	<0.005
	1.043	0.986	1.307	0.876	0.099	<0.005
Graševina	1.623	0.875	3.157	0.350	0.106	0.009
	1.443	0.774	4.123	0.331	0.086	0.005
	1.534	0.821	3.572	0.399	0.076	<0.005
	0.935	1.069	2.180	0.214	0.121	0.007
	1.442	0.733	2.337	0.287	0.104	0.007
Vranac	0.963	1.140	2.926	0.201	0.096	0.006
	0.762	0.814	3.216	0.281	0.086	0.005
	0.933	0.910	2.426	0.193	0.077	0.006
Prokupac	1.528	1.438	2.364	0.698	0.068	0.006
	1.338	1.137	1.742	0.595	0.064	0.007
	1.223	0.938	2.554	0.447	0.083	0.008
	1.326	1.332	2.068	0.613	0.065	0.005
Fruit wines						
Cherry	0.575	3.152	6.065	0.358	0.187	0.011
	0.595	3.002	4.064	0.287	0.102	0.005
	0.483	2.782	4.534	0.266	0.147	0.006
	0.477	2.835	3.675	0.298	0.153	0.007
Rasbery	0.388	0.324	1.147	0.264	0.038	0.005
	0.344	0.302	1.237	0.327	0.068	<0.005
	0.372	0.287	1.402	0.269	0.059	<0.005
Blackberry	0.563	2.482	2.421	0.181	0.106	0.009
	0.611	2.683	2.712	0.176	0.091	0.007
	0.456	2.724	2.122	0.168	0.094	0.008
Blueberey	0.371	1.378	5.351	0.207	0.102	0.008
	0.421	1.189	4.761	0.177	0.082	0.006

Table 3. Mean content of selected elements in grape wines and fruit wines

	Zn mg/L	Mn mg/L	Fe mg/L	Cu mg/L	Cr mg/L	Co mg/L
Mean values for grape wines	1.238	1.143	2.392	0.466	0.088	0.006
Mean values for fruit wines	0.404	1.469	2.821	0.213	0.087	0.006
p	<0.01	>0.01	>0.01	<0.01	>0.01	>0.01
p>0.01 – The difference between mean levels of elements determined in the samples prepared by dilution and those prepared by microwave digestion is not significant						

CONCLUSIONS

Fruit wines are a good source of oligoelements, as well as some grape wines from the market. The advantage can be given to fruit wines because of the lower ethanol content, making them suitable for the general population, as well as one good excipient for some supplements.

ACKNOWLEDGEMENTS

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INFLUENCE OF *THYMUS VULGARIS* ON INITIAL CELL ATTACHMENT AND BIOFILM OF *Salmonella* Enteritidis

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ABSTRACT

Bacteria within a biofilm have a distinct phenotype from planktonic cells and generally show higher tolerance to antimicrobial agents, such as disinfectant treatments and antibiotics. Due to the increased tolerance against antimicrobial treatments, biofilms are hard to eradicate and they cause all kinds of problems in medical and industrial settings.
Therefore, the purpose of this study was to examine the influence of *Thymus vulgaris* essential oil on the initial adhesion and preformed biofilm of *Salmonella* Enteritidis. Results for antimicrobial activity of *Th. vulgaris* essential oil obtained using broth microdilution method against *S.* Enteritidis were identical for all tested isolates (MIC/MFC=0.156/0.3125 µL/mL). In order to understand the anti-biofilm action of essential oil (EO), its effect was tested on both the initial cell attachment by planktonic cells as well as on preformed biofilms. Obtained results indicated that the effect of *Th. vulgaris* essential oil on initial cell attachment of tested isolates SE4, SE5, SE7 and SE9 was dosage dependent manner, although even at 0.5×MIC biomass attachment was reduced by 74.2%, 77.7%, 73.6% and 70.1%, respectively. Fairly high, but not complete, inhibition of cell attachment was achieved using 1×MIC of this EO. Using 1×MIC, biomass attachment of tested isolates SE4, SE5, SE7 and SE9 was reduced for 91.4%, 90.2%, 90.6% and 88.4%, respectively. When the same EO was tested against a preformed biofilm, its inhibitory effect was reduced greatly.
Keywords*: Salmonella* Enteritidis*, biofilm, thymus*

INTRODUCTION

The ability of biofilm formation is an ancient property of bacteria (and other prokaryotes) that represents, from the evolution point of view, the survival strategy in variable and often highly unfavorable conditions of the environment. Contrary to characteristics that bacteria show during their growth in the media abounding with nutritive substances, bacteria in biofilms show different properties in terms of genes expression and growth characteristics. Due to the presence of these differences, bacteria in biofilms show an increased resistance to antibiotics and disinfectants and that is why it is almost impossible today to treat infections caused by biofilms with conventional antibiotics, just as it is not possible to control bacteria in the hospital environment and places of food production using disinfectants in recommended concentrations. In medical and industrial microbiology, the concept of biofilms formed by bacteria was accepted during the 1990s and it is currently one of the greatest challenges in the field of protection of human health and production of healthy safe food.
A large number of studies confirmed that biofilms formed by bacteria on different surfaces in food industry plants make a long-term source of contamination of foodstuffs, not only with bacteria causing their spoilage but also with food-borne pathogen species such as *Salmonella* spp., *Campylobacter* spp., *Escherichia coli* and *Listeria monocytogenes*. It is known that some food-borne pathogens in food production plants may exist throughout several months, even years. These strains are known as "house strains", and the assumption is that the existence of such strains is enabled due to their ability to form biofilms (Møretrø *et al.*, 2012). Several studies confirmed the ability of adherence and biofilm formation of food-

borne pathogens on different types of materials that are usually used in food industry (Stepanović *et al.*, 2003; Lapidot *et al.*, 2006; Vestby *et al.*, 2009; Valeriano *et al.*, 2012). Biofilms of food-borne pathogens are found on conveyer belts, cutting and packing machines and other surfaces that get in contact with food. Having in mind the consequential effect that biofilm formation may have in food industry, the biofilm control in food industry plants becomes the imperative in production of healthy safe food.

The efficiency of disinfectants and recommended concentration are the results of tests conducted on broth bacteria cultures. While their application on broth cultures is efficient up to 100% (Lević *et al.*, 2011; Čabarkapa *et al.*, 2012) the application of the same concentrations on bacteria in biofilms shows inefficiency so that their elimination requires much larger concentrations. Because of these properties of biofilm-associated cells there is a large and increasing interest in substances, which inhibit specific processes in the initial phase of biofilm formation and therefore prevent the formation of mature biofilms with its elevated resistance against biocides and disinfectants (Szczepanski and Lipski, 2014). A large number of research are based on finding the potential biological solutions for biofilm prevention and elimination that include antimicrobial compounds of plant origin *(*Jadhav *et al.,* 2013), enzymes, phage, inter-species competitors, or antimicrobial compounds produced by microorganisms (Simões *et al.*, 2010).

This study was aimed to evaluate the influence of *Th. vulgaris* essential oil on initial cell attachment and preformed biofilm of *Salmonella* Enteritidis.

MATERIAL AND METHODS

Plant material
Plant material *Thymus vulgaris* used in this study was obtained from Institute of Medicinal Plant Research Dr. Josif Pančić, Belgrade, Serbia and voucher specimen was deposited in the herbarium of the Institute of Medicinal Plant Research Dr. Josif Pančić.

Isolation of the essential oil
The essential oil (EO) was isolated from dried plant material (100g) by hydro-distillation according to the standard procedure. Distillation was performed using Clevenger type apparatus, for 4 hours. The resulting EO was dried over anhydrous sodium sulfate and stored in sealed dark vials at 4°C. The yield of EO was expressed in volume percent (% v/w) that was calculated relative to 100 g of dried plant material.

Microorganisms
A total of four *S. enterica* strains of serovar Enteritidis (SE4, SE5, SE7 and SE9) were used in this study. Strains were isolated from fecal samples obtained from the Veterinary Institute in Kraljevo. Serological typing and verification of *Salmonella* isolates was carried out in the National Reference Laboratory for *Salmonella, Shigella, Vibrio cholera* and *Yersinia enterocolitica*, Institute of Public Health of Serbia "Dr Milan Jovanovic Batut", Belgrade, Serbia.

Preparation of bacterial suspension
S. Enteritidis isolates were cultured on Tryptone Soya Agar (TSA) at 37°C for 24h. The bacterial inoculates were prepared using 18h old cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

Resazurin powder preparation
A stock solution of the resazurin sodium salt (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide, Himedia) powder was prepared in sterile distilled water, concentration 0.01%. It was filter-sterilized and kept at 4°C.

Broth microdilution method

Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the National Committee for Clinical Laboratory Standards (NCCLS, 2002), with some modifications. The bacterial inoculates were prepared using overnight cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

All tests were performed in MHB (Muller-Hinton Broth, LabM). Propylene glycol (2 - (2 - hydroxypropoxy) - 1-propanol) was used to dissolve the EO and then diluted to the concentration (50 – 0.024 µl/mL). Twenty microliters aliquots of the EO were added to 96-well microtitre plates, in geometric dilutions, ranging from 50 to 0.024 µl/mL. Afterwards, aliquots of 160 µL of MHB, were added into each well. As the final step, 20 µL of $2{\times}10^6$ cfu/mL (according to 0.5 Mc Farland turbidity standards) of standardized bacterial suspensions were inoculated into each microplate. The test was performed in a total volume of 200 µL in each well with final EO concentrations of 5 – 0.0024 µl/mL. Plates were incubated at 37°C for 24 hours. The same tests were performed simultaneously for growth control (MHB + test organism) and sterility control (MHB + test oil).

After 24h of incubation, 20 µL of the resazurin solution (0.01%) was added to each well and the plate was incubated 6h at 37°C. After visual examination the plates were additionally incubated for 18h. A change of color from blue (oxidized) to pink (reduced) indicated the growth of bacteria. The MIC was defined as the lowest concentration of EO that prevented this change in color.

Referring to the results of the MIC assay, the wells showing complete absence of growth were identified and 100 µL solutions from each well was transferred to Plate count agar plates (PCA, LabM) and incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of the EO at which 99.9% of the inoculated microorganisms were killed.

Inhibition of initial cell attachment

The effect of EO on biofilm formation was evaluated as described by Jadhav *et al.* (2013). Solutions of EO (equivalent to 0.5×MIC and 1×MIC) were prepared. Twenty microliters of each solution were added to individual wells of a sterile flat-bottomed 96-well polystyrene microtitre plates (Greiner Bio-One). Afterwards, aliquots of 160 µL of TSB were added into each well. As the final step, 20 µL of $2{\times}10^6$ cfu/mL (according to 0.5 Mc Farland turbidity standards) of standardized bacterial suspensions were inoculated into each well to yield a final volume of 200 µL in each well. The cultures were added into the wells in quadruplicate. Control well contained all components except the inoculums (180 µl TSB + 20 µl of specific concentration of EO diluted in propylene glycol). Positive control well contained 160 µl TSB + 20 µl inoculums (in the same broth) + 20 µl of pure propylene glycol; this control is to reveal potential effect of solvent propylene glycol on *Salmonella* growth. Sterility control well contained 180 µl TSB + 20 µl pure propylene glycol; this control is to reveal possible contamination of solvents. The plates were sealed and incubated for 48h at 25°C under sterile conditions to allow cell attachment. Biofilm formation was assessed using the modified CV assay.

Inhibition of preformed biofilm

The effect of EO on biofilm growth and development was evaluated as described by Jadhav *et al.* (2013), with some modifications. Biofilms were allowed to be formed for 48h prior to addition of EO. Biofilm formation was achieved by transferring 160 µL of TSB into each microplate, followed by addition of 20 µL of bacterial culture (prepared as described above) into the wells of sterile flat-bottomed 96-well polystyrene microtitre plates in quadruplicates. The scheme of control samples was the same as described in previous section.

The microtitre plates were covered and incubated for 48h at 25°C to allow cell attachment and biofilm formation. Following incubation, 20 µL of each stock solution of EO was added to each well to yield a final volume of 200 µL. After the treatment of preformed biofilms with EO, the plates were incubated for 30 and 60 minutes. Following incubation, the biofilms were assessed for biomass attachment using the modified CV assay.

Biofilm biomass assay (modified CV assay)
Indirect assessment of cell attachment for *S.* Enteritidis was evaluated using the modified CV assay described by Agarwal *et al.* (2011). Following the 48h incubation (Section Inhibition of initial cell attachment) and (Section Inhibition of preformed biofilm), culture medium from each well was gently removed and the plates were washed three times with 250 μL sterile distilled water to wash away any loosely attached cells. The plates were air dried for 45 min. The cells in the biofilm were then stained with 250 μL 0.3% CV and incubated at room temperature for 15 min. The stain was removed by exhaustive washing with distilled water.
The plates were then allowed to dry. In order to quantify adhered cells, 250 μL of decolouring solution (ethanol/acetone, 80:20%) was added to each well for 15 min. The absorption of the eluted stain was measured at 595 nm using a microplate reader (ChemWel, Awareness Technology). The mean absorbance ($OD_{595 \ nm}$) was used for determining the percentage inhibition of biomass formation for each concentration of the oil according to the following equation:

$$\% \ inhibition = 100 - \left[\left\{ \frac{OD \ 595 \ experimental \ well \ with \ components \ of \ EO}{OD \ 595 \ control \ well \ without \ components \ of \ EO} \right\} \times 100 \right]$$

Statistical analyses
Statistical analysis was performed by Statistica 12 (StatSoft Inc., Tulsa, Oklahoma). Due to the size of the sample (n<30), the data from the assays were compared using the nonparametric Mann-Whitney test. Results were considered to be statistically significant at p<0.05.

RESULTS AND DISCUSSION

Results for antibacterial activity of *Th. vulgaris* obtained using broth microdilution method against *S.* Enteritidis were identical for all tested isolates (MIC/MBC=0.156/0.3125 μL/mL). Between the tested isolates of *S.* Enteritidis are not established differences in response to treatment with essential oil. This coincides with the findings of Lu and Wu (2010) who examined antimicrobial effect of *Th. vulgaris* essential oil, carvacrol and thymol on four different serotype of *Salmonella enterica:* Kentucky, Senftenberg, Enteritidis and Typhimurium. Obtained results for antimicrobial activity of *Th. vulgaris* essential oil are consistent with previous studies (Al-Bayati, 2008; Rota *et al.*, 2008; Roldán *et al.*, 2010; Stojković *et al.*, 2013). These studies reported MIC for serotype Enteritidis that ranged from 0.025 μL/mL (Stanković *et al.*, 2011) to 0.625 μL/mL (Roldán *et al.*, 2010), whereas MBC for these serotype ranged from 0.025 μL/mL (Stanković *et al.*, 2011) to 1.2 μL/mL (Rota *et al.*, 2008).
Ability of *Salmonella* spp. to form biofilms on different surfaces has been demonstrated in many researches (Stepanović *et al.*, 2003; Díez-García *et al.*, 2012; Castelijn *et al.*, 2013).
The CV assay indicated that the effect of *Th. vulgaris* essential oil on initial cell attachment of tested isolates SE4, SE5, SE7 and SE9 was dosage dependent manner, although even at 0.5×MIC biomass attachment was reduced by 74.2%, 77.7%, 73.6% and 70.1%, respectively. Fairly high, but not complete, inhibition of cell attachment was achieved using 1×MIC of this EO. Using 1×MIC, biomass attachment of tested isolates SE4, SE5, SE7 and SE9 was reduced for 91.4%, 90.2%, 90.6% and 88.4%, respectively (Figure 1). These results are consistent with the results of other researchers (Soni *et al.*, 2013; Burt *et al.*, 2014). Namely, Soni *et al.*, (2013) determined that sub-lethal concentrations up to 0.012% (approx. 0.12 μL/mL) of carvacrol, a component of thyme and oregano essential oil, significantly reduced biofilm formation in three strains of *S.* Typhimurium. In research of Burt *et al.*, (2014) significant reduction with carvacrol in *S.* Typhimurium biofilm formation was found between 0.75 mM - 1.25 mM (approx 0.11 - 0.19 μL/mL). Since they determined that the number of viable bacteria was not reduced using sub-lethal concentrations of carvacrol, these authors assumed that biofilm development can be inhibited without reducing bacterial viability, and that a mechanism other than growth inhibition or bacterial cell death may be involved in this antibiofilm activity of carvacrol. More recent studies show that carvacrol

reduces bacterial motility at sub-lethal concentrations owing to their ability to interfere with the quorum sensing (QS) signaling mechanism between bacterial cells, thereby also reducing the capacity for biofilm formation (Inamuco *et al.*, 2012; Burt *et al.*, 2014). But precise mechanism by which carvacrol inhibits enlargement of biofilm has not yet been fully established.
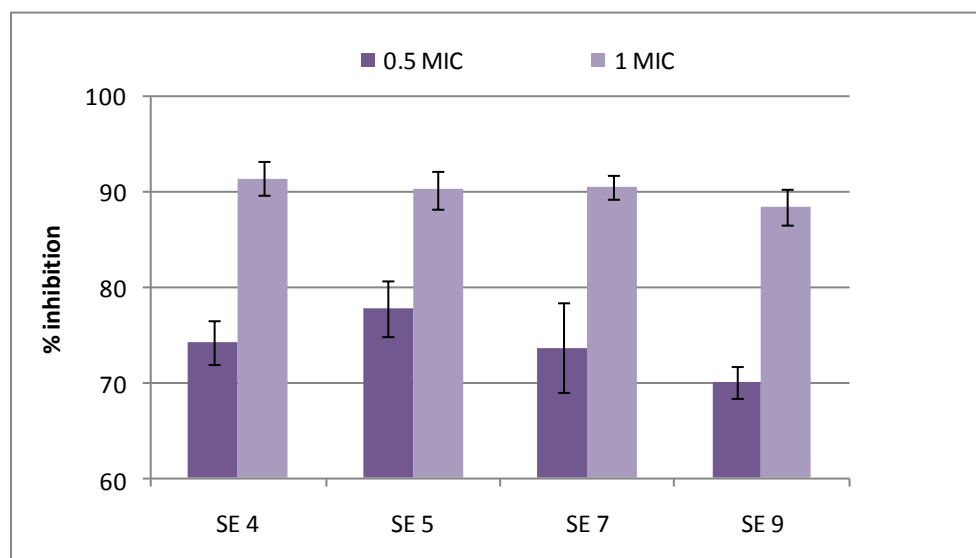
Figure 1. Effect of different concentrations of Th. vulgaris essential oil (expressed as percentage inhibition of biofilm formation) on initial cell attachment of tested isolates S. Enteritidis (SE4, SE5, SE7 and SE9)

Effect of tested concentrations of *Th. vulgaris* essential oil (0.5×MIC and 1×MIC) on pre-formed biofilms of tested isolates *S*. Enteritidis SE4, SE5, SE7 and SE9 is shown in Figure 2 and 3.
Following the first 30 minutes of incubation, preformed biofilm of tested isolates *S*. Enteritidis SE4, SE5, SE7 and SE9 with *Th. vulgaris* essential oil, only 27.8%, 18.5%, 19.4% and 28.1% inhibition occurred at 0.5×MIC level, respectively (Figure 2), while 1×MIC of this EO reduced preformed biofilm for 50.0%, 35.5%, 38.9% and 51.8%, respectively (Figure 3).
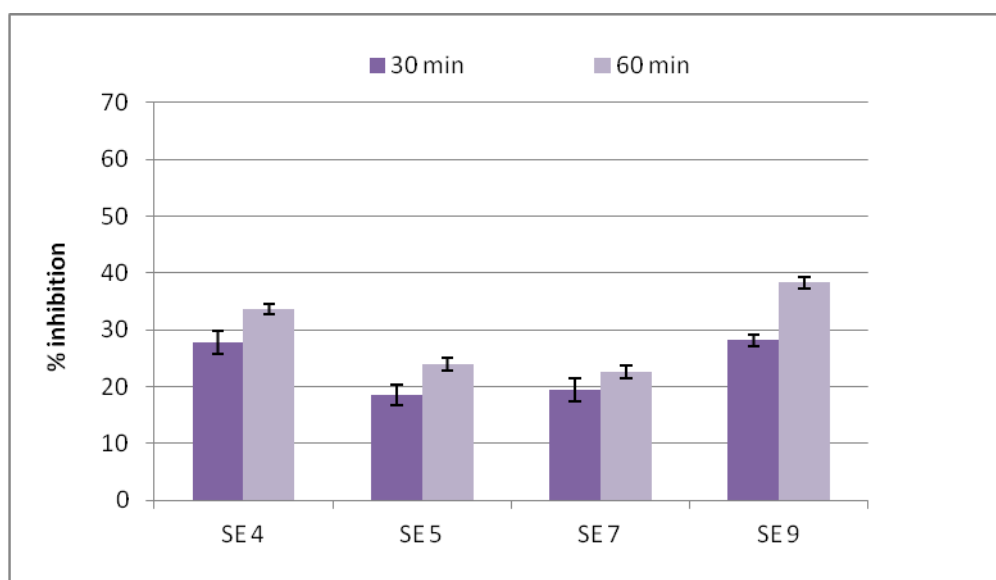
Figure 2. Effect of 0.5 MIC Th. vulgaris essential oil (expressed as percentage inhibition of biofilm formation) on preformed biofilm (48h) of tested isolates S. Enteritidis (SE4, SE5, SE7 and SE9)

After 60 minutes of incubation with *Th. vulgaris* essential oil with the preformed biofilm of *S. Enteritidis* tested isolates (SE 4, SE5, SE7 and SE9), only 33.7%, 23.8% 22.5% and 38.4% inhibition occurred at 0.5×MIC level, respectively (Figure 2). Likewise, inhibition of biofilm formation at 1×MIC was slightly higher than at 0.5×MIC, and amounted 54.0%, 40.7%, 41.8% and 51.8%, respectively (Figure 3).

Percent inhibition of preformed biofilms did not rise significantly after periods longer than 30 min ($p < 0.05$) (Figure 2 and 3).
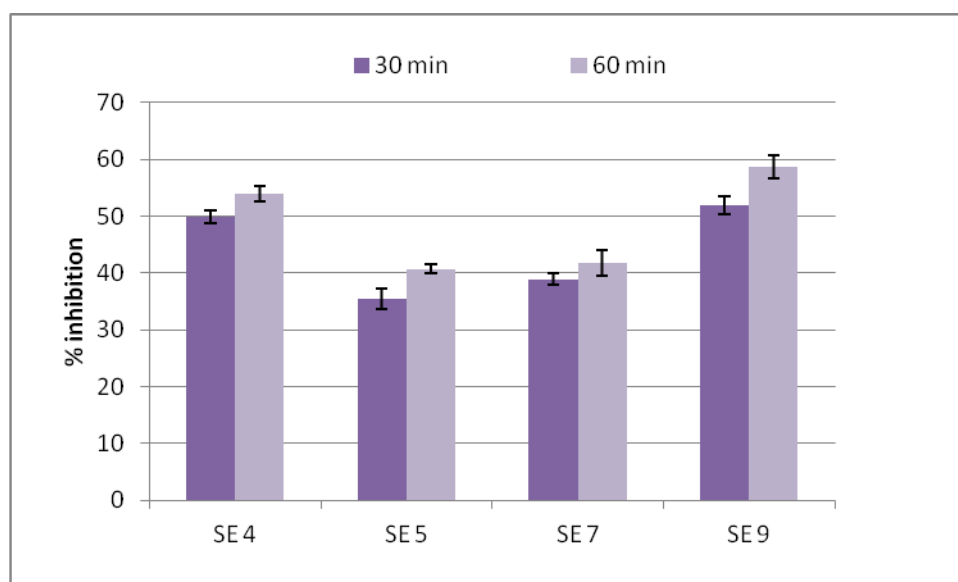
Figure 3. Effect of 1×MIC Th. vulgaris essential oil (expressed as percentage inhibition of biofilm formation) on preformed biofilm (48h) of tested isolates S. Enteritidis
(SE4, SE5, SE7 and SE9)

The ability of biofilm production by isolates applied in this study was previously characterized. Isolates SE4 and SE9 were characterized by moderate biofilm production whereas the phenotype on Congo red agar corresponded with BDAR (brown, dry, and rough) morphotype. SE5 and SE7 isolates were characterized by the strong biofilm production and the phenotype on Congo red agar corresponded with RDAR (red, dry, and rough) morphotype (data not shown). A large number of studies have reported that extracellular matrix component of *Salmonella* biofilm, cellulose, is an important characteristic for extracellular survival and is directly responsible for resistance to different antimicrobials (Solano *et al.*, 2002; White *et al.*, 2006). We noticed slightly lower percentage of inhibition of the isolates SE5 and SE7 for which we previously established to produce cellulose in biofilm matrix (RDAR morphotype), while the production of cellulose in the matrix of the biofilm was not determined (BDAR morphotype) for the isolates SE4 and SE9.

Overall, the data presented here show that resistance of a preformed biofilm can be associated with the presence of production of extracellular polymeric substance or biofilm matrix. The extracellular matrix limits the penetration of antimicrobial agents into the biofilm. This is partly due to diffusion limitation caused by the 3-dimensional structure, but primarily because of absorption or reaction of the antimicrobial agent with extracellular matrix components. This takes place at the outer part of the biofilm and neutralizes the antimicrobial agent. Therefore the innermost bacterial cells of the biofilm are not reached by the antimicrobial agent and survive the treatment. Another factor which may contribute to this increased resistance is that the majority of antimicrobial compounds are more effective against actively growing cells. The cells in a biofilm have a poor growth rate due to lack of nutrients and oxygen, which may reduce the antimicrobial effects of compounds against them (Sandasi *et al.*, 2010).

CONCLUSIONS

Present investigation demonstrated that *Th. vulgaris* essential oil is effective not only on planktonic cells but also on biofilms of *S.* Enteritidis. Overall, essential oil of *Th. vulgaris* was found to be more effective in inhibiting initial cell attachment compared to preformed biofilms. Due to excellent inhibitory effect of *Th. vulgaris* essential oil on initial cell attachment, the use of this essential oil and its components with a view to preventive inhibition of biofilm formation is a promising approach. Essential oil and its components would allow inclusion of these compounds in novel pharmaceutical products, disinfectant and sanitizer formulations.

ACKNOWLEDGEMENTS

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OSA STARCHES AS IMPROVERS IN GLUTEN-FREE BREAD FROM HEMP SEED MEAL

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ABSTRACT

By-products of vegetable, fruit and oilseed processing industry can been utilized for fortification of gluten-free products which, although being essential for people with celiac disease, usually lack important nutrients. However, their presence in gluten-free bakery formulations is often associated with negative technological quality of final product. According to recent studies, different hydrocolloids can be used as gluten mimetics. Due to the fact that feasibility of the most of them has already been investigated, the aim of this study was to test the role of starch sodium octenyl succinate (OSA starch) as gluten-free bread improver.

Therefore, in order to obtain both nutritionally and technologically improved gluten-free bread, a formulation in which up to 20% of rice flour was substituted with hemp seed meal (a by-product of cold pressed hemp seed oil processing) and up to 5% of single and dual modified OSA starches was developed. Obtained gluten-free mixture was tested for nutritional and rheological properties, as well as for quality attributes (specific volume, texture, crumb structure, sensory properties).

All samples with added hemp flour had much better nutritional quality than the one of solely rice flour in terms of higher protein, crude fibers, minerals, and essential fatty acids content. On the other hand, addition of pregelatinized OSA starch contributed to increased water absorption and viscosity of gluten-free batter in comparison to control sample. Moreover, gluten-free breads containing OSA starches have shown superior properties in term of specific volume and crumb texture. Developed gluten-free formulation was sensory acceptable, since addition of hemp flour contributed to pleasant nutty flavor.

Keywords: hemp processing by-products, OSA starch, gluten-free bread

INTRODUCTION

There is a trend in utilizing by-products of vegetable, fruit and oilseed processing industry for improving nutritional characteristic of different food products. Vergara-Valencia *et al.* (2007) demonstrated that mango dietary fiber concentrate from unripe fruit can be applied as bakery product ingredient in order to increase its antioxidant capacity. Moreover, apple pomace, a by-product of apple juice industry, can be used as a as a source of dietary fiber and polyphenols in cake production (Sudha *et al.*, 2007), while raspberry (Górecka *et al.*, 2010), white grape (Mildner-Szkudlarz *et al.*, 2013) and blueberry (Mišan *et al.*, 2014) pomace were used for cookies enrichment. Citrus by-products (lemon albedo and orange dietary fiber powder) were added to cooked and dry-cured sausages to increase their dietary fibers content (Fernández-López *et al.*, 2004), while orange juice fibers (peel, pulp and seeds) were used as a fat replacer in ice cream (Crizel de Moraes *et al.*, 2013).

This is especially important for gluten-free bakery formulations which, although being essential for people with celiac disease, usually lack important nutrients (Torbica *et al.*, 2010). However, addition of food industry by-products in bakery formulations is often associated with negative technological quality of final product. Investigation performed by Arora and Camire (1994) revealed that muffins with potato peels, although being significantly enriched with fiber content, were darker, lower in height, and more resistant to compression. According to recent studies, different hydrocolloids can be incorporated into gluten-free bakery products in order to increase their quality characteristics, since they can act as gluten mimetics (Lazaridou *et al.*, 2007).

Due to the fact that there are a numerous published papers regarding the implementation of different hydrocolloids as gluten mimetics, this research will focus on investigation of starch sodium octenyl succinates (OSA starch) as gluten-free bread improvers. The aim of this study was to obtain both nutritionally and technologically improved gluten-free bread based on rice flour enriched with hemp seed meal (a by-product of cold pressed hemp seed oil processing) and up to 5% of single and dual modified OSA starches. In order to indicate the role of rice flour substitution with hemp meal, nutritional profiles of both rice flour as well as hemp meal were determined. Moreover, dough rheological properties of obtained gluten-free mixtures as well as bread quality characteristics (specific volume, texture, crumb structure, sensory properties) of the final products were estimated.

MATERIAL AND METHODS

Material
Hemp cake, representing a by-product which remains after hemp seed oil cold-pressing, was donated by Svet konoplje, Kisač, Serbia. Hemp flour was prepared by grinding hemp cake using Foss Knifetec 1095 (FOSS, Hillerød, Denmark). Ground hemp meal was separated into two fractions >350 μm and <350 μm using a Universal Laboratory Sifter (Bühler AG, Uzwil, Switzerland) and the fraction below 350 μm was used for breadmaking.
The starch sodium octenyl succinate (C*EmTex 06328 – OSA MOD) and pre-gelatinized starch sodium octenyl succinate (C*EmTex 12688 – Pregel OSA MOD) were provided from Cargill, France. Salt, sugar, margarine and fresh yeast were purchased in local store.

Methods
Chemical analysis of flour
Protein, fat (total, saturated, and unsaturated), reducing sugar and crude fiber content was determined according to the standard AOAC methods (AOAC, 2006), while starch content was measured according to ICC standard (ICC, 1994). Mineral content of the samples (Na, Mg, Ca, Mn, Fe, Cu and Zn) was determined using atomic absorption spectrometer (Varian Spectra AA 10, Varian Techtron Pty Limited, Australia) equipped with flame furnace and operated with air-acetylene flame. Samples were prepared in two-step procedure using microwave oven (Milestone, Italy) and digested with nitric acid and hydrogen peroxide.

Breadmaking procedure
Control flour mixture consisted of 80% rice flour and 20% of hemp flour, while in other tested flour mixtures 5% of rice flour was substituted with 5% of OSA MOD or with 5% of Pregel OSA MOD, respectively. All other ingredients, including water were added calculated on a flour mixture mass (100g): margarine (4%), sugar (4%), salt (3%), fresh yeast (3%) and water 80%. Bread doughs, i.e. batters were prepared by mixing all ingredients with a 4-speed mixer (Gorenje MRP 275 EA, Slovenia) for 2 min. After that, fresh yeast was also added and mixed for additional 2 min. 120g of dough was then transferred into greased tin pans (90×60 mm in top, 80×50 mm in bottom and 50 mm in height) and proofed for 30 min at 30 °C and the relative humidity of 85 % for final fermentation. The baking tests were conducted at 230 °C using laboratory baking oven (MIWE Condo, Michael Wenz, Germany) until mass loss of 10%. After the baking procedure, loaves were removed from the pans and left to cool down for 2 h at 20°C and finally they were sealed in polyethylene bags prior bread quality determination.

Rheological properties of dough
Rheological measurements were conducted using Haake MARS rheometer (Thermo Scientific, Karlsruhe, Germany) equipped with cylinder sensor system Z20 DIN (bob diameter = 20 mm and inner cup diameter = 21.7 mm, gap 4.20 mm). Flow curves were recorded at 30 °C using a hysteresis loop method (shear stress versus shear rate) which was performed in the range of shear rate 0–100 1/s. Shear rate was increased to 100 1/s linearly during 2 min, afterwards it was held on 100 1/s for 2 min, and finally it was decreased to 0 1/s linearly

for 2 min. Obtained hysteresis area, representing a measure of structural breakdown, was determined using the following equation:

$$\text{Hysteresis loop area (A)} = A_{up} - A_{down} \tag{1}$$

where A_{up} and A_{down} are obtained surface areas under the ascending and descending flow curves, respectively. All the rheological measurements were performed in duplicates.

Bread quality characterization

Bread specific volume was determined in triplicates using VolScan Profiler 600 (Stable Micro Systems, UK).

Textural properties, i.e. breadcrumb firmness of the tested samples were investigated using Texture analyzer TA.XPplus (Stable Micro Systems, UK) equipped with a 30-kg load cell. Since the final product was smaller than the standard bread loaf, the modified standard method for determination of bread firmness AACC (74-09) was performed. Modification involved investigation of the breadcrumb textural properties using P/0.5 inch (d=12.7 mm) diameter cylinder probe instead of P/36R (d=36 mm). Textural properties were determined 2 h after baking and storage at room temperature on four slices of the each loaf and the obtained results were expressed as the hardness of the final product. Measurements were performed in compression mode using following settings: pretest speed 1 mm/s; test speed 1.7 mm/s and post-test speed 10 mm/s and strain 40%.

Bread crumb digital image analysis was performed after 8 h of storage, on four slices per loaf, according to Dapčević Hadnađev *et al.* (2014). For the particle size measurements the cell was defined as any form larger than 0.05 mm^2.

Sensory analysis

A sensory analysis was performed 2 h after baking by 20 untrained panelists. The following sensory attributes were evaluated: taste, appearance, softness and flavor using 5 - point hedonic scale (1 - Dislike very much, 2 - Dislike moderately, 3 - Neither like or dislike, 4 - Like moderately, 5 - Like very much).

Statistical analysis

All analyses were performed in replicates and the mean values with the standard deviations are reported. Analysis of variance and Tukey's multiple range test were performed using Statistica 10.0 (Statsoft, Tulsa, OK). Means were considered significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

Nutritional profiles of rice and hemp flour, used for gluten-free bread making, are presented in Table 1. The obtained results indicate that substitution of rice flour with hemp flour will result in higher protein and fat content and lower carbohydrate content of final product. This bread will also be a rich source of dietary fibers. The consumption of 100 g of this product daily will satisfy a 10% of EFSA dietary reference values for fibers (EFSA, 2010), since EFSA Panel considers dietary fiber intakes of 25 g/day to be adequate for normal laxation in adults. The hemp flour can also be considered as a rich source of macro- and micro-elements. According to Serbian legislative (based on European Union Directive), reference daily intake for Ca, Mg, Fe, Zn, Mn and Cu are 800, 375, 14, 10, 2 and 1 mg, respectively, meaning that 100 g of this gluten-free bread contains Ca, Mg, Fe, Zn, Mn and Cu in an amount which represents 5.7, 15.7, 20, 9.2, 81 and 22.8% of their reference daily intake, respectively. These values indicated that this product could have a food label with nutritional claims: "a source of Mg, Fe and Cu" and "high Mn". Hemp flour can be also considered as a rich source of polyunsaturated fatty acids, since they comprise 74.6% of total fatty acids.

Table 1. Nutritional value of rice and hemp flour

Nutrient content	Rice flour	Hemp flour
Total fat, g/100g	1.26	14.3
Saturated fat, g/100g	0.41	1.7
Monounsaturated fat, g/100g	0.46	1.9
Polyunsaturated fat, g/100g	0.39	10.7
Total carbohydrate, g/100g	80.3	31.7
Dietary fibre, g/100g	2.5	10.7
Sugars, g/100g	0.12	3.6
Protein, g/100g	5.7	36.0
Ca, mg/100 g	9.7	341
Mg, mg/100 g	33.9	351
Na, mg/100 g	n.d.	9.21
Fe, mg/100 g	n.d.	22.11
Zn, mg/100 g	0.7	4.77
Mn, mg/100 g	1.1	8.94
Cu, mg/100 g	n.d.	1.48

Concerning the gluten-free bread technological quality, formulation containing 80% rice flour and 20% hemp flour (calculated on flour basis) resulted in viscous batter which expressed thixotropic behavior (viscosity at 100 s-1 = 3.171 Pas and hysteresis loop area (A) =2818 Pa/s). Addition of OSA MOD slightly decreased viscosity (2.770 Pas) and hysteresis loop area (2713 Pa/s) values, while presence of Pregel OSA MOD significantly increase both viscosity (9.572 Pas) and thixotropy (48470 Pa/s) values. This was influenced with increased water absorption capacity of Pregel OSA MOD (Dapčević Hadnađev *et al.*, 2014).

Influence of OSA starch addition on gluten-free bread quality was determined by measuring their specific volume and breadcrumb texture and structure. The obtained results are summarized in Table 2.

Table 2. Bread quality parameters

Sample	Control	OSA MOD	Pregel OSA MOD
Specific volume (cm^3/g)	1.595±0.004[a]	1.623±0.003[b]	1.743±0.003[c]
Bread crumb hardness (g)	2059.95±41.66[c]	1506.93±59.35[b]	718.59±67.11[a]
Cells/cm^2	40.36±1.73[b]	41.40±1.49[b]	34.94±3.64[a]
Mean cell area (mm^2)	0.883±0.048[a]	0.848±0.025[a]	1.080±0.146[b]
Cell/total area'ratio (%)	35.55±0.56[a]	35.06±0.61[a]	37.32±1.24[b]

Values are the mean±standard deviation. Values followed by the same letter in the row are not significantly different (p>0.05)

In general, the addition of OSA starches resulted in specific volume increase and hardness decrease and this effect was more pronounced for Pregel OSA MOD. Concerning crumb grain features, the addition of Pregel OSA MOD significantly affected cell size and distribution, i.e. the breadcrumb pores were larger and therefore the structure was less dense which was in accordance with the results of texture analysis and specific volume. Finally, the sensory analysis revealed that there were no significant differences between tested samples concerning taste (3.02-3.26) and flavour (3.91-4.12), while bread with Pregel OSA MOD was characterized with the highest scores for softness (4.3) and appearance (3.5). Although the addition of hemp flour contributed to pleasant nutty flavour, lower scores for taste were influenced by slight bitterness which is uncommon for bread.

CONCLUSIONS

According to determined nutritional profiles of rice and hemp flour, it was concluded that partial substitution of rice flour with hemp flour resulted in gluten-free bread of significantly improved protein, dietary fiber, polyunsaturated fatty acids and mineral content. Further

substitution of rice flour with OSA starches (especially pregelatinized OSA starch) yielded gluten-free bread of improved quality characteristics concerning bread specific volume and crumb texture, which was influenced with altered rheological properties of dough prepared from formulation containing pregelatinized OSA starch.

ACKNOWLEDGEMENTS

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DEAERATION METHOD FOR IMIDACLOPRID DETERMINATION ON GLASSY CARBON ELECTRODE

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ABSTRACT

In this work deaeration methods were investigated for chronopotentiometric determination of pesticide imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine] on glassy carbon electrode. As a supporting electrolyte, a Britton-Robinson buffer was used. All investigations were carried out in model systems. Cathodic peak of imidacloprid could not be registered only in Britton-Robinson buffer, due to electrochemical reduction of dissolved oxygen. For this reason, it was necessary to investigate different deaeration methods for removing oxygen from the solution. Within this, deaerations by various reductants and by passing a nitrogen stream were compared. Investigated reductants were sodium sulfite, ascorbic acid, oxalic acid, potassium bromide and sodium bromide. Combinations of a certain reductant and nitrogen were compared as well. Addition of a saturated solution of sodium sulfite directly in the tested solution proved as the best deaeration method. This method provided fast deaeration step as it took less than a minute for completely removing the oxygen. An optimal concentration of the solution of sodium sulfite was also investigated. Concentrations of sodium sulfite were tested in the range of 0.6 g/dm^3 to 230 g/dm^3. Considering the height of the analytical signal and its reproducibility, concentration of 8.8 g/dm^3 showed as optimal. This method could be applied for the determination of imidacloprid in commercial formulations and some real samples.
Keywords: *imidacloprid, glassy carbon, deaeration method*

INTRODUCTION

Imidacloprid belongs to group of neonicotinoids, relatively new group of insecticides, characterized by low toxicity to warm-blooded animals, and good efficiency against potato beetle, thrips, aphids, as well as many other insects (Šovljanski and Lazić, 2007). Its specific mode of action interrupts nervous functions of insects, which brings to paralysis and death (Šovljanski and Lazić, 2007). Wider application of imidacloprid leads to its more frequent occurance in food and environment, which imposes development of rapid and simple methods for its detection.

In this work, chronopotentiometric determination of imidacloprid on a glassy carbon electrode as a working electrode was proposed. This electrochemical method is based on oxidation/reduction of the analyte on the electrode surface, in steady solution with the constant current. Qualitative and quantitative characteristics of the analyte were obtained by measuring the oxidative/reductive potential and oxidative/reductive time. Given that imidacloprid in its structure has a nitro group, it reduces in two steps. The first step is based on irreversible reduction of nitro group to hydroxylamine and then to the corresponding amine in the second step (Guiberteau *et al.*, 2001). In previous research on reduction of imidacloprid on glassy carbon electrode, when voltammetry was used, only one reduction peak could be registered at -1,2 V (*vs. saturated calomel electrode*) (Guzsvány *et al.*, 2005). Electrochemical detection of imidacloprid was not possible without previous deaeration of the tested solution, due to reduction of dissolved oxygen on the working electrode, resulting in high residual current, which interfered with the measurement of many reducible analytes (Walace, 1985; Wang, 2006). In addition, species that were formed during this reaction (H_2O_2 and OH^-) might affect the electrochemical process being studied (Walace, 1985).

There are many different methods for removing dissolved oxygen. Currently, the most popular method is purging samples with an inert gas, usually nitrogen. Time of purging depends on the volume of the solution, flow rate and bubble size of deaeration gas, and technique that is used. Deaeration step by inert gas usually lasts 10-15 minutes.

Another method of oxygen removal is chemical deaeration, by adding reductant in the tested solution, which will react with dissolved oxygen from the solution. Addition of sulfite ion represents the oldest method for oxygen removal. Due to electroactivity of the sulfite ion at pH ≥ 7, this procedure is not commonly used in analytical practice (Walace, 1985).

According to literature, standard deaeration method that is used within different electroanalytical methods for pesticides determination is passing a nitrogen or argon stream (El-Shahawi and Kamal, 1998., Farzinnejad *et al.*, 2005; Guibertau *et al.*, 2001; Guzsvány *et al.*, 2005; Pushpalatha *et al.*, 2011; Pushpalatha *et al.*, 2013; Navalón *et al.*, 1999; Nigović *et al.*, 2011; Papp *et al.*, 2010; Papp *et al.*, 2011; Papp *et al.*, 2009; Zuman *et al.*, 2000). This deaeration method is time consuming, as it lasts up to 15 minutes, much longer than the analysis. Nowadays, the development of rapid and simple deaeration method is necessary. Therefore, in this study, different deaeration methods for chronopotentiometric determination of imidacloprid were compared. Investigation included comparison of passing a nitrogen stream, addition of different reductants, combinations of certain reductants, and combination of nitrogen and reductant.

MATERIAL AND METHODS

Apparatus

All chronopotentiometric experiments were carried out by automatic system for potentiometric and chronopotentiometric stripping analysis, constructed by our laboratory. Electrochemical cell consisted of three electrodes and electrical stick stirrer. Glassy carbon disc electrode was used as a working electrode (total surface area of 7.07 mm^2), a platinum wire (φ=0.7 mm, l=7mm) served as a counter electrode, and Ag/AgCl (KCl, 3.5 mol/dm^3) was used as the reference electrode. The three-electrode system and electrochemical stick stirrer were placed in a process glass. It was a glass vessel of 50 cm^3, with tapered bottom. Before each measurement, the surface of the glassy carbon was washed with acetone and doubly distilled water. All measurements were carried out at room temperature (23 ± 2 °C). All values of the potential were shown versus Ag/AgCl (KCl, 3.5 mol/dm^3) reference electrode.

Reagents and solutions

All chemicals used were of analytical reagent grade purity. For all dilutions and dissolutions doubly distilled water was used. Stock solution of imidacloprid (0.4 g/dm^3), was prepared by exact weighing of the reagent (Bayer AG, Leverkusen, Germany) and dissolution in doubly distilled water. This solution was stable for a three-week period when stored in the dark at 4°C. Britton-Robinson buffer was used as a supported electrolyte. It was prepared from equimolar 0.04 mol/dm^3 stock solutions of orthophosphoric (Zorka, Šabac, Serbia), boric (Zorka, Šabac, Serbia) and acetic (Lach-Ner, Brno, Czech Republic) acids. Required pH value of the buffer (pH 7.5) was set by adding 0.2 mol/dm^3 sodium hydroxide (Donau Chemie, Wien, Austria).

General procedure for investigation of deaeration method implied introducing of a certain volume of analyzed solution (usually 20 cm^3) into the process glass, followed by adding certain mass of reductants, stirring the solution, and after a brake of 10 seconds, the analytical step was performed from –0.91 V to –1.42 V. The tested reductants were: sodium sulfite (Centrohem, Stara Pazova, Serbia), oxalic acid (Lachema, Brno, Czech Republic), ascorbic acid (Lach-Ner, Brno, Czech Republic), potassium bromide (Merck, Darmstadt, Germany) and sodium bromide (Carlo Erba, Milano, Italy). Combinations of certain reductants were also investigated. When deaeration was performed by passing a nitrogen stream, the procedure was similar with the blank (20 cm^3 of Britton-Robinson buffer). Deaeration usually lasted for 10 minutes. Any additional measurements in the same solution implied additional deaeration for 2 minutes.

RESULTS AND DISCUSSION

At the beginning of the study, preliminary experiments were performed by comparing chronopotentiogram recorded in supporting electrolyte with a certain amount of various reductant. After a period of stirring (30-60 s), the analytical step was performed. Afterwards, certain amounts of standard solution of imidacloprid were added, and analytical signals of analyte were compared. The first tested reductant was sodium sulfite. After addition of sodium sulfite, deaeration took only 30 seconds, analysis was fast (1-2 s), and concentration of 15 mg/dm^3 of imidacloprid was detected. Ascorbic acid (Figure 1.a), oxalic acid, potassium bromide (Figure 2.b) and sodium bromide were not able to completely deaerate the solution, so analytical signal of imidacloprid could not be registered, even at higher concentrations (40 mg/dm^3 and 60 mg/dm^3). Increasing the amounts of ascorbic acid, oxalic acid and potassium bromide did not lead to improvements. By increasing the amount of oxalic acid, the pH of the tested solution was very low (pH 2). At this pH value imidacloprid could not be detected at all. When sodium sulfite was added to the solution containing ascorbic acid, sodium bromide or potassium bromide, the final potential was reached, and concentration of 20 mg/dm^3 was detected. Addition of higher concentrations of sodium bromide in blank was enough for achieving the final potential of -1.35 V, but the concentration of 20 mg/dm^3 of imidacloprid could not be detected.
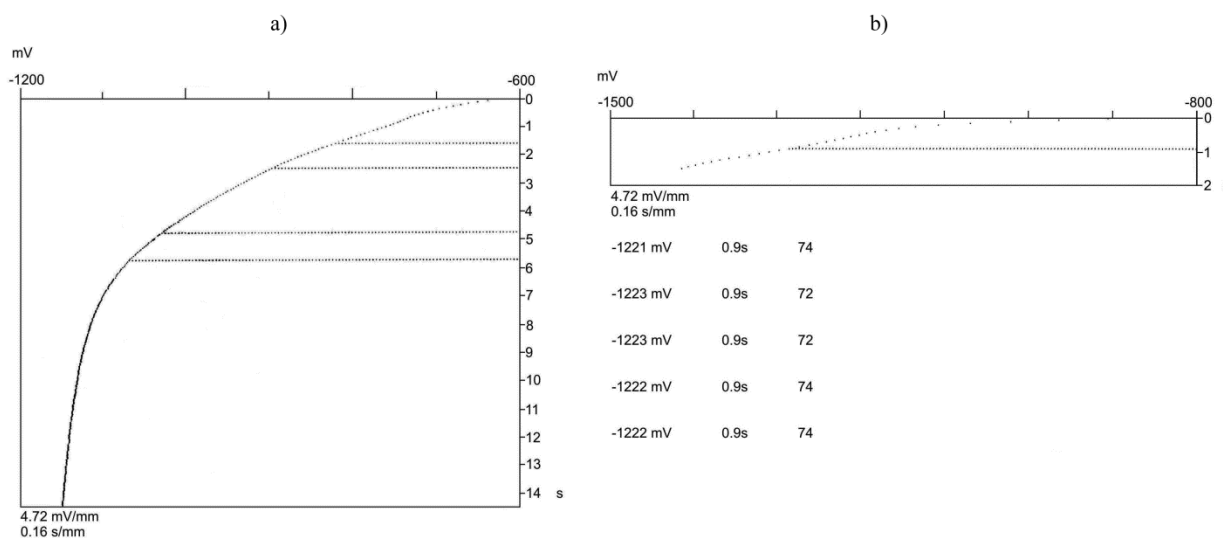
Figure 1. Original chronopotentiograms obtained by deaeration with (a) ascorbic acid, and (b) 8.8 g/dm^3 sodium sulfite

When nitrogen was used for deaeration, usual time for deaeration was 10 minutes. Analysis of the blank was fast (2-3 s), and there were no signals. After addition of standard solution of imidacloprid, in this case, the analysit had to be very agile, because in a short time when an airtight apparatus was opened, oxygen re-enetered the system, so additional deaeration for 2 minutes between adding a standard solution was performed. When nitrogen was used for deaeration, concentration of imidacloprid that was detected was 10 mg/dm^3. Sometimes, analysis of the blank and different concentrations of imidacloprid could not be performed at all, due to blocking the glassy carbon surface with bubbles of nitrogen (Figure 2.a).
This behavior was similar to the situation when oxygen was present in solution, which led to blocking of the analyzer at the potential of -1.3 V. After wiping of the glassy carbon surface with acetone and doubly distilled water, the analysis could be performed, but conditions were not reproductive, as demonstrated by slightly lower analytical signal. The same thing happened when the combination of nitrogen stream and certain concentration of sodium sulfite were studied, but in this case, deaeration lasted only 5 minutes. Considering all these results, as well as difficulties when nitrogen was used, all reductants, except sodium sulfite,

were excluded from further research. However, in order to achieve the best sensitivity with satisfactory reproducibility, it was necessary to investigate an optimal concentration of sodium sulfite. Saturated solution of sodium sulfite was prepared (230 g/dm^3), and different volumes of this solution were added to the Britton-Robinson buffer. Tested concentrations of sodium sulfite were in the range: 0.6 to 230 g/dm^3, in order to compare analytical signal of imidacloprid concentration of 15 mg/dm^3. The concentration of 0.6 g/dm^3 of sodium sulfite was sufficient for deaeration, but the concentration of 15 mg/dm^3 of imidacloprid could not be detected. When only saturated solution of sodium sulfite was used as supporting electrolyte, concentration of 15 mg/dm^3 of imidacloprid was detected, and this solution gave the highest analytical signal, but also outstretched chronopotentiograms, with very poor reproducibility (RSD = 19.63%). Results of investigations are shown in Table 1. It is evident that there is no significant difference in the height of the analytical signals when different concentrations of sodium sulfite were used. In terms of reproducibility, there was a great difference when different concentration of sulfite was used. Due to the satisfactory sensitivity and the best reproducibility of determination, concentration of 8.8 g/dm^3 of sodium sulfite was accepted as optimal (Figure 1.b).

Table 1. Overview of the reduction time in function of the concentration of sulfite

Concentration of sodium sulfite (g/dm^3)	Reduction time (s)	RSD (%)
0.6	/	/
1.1	0.78±0.08*	5.73
1.7	0.78±0.16	10.73
2.3	0.72±0.08	6.21
2.8	0.74±0.10	7.40
3.4	0.72±0.08	6.21
4.0	0.72±0.08	6.21
4.5	0.7±0.00	0.00
6.7	0.88±0.16	9.51
8.8	0.90±0.00	0.00
11.0	0.94±0.22	12.13
57.5	0.78±0.16	10.73
76.7	0.90±0.20	11.11
115.0	0.94±0.18	9.52
230.0	0.98±0.38	19.63

*Xmean±2SD
RSD – Relative standard deviation
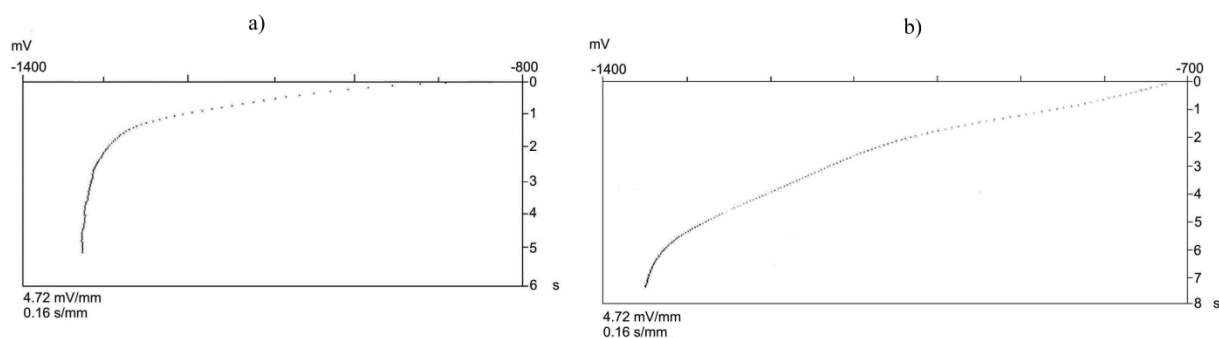
Figure 2. Original chronopotentiograms obtained by deaeration with (a) nitrogen, and (b) potassium bromide.

CONCLUSIONS

This study suggests the importance of chemical deaeration for electrochemical determination of imidacloprid, due to the fact that proper selection of deaeration method can lead to faster analysis and better reproducibility. When solution of sodium sulfite was used for deaeration, no blocking the glassy carbon surface was observed. This method is fast as it takes only 30 seconds to completely remove the oxygen from the solution. The proposed deaeration method can be used for development of chronopotentiometric determination of imidacloprid on glassy carbon electrode, or some other solid electrode, which can be applied to commercial formulations and real sample analyses.

ACKNOWLDGEMENTS

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RHEOLOGICAL METHODS TO CHARACTERIZE SPELTA FLOUR FOR PASTA MAKING

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ABSTRACT

Spelt wheat is suitable raw material for flour based product characterized by altered nutritional characteristics and health benefits compared to conventional wheat products. This paper investigates rheological methods for determining the quality of 5 different spelt samples of flour as a raw material for pasta making. Correlation between rheological methods for flour (Mixolab data and Farinogram of semolina) pasta quality is defined. Sample 1 has the lowest speed of protein network weakening due to warming -0.053 (Nm/min), the lowest warm paste stability (0.173 Nm/min) and retrogradation of starch (2.94 Nm) and the best score for semolina farinogram. These data are in direct correlation with quality of pasta (time of cooking 12 min and adhesiveness 10.25 gsec). Negative correlation between water absorption and Farinogram of semolina, as well as between water absorption and adhesiveness was observed (r=-0.98, statistically significant at p<0.01 level), while positive correlation was noticed between water absorption and gluten strength (r=-0.98, p<0.01). Positive correlation between warm paste stability and Farinogram of semolina (r=0.98, p<0.01), as well as negative correlation between warm pasta stability and gluten strength was observed (r=-0.97, p<0.01). Retrogradation of starch and cooking time are negatively correlated (r=-0.99, p<0.01). Based on the rheological methods such as Mixolab and Farinogram data quality of pasta can be predicted.
Keywords: *Mixolab data, Farinogram of semolina, pasta quality*

INTRODUCTION

Spelt wheat shows a very good adaptability, growing without use of pesticides, so it is suitable as an organic material. Spelt have shown potential in various food applications, including bread, pasta, breakfast cereal and other products of altered nutritional characteristics compared to conventional wheat products (Bonafaccia *et al.*, 2000, Bojanska and Frančakova, 2002). Rheological methods for flour (Mixolab data and Farinogram of semolina) are determining the quality of pasta. The Mixolab technique can be considered as an empirical method that record the dough changes when subjected to large deformations and to temperature sweeps particularly experienced for characterizing bread dough (Ozturk *et al.*, 2008, Rosell *et al.*, 2010).
This paper investigates rheology methods (mixolab and alveograph data) of five spelt samples to define possibility of correlation with the pasta quality.

MATERIALS AND METHODS

Five spelt samples growing in Serbia were used. Dough rheology investigations were performed by Mixolab (Chopin, Tripette et Renaud, Paris, France). The method was described by Filipović *et al.*, (2013). Semolina Farinogram was determined using 50g Farinograph mixing bowl according to Kaluđerski and Filipović (1998). Pasta was made using the device "La Parmigiana D45" MAC 60 according the procedure described by Filipović *et al.* (2013). Quality of pasta cooking characteristics (time of cooking) was determined according to Kaluđerski and Filipović (1998). Textural properties of cooked pasta were measured with Texture analyzer TA.HD plus (Stable Micro System, U.K.) equipped with a 5-kg load cell. Adhesiveness was described by Filipović *et al.*, (2014). Principal component

analysis (PCA) was applied successfully to classify and discriminate the different samples, according to technological parameters. The evaluation of principal PCA of the obtained results was performed using StatSoft Statistica 10.0® software.

RESULTS AND DISCUSSION

Different samples of Spelt flour are used to find relationships between parameters characterizing the main dough proteins and starch. Correlation is established between Mixolab parametars (Table 1) and quality of pasta (time of cooking and adhesiveness) (Table 2). In Table 1, there are 5 different samples with diverse physical responses during mixing-pasting-gelling, which vary substantially from one sample to other. Increasing water absorption (53.7 %) in sample 1 (Table 1) is in direct correlation with quality of pasta (time of cooking 12 min and adhesiveness 10.25 gsec) (Table 2). Lowest speed of protein network weakening due to warming -0.053 (Nm/min), the lowest speed of starch gelatinization (0.173 Nm/min) and retrogradation of starch (2.94 Nm) positive affected on the best score for semolina Farinogram and quality of pasta (Table 2).

In Table 2, there are five flours of spelt samples with defining quality. There is trend of increasing of mixing time and gluten strength, reduction of maximum consistency, mixing tolerance, and dough elasticity between samples 1 to 5 (Table 2).

Table 1. Mixolab properties of different samples of flour

Mixolab data	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Water absorption (WA), %	53.7	52.9	52.8	52.1	50.9
Dough development (DD), min	8.73	8.86	8.93	9.03	10.2
Dough elasticity (DE), Nm	0.05	0.05	0.05	0.05	0.05
Dough stability (DS), min	7.57	8.20	7.67	8.17	6.83
Speed of weakening protein networks due to warming (SW), Nm/min	-0.053	-0.051	-0.051	-0.052	-0.049
Maximum torque (MT),Nm	1.37	1.35	1.54	1.66	1.69
Speed of starch gelatinization (SSG), Nm/min	0.173	0.192	0.219	0.242	0.227
Speed of enzymatic degradation (SED), Nm/min	0.097	0.103	0.089	0.083	0.1
Warm paste stability (WPS), Nm	2.05	2.07	2.16	2.24	2.34
Retrogradation of starch (RS), Nm	2.94	2.95	3.10	3.29	3.38

Sample 1 has the lowest mixing time (4 min), the lowest gluten strength (7.75 min), highest maximum consistency (560 FU), highest mixing tolerance (1.75 min) and highest dough elasticity (140 mm). Sample 1 has the best score for semolina Farinogram, while sample 5 has the worst score, which are in direct correlation with quality pasta. Sample 1 has the best quality of pasta (time of cooking increasing 12 min and adhesiveness reducing 10.25 gsec) and sample 5 has the worst quality of pasta (time of cooking reducing 7 min and adhesiveness increasing 33.97 min). Quality and textural of pasta are very important product attributes which affect on acceptance of the product by the consumers.

Table 2. Quality of semolina and pasta of different samples of flour

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Faringogram of semolina, FS					
Mixing time (MIT), min	4	4.5	5.5	6	8
Gluten strength (GS), min	7.75	8.5	11	10.5	12
Maximum consistency (MC), FU	560	500	450	420	340
Mixing tolerance (MTL), min	1.75	1	0.5	0.5	0.5
Dough elasticity (DE),mm/FU	140	100	100	100	80
Quality/ texture of pasta					
Time of cooking (CT), min	12	12	10	7	7
Adhesiveness (AD), gsec	10.25	18.32	22.28	24.12	33.97

Correlation analysis (Table 3), showed that WA is negatively correlated to FS and AD and positively correlated to GS, statistically significant at p<0.05 level. DD relates to FS, while MT is in positive correlation to FS and MIT and negative correlation to GS and CT, statistically significant at p<0.05 level. SSG is negatively correlated to MC and CT, while WPS and RS are positively correlated to FS, MIT and AD, and negatively correlated to GS and CT.

Table 3. Correlation analysis of Mixolab properties and the quality of semolina and pasta of different samples of flour

		**Quality of semolina and pasta of different samples of flour**						
		FS	MIT	GS	MC	MTL	CT	AD
	WA	-0.98*	-0.86**	0.98*	0.75	0.87**	0.87**	-0.98*
		p=0.004	p=0.061	p=0.004	p=0.141	p=0.053	p=0.056	p=0.004
	DD	0.94*	0.76	-0.87**	-0.51	-0.72	-0.70	0.88*
		p=0.019	p=0.139	p=0.054	p=0.378	p=0.168	p=0.186	p=0.047
	DS	-0.64	-0.50	0.50	0.09	0.25	0.34	-0.51
		p=0.241	p=0.386	p=0.391	p=0.887	p=0.680	p=0.573	p=0.385
Mixolab properties	SW	0.82**	0.76	-0.83**	-0.66	-0.89*	-0.50	0.88**
		p=0.087	p=0.139	p=0.086	p=0.224	p=0.042	p=0.394	p=0.050
	MT	0.90*	0.91*	-0.91*	-0.80	-0.68	-0.99*	0.86**
		p=0.037	p=0.031	p=0.031	p=0.107	p=0.206	p=0.002	p=0.060
	SSG	0.76	0.86**	-0.85**	-0.93*	-0.75	-0.92*	0.81**
		p=0.135	p=0.061	p=0.070	p=0.024	p=0.142	p=0.028	p=0.099
	SED	-0.08	-0.32	0.16	0.42	-0.01	0.48	-0.07
		p=0.896	p=0.600	p=0.795	p=0.483	p=0.986	p=0.412	p=0.907
	WPS	0.98*	0.92*	-0.97*	-0.77	-0.78	-0.95*	0.95*
		p=0.003	p=0.028	p=0.005	p=0.128	p=0.121	p=0.012	p=0.014
	RS	0.95*	0.89*	-0.95*	-0.76	-0.73	-0.99*	0.91*
		p=0.015	p=0.044	p=0.015	p=0.135	p=0.164	p=0.002	p=0.034

*Significant at p<0.05 level, **Significant at p<0.10 level, 95% confidence limit

The PCA allows a considerable reduction in a number of variables and the detection of structure in the relationship between measuring parameters and different samples that give complimentary information. The full auto scaled data matrix were submitted to PCA. For visualizing the data trends and the discriminating efficiency of the used descriptors a scatter plot of samples using the first two principal components (PCs) from PCA of the data matrix is obtained (Fig. 1). As can be seen, there is a neat separation of the five samples of pasta

formulations, according to Mixolab properties and the quality of semolina and pasta of different samples of flour.

Quality results show that the first two principal components, account for 91.64% of the total variability and can be considered sufficient for data representation. Concerning observed technological characteristics, WA (with 7.6% contribution based on correlation), WPS (7.9%), RS (7.5%), FS (7.9%), MT (7.4%), GS (8.1%) and AD (7.9%) contributed the most to first factor calculation, DD (9.4%), DS (17.0%), SW (10.6%), SSG (10.5%) and SED (37.4%) contributed more to second factor coordinate calculation. PCA plot also shows the good correlation between MC and CT, GS, MTL and WA; SW and DD; FS and AD, and also WPS, MIT and RS. As can be seen (Fig. 1), samples 1 and 2 shows high MC and CT, as well as GS, MTL and WA values, while samples 3 and 4 exerted somewhat greater SSG and SED and lesser SED values compared to other samples. Sample 5 shows larger SW and DD, as well as FS, AD, WPS, MIT and RS values compared to other samples.
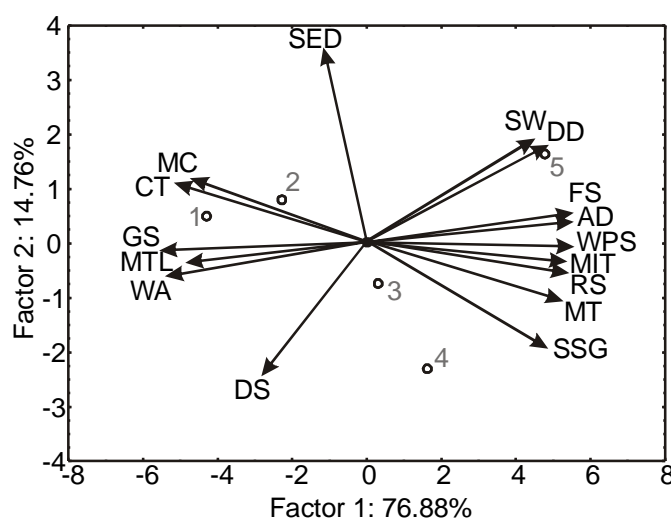
Figure 1. Biplot diagram of semolina and pasta of different samples of flour

CONCLUSIONS

Based on investigated data of five flours samples and mixolab and alveograph data of pasta quality, it can be concluded:

- Sample 1 has the lowest speed of protein network weakening due to warming -0.053 (Nm/min), the lowest warm paste stability (0.173 Nm/min) and retrogradation of starch (2.94 Nm) and the best score for semolina Farinogram and quality of pasta.
- Mixolab data, Farinogram data of semolina are in direct correlation with quality and texture of pasta.
- Sample 1 has the best quality of pasta (time of cooking 12 min and adhesiveness 10.25 gsec) and sample 5 has the worst quality (time of cooking 7 min and adhesiveness 33.97 gsec).
- Negative correlation was observed: between water absorption and Farinogram of semolina, water absorption and adhesiveness ($r=-0.98$, statistically significant at $p<0.01$ level), warm pasta stability and gluten strength ($r=-0.97$, $p<0.01$), retrogradation of starch and cooking time ($r=-0.99$, $p<0.01$)
- Positive correlation was noticed between water absorption and gluten strength ($r=-0.98$, $p<0.01$), warm paste stability and Farinogram of semolina ($r=0.98$, $p<0.01$),

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TRADITIONAL DAIRY PRODUCTS: RICH AND DIVERSE MICROBIOTA WITH HELTH-PROMOTING PROPERTIES

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ABSTRACT

Understanding the effect of beneficial microorganisms in maintaining the human health could shed new light on rare diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), ulcerative colitis, colorectal cancer (CRC), diabetes etc. as well as lead to new therapeutic strategies for their treatment. Hence, we aim to study the health-promoting potential of natural isolates of lactic acid bacteria (LAB) originating from traditional dairy products manufactured in households in Western Balkan Countries (WBC). The ultimate goal is to identify the autochthonous LAB isolates with anticancerogenic or immunomodulatory effect.

WBC is a distinct geographical area in Europe with the long tradition of manufacturing artisanal dairy products. Using molecular genetics methodology (rep-fingerprinting analysis, 16S rDNA sequencing) and detailed technological and probiotic characterisation of LAB isolates, including milk protein coagulation, proteinase and antimicrobial activity, exopolysaccharide production, production of aroma and taste precursors, as well as survival in simulated conditions of gastrointestinal tract, adhesion to epithelial intestinal cells (EIC), proliferation of gut associated lymphoid tissue (GALT) and peripheral blood mononuclear cells (PBMC), revealed the huge diversity of LAB strains present in the artisanal dairy products of WBC. Interestingly, our results showed that the autochthonous LAB isolates produce bioactive substances involved in immunomodulation-reducing directly or indirectly the level of cholesterol, triglycerides and blood sugar, or having hypoallergenic and immunosuppressive effect.

In conclusion, the characterisation of indigenous LAB strains in the artisanal cheeses, as well as characterisation of their probiotic and technological potentials makes possible formulation of defined functional starter cultures for novel dairy foods with geographical origin. In addition, such concept would lead to the isolation and characterisation of bioactive compounds suitable for the development of new therapeutics (nutraceuticals).

Keywords: *microbial diversity, artisanal dairy products, lactic acid bacteria, health-promoting properties*

INTRODUCTION

Interest in the role of traditional food and their associated microorganisms in human health is significantly increased, especially in term of their implication in the disease development and prevention. Modern Western diet includes the consumption of increased amounts of industrially processed and ready-to-eat food products containing numerous chemicals and additives. Recently it has been noted the correlation between the Western diet and the increased incidence of obesity, high blood cholesterol levels, high blood pressure, diabetes and many other health problems including certain types of cancers. In contrast, the value of the traditional food for the human health is supported by the fact that people inhabiting rural mountain areas, having traditional diet based on fermented food manufactured in households, are well known for good health and longevity.

Western Balkan Countries (WBC) region has a long and rich experience in traditional dairy products manufactured by spontaneous or controlled fermentation from cow's, ewe's and goat's milk resulting in traditional regional cheeses and other fermented dairy products. These products harbour a remarkable diversity of lactic acid bacteria (LAB) and yeasts (Golic *et al.,* 2013). Large quantities of various spontaneously fermented dairy foods are

manufactured in households of this region in specific way without use of commercial starter cultures.

Preliminary research on autochthonous LAB isolated from traditionally manufactured cheeses in WBC strongly indicates the considerable diversity among microorganisms in these products (Golic *et al.*, 2012; Terzic-Vidojevic *et al.*, 2014). Natural isolates of LAB are shown to be a valuable source of antimicrobial peptides targeting food pathogens without adverse effects (Topisirovic *et al.*, 2011; Veljovic *et al.*, 2014). Hence, they have a great potential for application in microbial food safety. Moreover, previous results revealed that many LAB isolated from traditional cheeses showed good milk protein coagulation, produce proteinases and exopolysaccharides. On the other hand, our recent results showed that LAB natural isolates originating from traditional dairy products have significant probiotic potential (Lukic *et al.*, 2012; Nikolic *et al.*, 2012a; Uroic *et al.*, 2014).

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). Probiotics express their positive traits through the following mechanisms: (a) competition with pathogenic bacteria for nutrients and binding sites in the gut epithelium, (b) inactivation of the toxins and metabolites produced by pathogens, (c) the production of antimicrobial substances which inhibit the growth of pathogenic microorganisms, (d) stimulation/modulation of the immune response, or (e) anticancer action (Boirivant and Strober, 2007). Nowadays, it is well known that probiotics impact on the immune function of humans and animals throughout the regulation of the immune response. Although, it is necessary to underline that the immune modulation ability is strictly species- and strain dependent (Díaz-Ropero *et al.*, 2007; van Hemert *et al.*, 2010) and that the individual strains exhibit specific probiotic traits effective for the treatment of a specific disease (Delcenserie *et al.*, 2008). However, the molecular basis of the mechanisms behind the probiotic action is still not fully characterized. Accordingly, our current work has been focused on the interactions of LAB natural isolates with the host immune system in order to detect the unique health-promoting properties of the individual strains.

MATERIAL AND METHODS

Bacterial strains. The LMM (Laboratory for Molecular Microbiology, IMGGE) collection has several thousands of fully characterized LAB isolates. Among them, up to 100 have been characterized according to FAO/WHO (2006) selection criteria as potential probiotic strains. LAB strains from LMM collection used in this study were isolated previously from white – pickled and semi-hard cheeses traditionally manufactured in rural regions of Serbia and Montenegro.

Host-microbe interactions. The epithelial intestinal cells (EIC) Caco-2 (purchased from the European Collection of Cell Cultures [ECACC No. 86010202] were used to determine the adhesion ability of the isolates to mucosal surfaces in the intestine. The pathogen exclusion experiments were carried out following the procedure described by Nikolic *et al.* (2012b). The isolation and proliferation of gut associated lymphocyte tissue (GALT) from rats were carried out as described by Hidalgo-Cantabrana *et al.* (2014). This study was approved by the Animal Experimentation Ethical Committee of the Faculty of Pharmacy, University of Belgrade (Serbia), strictly following the International Directives.

Statistical analysis. After checking the normal distribution of the proliferation data, one-way ANOVA tests were used to determine differences between each factor and negative control. Finally, one-way ANOVA tests together with the mean comparison test LSD (less significant difference) were used to compare differences between the three strains. Results were represented by mean ± standard deviation or standard error. The SPSS 15.0 statistical software package (SPSS Inc) was used for all determinations and $p < 0.05$ value was considered significant.

RESULTS AND DISCUSSION

Antimicrobial activity. As defined by WHO/FAO (2006), one of the important health promoting properties of probiotic strains is their capability to counteract the negative effects of gut pathogens. The exclusion of pathogens by probiotics is a strain-dependent characteristic. Several mechanisms of this action have been proposed: a) production of the antimicrobial substances, b) colonization competition, providing a physical barrier blocking the pathogen entry, c) induction of the mucus production, d) increasing the tight-junctions among the EIC, or e) stimulation of the innate immune response (Liévin-Le Moal and Servin, 2014).

Our previous results showed that dairy products microbiota contain promising probiotic candidates producing one or more bacteriocins with broad inhibitory spectrum both against Gram$^+$ and Gram$^-$ pathogens (Topisirovic *et al.*, 2011). In this study, we have found that among the analysed LAB strains, the strain *Lactobacillus paracasei* BGBUK2-8 produces antimicrobial substances active against *Helicobacter pylori*, while the strain *Lc. lactis* BGBU1-4 shows, among others, antimicrobial activity against food pathogen *Listeria monocytogenes.* In addition, bacteria of the genus *Enterococcus*, frequently found in many different types of fermented foods produce bacteriocins, designated as enterocins, active against food-born pathogens such as *L. monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* and could be used as food "biopreservatives" (Veljovic *et al.*, 2014). The dairy natural isolates of enterococci from LMM collection are found to exhibit antimicrobial activity against *L. monocytogenes, Escherichia coli* and *S. aureus.*

It is assumed that the probiotic properties are in large part derived from the specific surface molecules present on the surface of bacterial cells (exopolysaccharides - EPS, aggregation proteins - Agg, collagen binding proteins, proteinases) (Kleerebezem *et al.*, 2010). Hence, we have tested the ability of the lactobacilli natural isolates from LMM collection, producing various cell surface molecules, to reduce the adhesion of *Salmonella* 654/7E isolate to the Caco-2 EIC (Figure 1). The results revealed that the strain *L. paracasei* BGNJ1-64 Agg$^-$, has the ability to reduce the adhesion of *Salmonella* 654/7E. Since the strain BGNJ1-64 Agg$^-$ is a non-aggregating derivative of the strain BGNJ1-64, it could be assumed that some factors, other than aggregation factor, present on the cell surface, are involved in the reduction of *Salmonella* 654/7E. In addition, it was shown that the strain *Lactobacillus paraplantarum* BGCG11, the EPS-CG11 producer, and its polymer EPS-CG11 reduced the cytotoxic effect of several pathogens: *Clostridium difficile*, *Yersinia enterocolitica*, *E. coli* and *L monocytogenes* (data not shown).
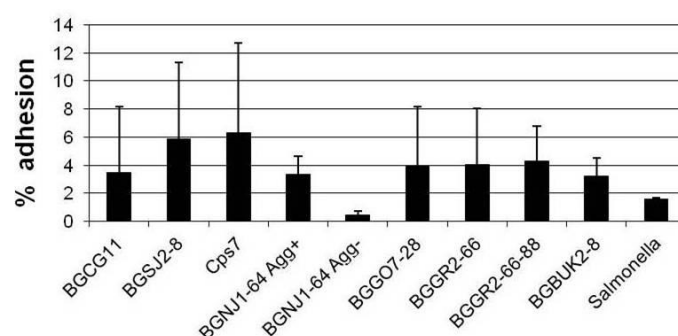
Figure 1. Percentage of adhesion of Salmonella 654/7E in the presence and absence of lactobacilli strains

Modulation of the immune response. The next important characteristic of potential probiotic candidates is the capacity to modulate the immune response of the host. Previously, we have shown that EPS-producing strain *Lb. paraplantarum* BGCG11 or its EPS-CG11 polymer exhibit an anti-inflammatory and immunosuppressory profile. The results revealed that this natural isolate increased cytokine ratios suggestive of Th2-Treg response

involved in suppressive and immunoregulatory functions, as well as Th17 response, relevant for the immune mucosa homeostasis. Interestingly, the non-EPS-producing derivatives of BGCG11 triggered a Th1 (pro-inflammatory) response type, possibly due to the exposure of other surface molecules in the absence of the EPS-CG11 polymer (Nikolic *et al.*, 2012a). Since the strain BGCG11 induced higher percent of the PBMC proliferation than the EPS-CG11 polymer, it can be concluded that the other specific cell surface molecules are involved in the proliferation of the lymphocytes (Nikolic *et al.*, 2012a).
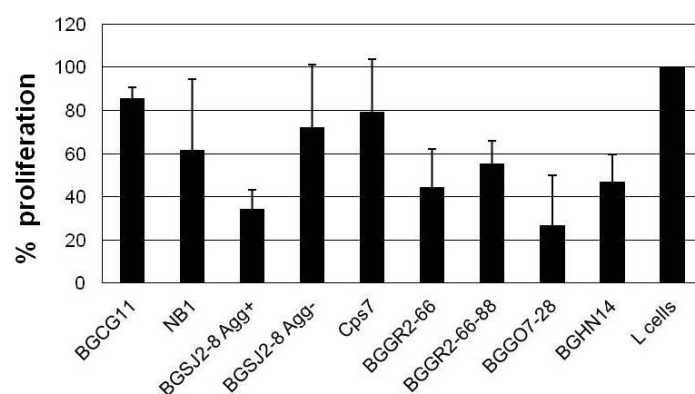
Figure 2. Percentage of GALT proliferation in the presence of lactobacilli strains

Recent studies showed that probiotics exhibit the health beneficial effects by directly modulating or down-regulating immune system through modification of immune response in GALT preventing in that way the symptoms of inflammatory bowel disease, allergies and asthma (Ouwehand *et al.*, 2007). In order to monitor the effect of cell surface properties on immunomodulatory potential of dairy isolates, the proliferation of the GALT cells was followed. The results showed that the strain *Lactobacillus sucicola* BGGO7-28, producing S-layer protein on the cell surface, exhibits the lowest level of the GALT proliferation comparing to the control, suggesting the immunosupressing activity of this strain. Interestingly, the reduction of GALT proliferation was also seen in the presence of the wild type strain *Lb. paracasei* BGSJ2-8, producing cell surface polysaccharide Cps and aggregation factor, leading to the conclusion that these surface molecules could be involved in suppression of the immune response.

Diabetes prevention and therapy. Finally, in our pilot experiments, the strain *Lb. paraplantarum* BGCG11 was shown to have therapeutic effects on rats with diabetes type 1 (streptozotocin induced). The level of blood sugar, BUN (Blood Urea Nitrogen), as well as AST (aspartate aminotransferase) and ALT (alanine aminotransferase) levels from liver were reduced when the rats were fed with BGCG11 strain resuspended in milk (Table 1).

Table 1. The influence of Lactobacillus paraplantarum BGCG11 probiotic strain on standard parameters of diabetes type 1

	Glucose	BUN	AST	ALT	Cholesterol	Triglycerides
Control	3.85	4.9	167	57.7	1.55	1.15
Control+BGCG11	5.85	8.45	144.5	63.35	1.45	0.8
Diabetes	27.5	20.7	360.7	223.2	1.8	1.8
Diabetes+BGCG11	11.3	14.3	229.4	141	1.6	1.2

Moreover, the improvements in the crypts architecture in duodenum and the Langerhans islets in pancreas were noticed, as well as less damage in DNA level of liver and kidney cells in the diabetic rats fed with BGCG11. Differences in the level of IL-6 and IL-10 production from both spleen lymphocytes and lymphocytes from mesenteric lymph nodes were detected when rats were fed with BGCG11 in milk, in comparison with diabetic rats drinking milk without BGCG11 strain (Figure 3). The results indicate that BGCG11 is able to ameliorate the effect of induced diabetes in rats.
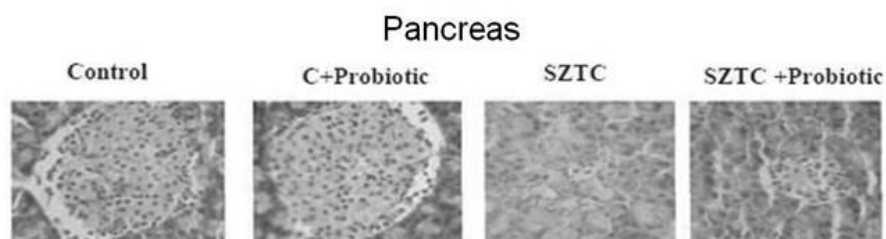
Figure 3. Role of Lactobacillus paraplantarum BGCG11 probiotic strain on the improvements in the Langerhans islet architecture in diabetic rats

CONCLUSIONS

In conclusion, the traditional fermented foods represent rich source of new LAB strains with a considerable genetic, metabolic, technological and probiotic potential. The autochthonous dairy isolates described in this paper are found to have strong health-promoting potential and could be used eventually in formulation of functional starter cultures for production of the innovative foods designed for diverse patients.

ACKNOWLEDGEMENTS

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TRENDS IN ASH AND MINERAL CONTENT OF MILK FROM DOMESTIC BALKAN DONKEY BREED THROUGHOUT LACTATION

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ABSTRACT

Ten female donkeys belonging to Domestic Balkan donkeys, autochthonous breed, were controlled throughout a whole lactation period. Subjected animals were grown at Valjevac pasture of Special Nature Reserve "Zasavica". The milk samples were taken by hand milking of two glands at days 45, 60, 80, 100, 125, 150, 170 and 200 *postpartum*. From days 80 to 170 *postpartum*, the ash content increased from 0.38 to 0.76 g/100mL of milk ($P < 0.05$). In the same period, the content of the mineral elements also increased significantly ($P < 0.05$): Ca from 157 to 224 mg/100mL; Na from 34 to 36 mg/100mL; Zn from 0.38 to 0.57 mg/100mL. Similarly, the concentrations of P, K and Mg increased from 50 to 162 g/100mL, from 117 to 135 g/100mL and from 8 to 10 g/100mL, respectively, at day 80, day 125, and day 170 of lactation. Thus, the concentration of the investigated minerals was affected by the stage of lactation. The obtained results for the mineral content of the donkey milk characterized showed that it can be considered as nutritionally valuable and healthy food.
Keywords: Balkan donkey milk, lactation stage, ash, mineral elements

INTRODUCTION

The minerals are quantitatively minor compounds essential for life, because they contribute to multiple and different vital functions in the organism, like bone structure, muscular contraction, metabolism via the enzymatic systems, etc. The mineral fraction of milk (approximately 8–9 g/l) is composed of macro elements (Ca, Mg, Na, K, P and Cl) and oligoelements (Fe, Cu, Zn and Se) (Gaucheron, 2005, 2011). Mineral metabolism particularly that of calcium and phosphorus, plays a fundamental role in the rapid skeletal development of the foal. The essential element is required for the various physiological functions of the organism: Ca and P for skeletal development, Mg for bone mineralization, Na as a cation in blood and extracellular fluid, K for the maintenance of fluid integrity within the cell (Laires *et al.*, 2004).

Donkey milk has unique nutritional features similar to human milk in total proteins and protein profiles, lactose contents, fatty acid, as well as fairly low mineral content (Medhamar *et al.*, 2011). Recently, the interest in donkey's milk has increased due to the possible health promoting characteristics. Donkey milk has become attractive as substitute to cow milk for human infants with allergic reactions, and also in the treatment of complicated cases with multiple food intolerance (Carroccio *et al.*, 2000; Monti *et al.*, 2007).

Milk composition of mammalian species varies widely with reference to genetic, physiological and nutritional factors, as well as environmental conditions (Sabahelkhier *et al.*, 2012). Although several authors have examined the nutritive value of donkey's milk taking into consideration various factors (Salimei *et al.,* 2004; Guo *et al.*, 2007; Martini *et al.*, 2014), little is known about its minerals composition in relation to the stage of lactation (Fantuz *et al.*, 2009, 2012). Aiming to increase the knowledge of mineral composition of milk from Domestic Balkan donkey breed, the concentrations of Ca, Mg, P, K, Na and Zn were studied throughout the whole lactation period.

MATERIAL AND METHODS

Milk collection

Donkeys' milk samples were collected from 10 female Domestic Balkan breed, after parturition in the Zasavica Special Nature Reserve (http://www.zasavica.org.rs/en/o-magarcima/). Zasavica is located in the north-west region of Serbia, and currently it is home to herd of over 100 female donkeys'. During the experimental period, donkeys were daily fed on pasture meadow plants with the addition of corn before the milking. Milk samples were collected using hand milking at the same time of day (in the morning between 08.00 and 10.00 a.m.), preserved in labeled bottle and kept in the refrigerator at 4^oC.

Chemical Analysis

Ash content was determined after mineralization of milk, at 550 ºC for 4 h according to standard methods (Carić *et al.*, 2000). Mineral contents as calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and Zinc (Zn) were measured in the ash using Atomic Absorption Spectrophotometer, Varian spectra AA-10 using IDF method 119:2007(E) (IDF/ISO, 2007). Phosphorous (P) was estimated using GBC CINTRA 303UV/VIS Spectrophotometer (IDF 42: 2006).

Statistical analysis

Average results of triplicate samples were submitted to statistical analyses. Results were analyzed using analysis of variance of the Statistica 10 for Windows, Stat Soft, Tulsa, Oklahoma, USA, 2009. Significant differences between means were determined at $p \leq 0.05$.

RESULTS AND DISCUSSION

The trends of ash and minerals composition of the Balkan donkey milk during the 200 day lactation period are shown in Figure 1.

During lactation, from days 80 to 170 postpartum, the ash content increased from 0.38 to 0.76 mg/100mL of milk (P < 0.05). On the average, donkey's milk has lower ash content compared to cow's milk (0.75 mg/100 ml of milk) and higher than in woman's milk (0.22 mg/100 ml of milk) (Csapo´ *et al.*, 1996; Malacarne *et al.*, 2002). Values of ash content obtained in this study was higher with the previous reports of some researchers for ash content in donkeys' milk (Salimei and Chiofalo, 2006; Guo *et al.*, 2007; Polidori *et al.*, 2012).

All macro minerals in horse and donkey milk vary significantly during lactation, which is in agreement with previous research of other donkey breeds (Summer *et al.*, 2004; Fantuz *et al.*, 2012). In our study increasing trends during lactation for for Ca, Na and Zn were observed, what is consistent with the increase of ash content from 80. to 170. day of lactation. In the same period, the content of the mineral elements also increased significantly (P < 0.05): Ca from 157 to 224 mg/100ml; Na from 34 to 36 mg/100ml and Zn from 0.38 to 0.57 mg/100ml. Other author reported decreasing Ca, P and Mg concentrations during lactation, what were also observed for Martina Franca breed (Fantuz *et al.*, 2012) Similarly, the concentrations of P, K and Mg increased from 50 to 162 g/100ml, from 117 to 135 g/100mL and from 8 to 10 g/100ml, respectively, at day 80, day 125, and day 170 of lactation. Thus, the concentration of the investigated minerals was affected by the stage of lactation**.**

The level of ash in equine and donkey milk is similar to those in human milk except for higher concentration of Ca and P (Holt and Jenness, 1984; Uniacke-Lowe, 2011). The concentrations of K, Na, and Mg in horse and donkey milk appear to be similar to those in human milk. The concentration of K, are higher in equine and donkey milk than in human milk, but all are considerably lower than in bovine, caprine, ovine and porcine milk (Uniacke – Lowe, 2011).
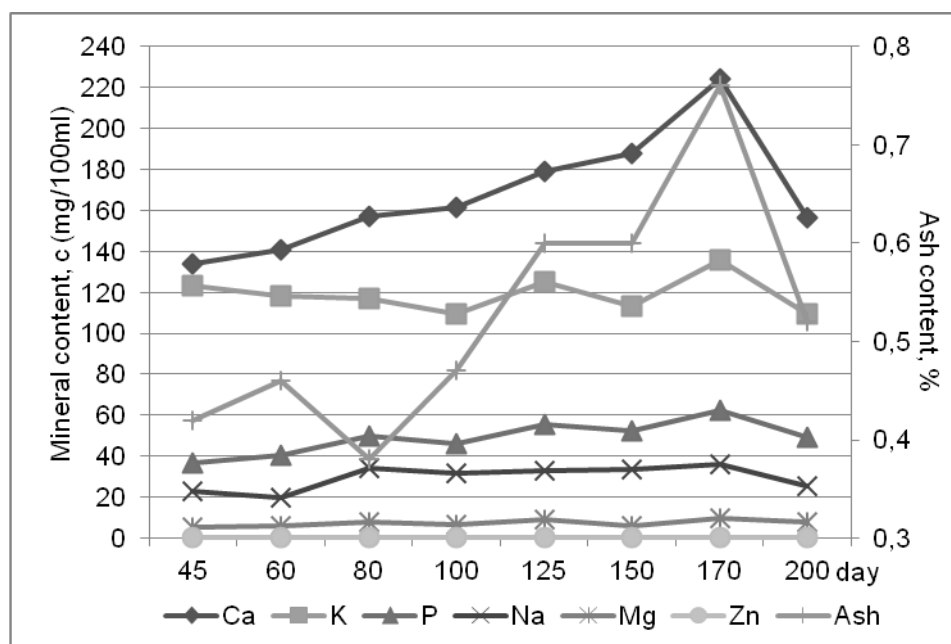
Figure 1. The content of ash and mineral elements in donkey milk during lactation period

Concentrations of all elements were within the ranges for Littoral-Dinaric breed donkey reported in previous study (Bilandžić *et al.*, 2014), although they are kept under similar conditions.

CONCLUSIONS

This study of milk from Domestic Balkan donkeys, autochthonous breed, provides a better understanding of donkey's milk mineral composition, and its variations throughout the main stages of lactation.

The content of ash and all mineral elements in donkey's milk vary significantly throughout the whole lactation period. In Balkan donkey milk, the obtained mineral concentrations were higher in comparison to previously reported levels for different donkey breeds. The concentration of the studied minerals was affected by the stage of lactation, showing an increasing trend until the beginning of the late phase. As a consequence, when describing donkeys milk composition the stage of lactation should be specified. Data provided by the current study can be also used to support the assessment of macro mineral nutritional requirements of different donkey breeds.

ACKNOWLEDGEMENTS

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PREDICTION OF STRUCTURE AND CATALYTIC RESIDUES OF β -GALACTOSIDASE FROM *Streptococcus thermophillus*

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ABSTRACT

As there are no data on *S. thermophillus*, β-galactosidase's 3D structure, the aim of this study was to make 3D model of β-galactosidase with its active site and to predict its catalytic residues.

Sequence were retrieved from NCBI, PubChem and PDB databases. β-galactosidase from E. coli used as the template sequence. Sequences alignment was performed using MUSCULE program. Homology models were made using Modeller 9.11 software. The modelled structures were minimized using AMBER7 force and fields and AMBER atomic charges. Docking studies were performed using Surflex-Dock program. Protein structures were prepared for docking studies using various parameters such as: polar hydrogen atoms added, protonation types and termini treatment.

BlastP search revealed its similar structure with *E. coli* β-galactosidase. As they are distant related species, it is evident horizontal gene transfer. Docking analysis revealed active sites position, residues involved in substrate binding and active residues. According to our model, putative catalytic residues are GLU458 and GLU546. These results were verified by sequence alignment with closely related β-galactosidases with known structure and active residues. Both catalytic residues are positioned close to lactose cleavage site and make hydrogen bonds with it. Substrate binding residues are also ASN98, ASP201 and HIS380.

Keywords: *β-galactosidase, yoghurt, docking, catalytic residues*

INTRODUCTION

Streptococcus thermophillus is one of the first most used strains in dairy industry, as it ferments lactose in milk during yoghurt production. In dairy industry it is used for yoghurt production since 1900s (Delcour *et al.,* 2000). Milk fermentation by *S. thermophilus* leads to numerous organoleptic and health benefits of the final product. The final product of lactose fermentation in milk by *S. thermophilus* is lactic acid. *S. thermophilus* produce enzyme β-galactosidase which leads the first reaction in lactose degradation and catalyse it to galactose and glucose (Farnworth, 2003).

β-galactosidase (EC 3.2.1.23), catalyzes the hydrolysis of β(1-3) and β(1-4) galactosyl bonds in oligo- and disaccharides. Apart from its benefits in fermented dairy technology, it has a great role in low lactose and lactose free dairy products, which allow lactose intolerant persons to consume milk and enjoy its beneficial properties (Chen *et al.,* 2008, Ladero *et al.,* 2003). All known β-galactosidases belong to the GH-A superfamily of glycoside hydrolases, and to the 1, 2, 35 and 42 subfamilies (Cantarel *et al.,* 2009). They have two catalytic glutamic acid residues, one proton donor and one nucleophile. β-galactosidase from *S. thermophilus* belongs to GH-2 subfamily. Nucleophilic and proton donor residues catalyse substrate by nucleophile, attack on the anomeric centre to displace the aglycon and form a glycosyl-enzyme intermediate. Proton donor acts as an acid catalyst and protonates the glycosidic oxygen as the bond cleaves. Binding residues play an important role in substrate catalysis as they stabilise the enzyme-substrate complex. The strength of their interaction with the substrate could be correlated with reaction rate due to feedback effect of reaction products.

There are several crystal structures of β (1-4)-galactosidases in Protein Data Bank (PDB) database (www.rcsb.org) (Table 1). As 3D structure and active residues of *S. thermophilus* β-galactosidase has not been experimentally determined the aim of this work was to make its 3D model and to predict active and biding resides.

Table 1. β (1-4)-galactosidases of known crystal structure

Organism	GH-subfamily	PDB code	Reference
Sulfolobulus solfataricus	GH-1	1UWQ	Aguilar *et al.*, 1997
Escherichia coli	GH-2	1DP0	Jacobson *et al.*, 1994
Arthrobacter sp.	GH-2	1YQ2	Skalova *et al.*, 2005
Klyveromices lactis	GH-2	3OBA	Pereira-Rodrıguez *et al.*, 2012
Aspergilus oryzae	GH-35	4IUG	Maksimainen *et al.*, 2013
Penicillium sp.	GH-35	1TG7	Rojas *et al.*, 2004
Bacteroides thetaiotaomicron	GH-35	3D3A	Unpublished[a]
Trichoderma reesei	GH-35	3OG2	Maksimainen *et al.*, 2011
Homo sapiens	GH-35	3THC	Otho *et al.*, 2012
Thermus thermophilus	GH-42	1KWG	Hidaka *et al.*, 2002
Bacillus circulans ssp. *alkalophilus*	GH-42	3TTS	Maksimainen *et al.*, 2012

[a]*K. Palani, D. Kumaran, S. K. Burley, and S. Swaminathan, unpublished results (www.rcsb.org)*

MATERIAL AND METHODS

Protein sequence of *Streptococcus thermophilus* β-galactosidase used as the query sequence was retrieved from NCBI database (GenBank: ACA96932.1) (http://www.ncbi.nlm.nih.gov/). Lactose structure data file was retrieved from the PubChem database (http://pubchem.ncbi.nlm.nih.gov).

The NCBI BlastP was performed against the Protein Data Bank database (PDB database) for identification of highly similar sequence templates for template sequences. PDB file of the β-galactosidase from *E. coli* used as the template sequence was retrieved from (PDB ID: 1DP0:A). Sequences alignment was performed using MUSCULE program included in the UGENE 1.11.3 software package (Okonechnikov *et al.*, 2012, Edgar, 2004). After aligning, the homology models of *Streptococcus thermophilus* β-galactosidases were made using Modeller 9.11 software (Eswar *et al.*, 2007). The best model was selected by the smallest value of the normalized discrete optimized molecule energy (DOPE) (Sali, 2006). The final 3D models were verified using Ramachandran plot, the Structural Analysis and Verification Server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES).

Downloaded protein structures and ligands were minimized using MMFF94 force field. Atomic charges were calculated using the MMFF94 method. The modelled structures were minimized using AMBER7 force and fields and AMBER atomic charges. Powell method, distance dependent dielectric constant and convergence gradient method with a convergence criterion of 0.005 kcal/mol were used. Protein structures were prepared for docking studies using various parameters such as: polar hydrogen atoms added, protonation types and termini treatment. Docking studies were performed using Surflex-Dock program with flexible H atoms (Jain, 2003).

RESULTS AND DISCUSSION

BlastP analysis of *S. thermophilus* β-galactosidase, revealed its highest similarity with *E. coli* β-galactosidase structure retrieved from PDB database (PDB ID: 1DP0:A), with query cover 98%, E value $2e^{-163}$ and identity 33%.

Sequence alignment revealed high conservation of residues in the active site area (Figure 1). Active residues of *E. coli* β-galactosidase were experimentally determined in previous literature (Jacobson *et al.*, 1994). According to their findings proton donor residue is GLU

462, while nucleophile residue is GLU538. According to our results active residues are conserved in both enzymes. Proton donor residue in *S. thermophilus* β-galactosidase is GLU 458, while nucleophile residue is GLU546.
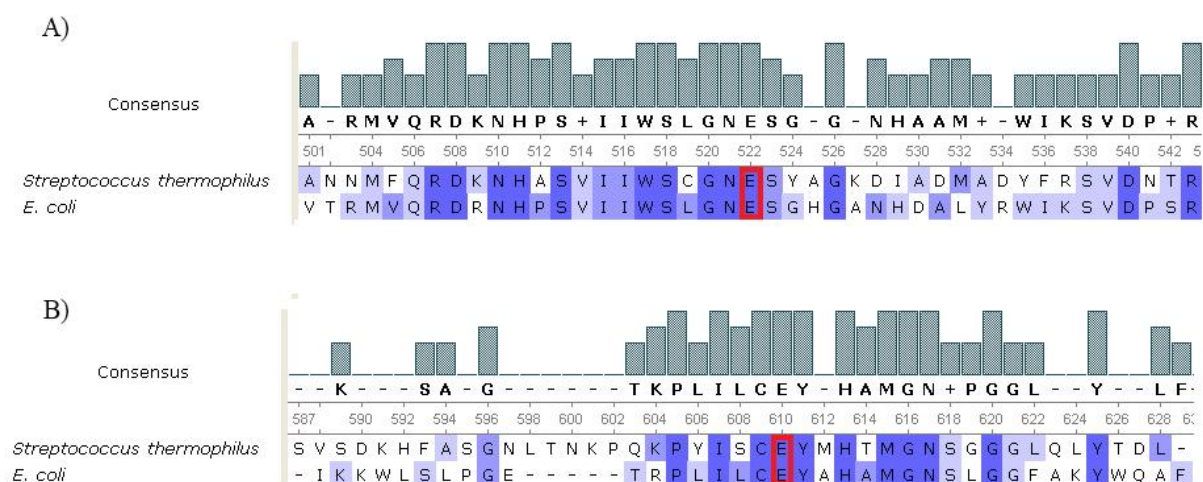
Figure 1. Sequence alignment of active site area; A) Sequence around proton donor residue, B) Sequence around nucleophile residue

Modelled enzyme showed high 3D alignment with its template sequence (Figure 2A). It can be seen that although peptides differ in their sequences (red color), overall 3D structure remained mainly conserved. Furthermore, residues that build active site remained highly conserved (Figure 2B).
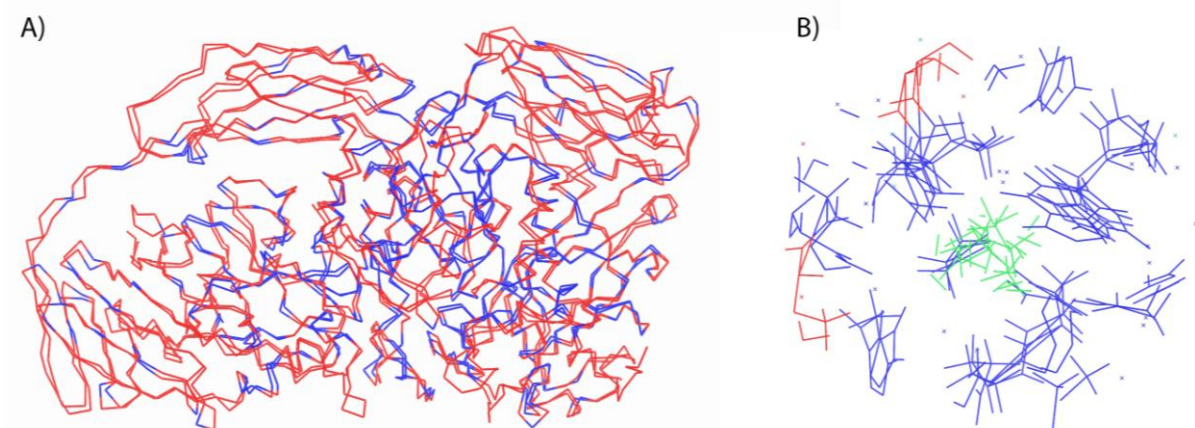
Figure 2. Alignment of S. thermophillus and E. coli β-galactosidases; Red color – differences in sequences, blue color – matched residues, green color – protomol; A) Complete sequence alignment, B) Active site alignment

Docking analysis was performed for *S. thermophilus* and *E. coli* β-galactosidases with lactose molecule. The active site has been set using both potential active residues (GLU461 and GLU537) with additional spheres of 5 Å, in order to include all possible binding residues and to predict the most probable active site conformation and ligand docking. Docking results revealed that both active residues of *E. coli* β-galactosidase (1DP0) make H bonds with lactose and both are close to its cleavage site (Figure 3A). Glu537 is thought to be the catalytic nucleophile, forming a covalent bond with the substrate (Gebler *et al.*, 1992). Other binding residues are ASN102, ASP201 and ASN460. Model of *S. thermophillus* β-galactosidase docking showed that both putative catalytic residues (GLU458 and GLU546)

make hydrogen bonds with lactose and are closely positioned to lactose cleavage site (Figure 3B). These results confirmed predicted active resides made by phylogenetic analysis. Substrate binding residues are also ASN98, ASP201 and HIS380 and therefore are crucial for the enzyme function. It can be seen that the enzymes differ in only one residue which is included in substrate catalysis. In *E. coli* it is ASN460, while in *S. thermophillus* it is HIS 380. This high conservation of important residues resulted in conserved enzyme function through their evolution.
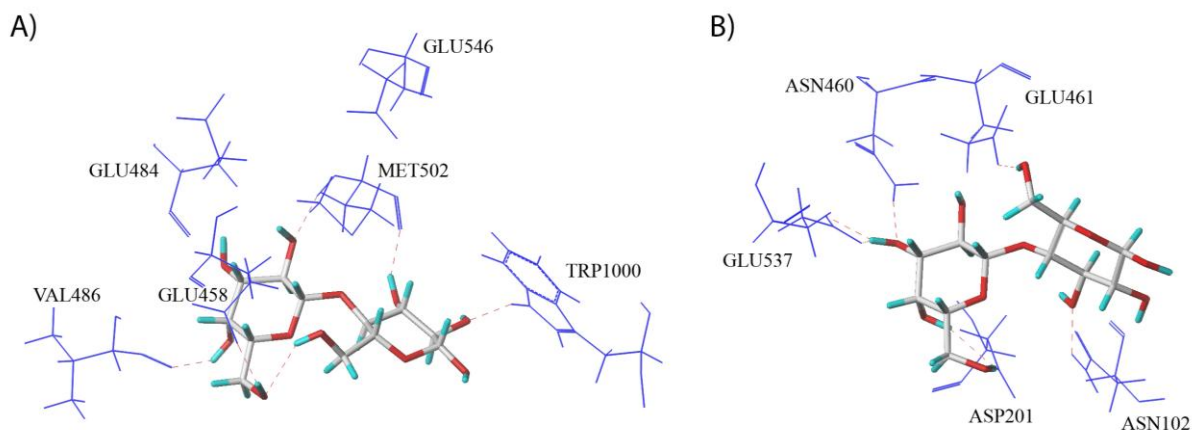
Figure 3. Docking results with lactose molecule; A) β-galactosidase from S. thermophillus, B) β-galactosidase from E.coli

CONCLUSIONS

Model of β-galactosidase from *S. thermophillus* was built using β-galactosidase from *E. coli* as a template sequence. Although residues differ in their sequences, overall 3D structure remained mainly conserved. Our results revealed high conservation of residues involved in enzymes catalitic activity. Predicted proton donor residue in *S. thermophilus* β-galactosidase is GLU 458, while nucleophile residue is GLU546. Substrate binding residues are also ASN98, ASP201 and HIS380.

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HYDROLYSIS OF LACTOSE IN THE PERMEATE OF MILK UNDER THE ACTION OF THE ENZYME GALACTOSIDASE

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ABSTRACT

Nowadays, food industry develops dairy products free of lactose to satisfy the needs of the people suffering from lactose intolerance. Lowering or removing lactose from milk, these products become available for intolerant persons.
Galactosidase is one of the widespread enzyme in nature and very important enzyme in food processing.
Hydrolysis of the lactose by β-galactosidase into glucose and galactose, increases the possibility of commercial use of the permeate – byproduct obtained by ultrafiltration of milk during production of cheese or whey.
The objective of this research was to examine the hydrolysis of lactose by applying the enzyme % β-galactosidase in milk permeate.The effect of β-galactosidase (isolated from *Klyveromyces lactis)* at various concentrations (0.1, 0.3 and 0.5 % (w/w)) and reaction temperature 30°C on the degree of lactose hydrolysis in permeate during 60 minutes was investigated.
The hydrolysis of lactose by addition of 0.1% enzyme contributed to 95.15% degree of hydrolyses, but 100 % degree of hydrolysis was achieved after 20 min with 0.3% β-galactosidase and after 10 min when 0.5% enzyme was added. The maximum yield of glucose and galactose was 3.02 and 3.5 2g/100g, respectively.
***Keywords**: lactose, permeate, β-galactosidase, hydrolysis*

INTRODUCTION

Lactose (milk sugar, 4-0-β-galactopyranosyl-D-glucopyranose, $C_{12}H_{22}O_{11}$) is a disaccharide comprising one glucose molecule linked to a galactose molecule. Lactose is synthesized in the epithelial mammary cells from two molecules of glucose absorbed from the blood. The concentration of lactose in milk is inversely related to the concentration of lipids and to the concentration of casein. The content of lactose in mature bovine, buffalo, ovine and caprine milk is about 4.8., 4.8, 4.6 and 4.1% w/w, respectively. It increases slightly during the early stages of lactation but then decreases to about 70% of the maximum at the end of lactation. In contrast, the lactose content of the milk of equidae (horse, donkey and zebra) increases during lactation, reaching values in the range 6.0–7.4%, w/w, with considerable interindividual variation. Human milk contains 7.5%, w/w, lactose (Fox, 2011).
Lactose has a low level of sweetness. It is only about 16% as sweet as sucrose at 1% in solution and hence has limited value as a sweetening agent (Ganzle *et al*, 2008). During hydrolysis, the lactose is cleaved into its constituent absorbable monosaccharides, glucose and galactose by enzyme β-galactosidase (lactase). Hydrolysis of lactose leads to the improvement of properties (sweetening, solubility, fermentability, etc.), and the possibilities of the industrial applications (Dekker and Daamen, 2011; Ilić Udovičić *et al.*, 2013a). The application of β-galactosidase in enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the food industry.
β-galactosidase is very widely distributed in nature because of its multiple functions. β-galactosidase is thus essential for the nourishment of newborn mammals, whose sole source of nutrition is milk. Most of the world populations lost part of their β-galactosidase activity in

the small intestine after the childhood (Swallow, 2011). So, nowadays the production of lactose-free dairy products are of great interest for lactose intolerant persons (Ladero *et al.*, 2000; Jankowiak and Ludwig, 2008; Mlichova and Rosenberg, 2006; Jurado *et al.* 2004); Harju *et al.* 2012; Brown-Esters *et al* 2012).

The aim of this study was to examine the hydrolysis of lactose by applying the enzyme β-galactosidase in permeate obtained by ultrafiltration of milk during cheese production.

MATERIAL AND METHODS

Substrate

Permeate was obtained, during the manufacture of feta cheese by ultrafiltration of milk with 3.7% fat (manufacturer "DAIRY Šabac", Serbia). Permeate has the following composition: dry matter (g/100g), 5.55, milk fat (g/100g) <0.1, total proteins (g/100g) 0.20, lactose (g/100g) 5.72, ash (g/100g) 0.48 and pH 6.46.

Enzyme

Enzyme Maxilact® LG5000 (DSM Food Specialties, The Netherlands) – β-galactosidase derived from the yeast *Kluyveromyces lactis* was used for lactose hydrolysis.

Enzymatic hydrolysis

Different parameters such as time, temperature and the enzyme levels were optimized to obtain lactose hydrolysis in permeate. Enzyme preparation was added to permeate at the temperature of 30 °C in concentrations of 0.1% (w/w), 0.3% (w/w) and 0.5% (w/w). Samples for determination of lactose, glucose and galactose content were taken at each 10 minutes during 60 minutes of hydrolysis process. The inactivation of the enzyme was accomplished by heating in a water bath on temperature 85°C. The technological process of lactose hydrolysis in milk permeate is presented in Figure 1.
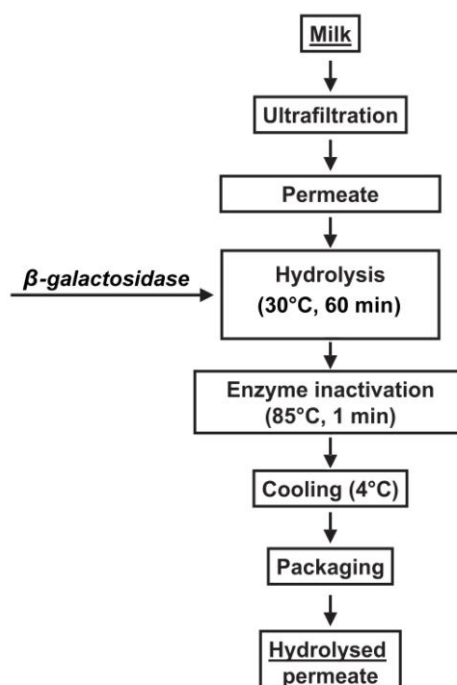
Figure. 1. Technological process of lactose hydrolysis in milk permeate

Physicochemical analysis

The following physicochemical characteristics were determined in milk, permeate and hydrolysed permeate: total solids (TS) by oven drying (ISO 6731, IDF 21: 2010); and pH was measured with a pH-meter (EcoScan pH 6 Eutech Instruments, Netherlands).

Sugar content was analyzed in all samples by Liquid Chromatograph Agilent Technologies 1200 Series with ELSD (Evaporative Light Scattering Detector) and Zorbax Carbohydrate Column (4.6x250mm. 5 μm) (Agilent Technologies). Samples (5g of each sample) were diluted in 25 mL volume flasks with 10 mL distilled water. The solutions were incubated in a water bath at 50°C for 15 min. After cooling 0.5 mL of Karez I, 0.5 mL of Karez II and 1 mL of 100 mM NaOH were added. The flask was amended with distilled water, mixed and samples were filtered through filter paper No.381. The filtrates (10μL) were injected using autosampler. The flow rate was 1.000 mL/min, at ambient temperature and run time was 15 min. The mobile phase with isocratic flow was a cetonitrile/water (70/30. v/v). ELSD parameters were: temperature 40±1°C, nitrogen pressure: 4.5±0.1 bar.

The degree of lactose hydrolysis (DH) was calculated using formula:

$$DH = (lactose_{total} - lactose_{after\ hydrolysis})*100/\ lactose_{total}$$

Statistical analysis

All experiments and standard deviation were carried out in triplicate and all data were expressed as mean values. The results were statistically processed by analysis of variance at the significance level α= 0.05. The adequacy of the model was evaluated by coefficient of determination (R^2) and model p-value. For the description of the responses Y (glucose content, galactose content), a second degree polynomial model was fitted to data (Eq.1):

$$Y=b_0+\sum b_1 X_1+\sum b_2 X_2+\sum b_{11}^2 X_{11}^2+\sum b_{22}^2 X_{22}^2+\sum b_{12}X_1 X_2 \qquad (1)$$

where b_0 is intercept, b_1 represents the linear, b_{11} and b_{22} the quadratic and b_{12} the interaction effect of the factor. The factor variables and their values are: X_1 time of hydrolysis (0, 10, 20, 30, 40, 50, 60 min) and X_2 concentration of enzyme (0.1, 0.3 and 0.5% (w/w)).

Statistical and graphical analyses of results were carried out with the computer software program "Statistica 9.1" (Statistica, 2009). Plotting responses as a function of two factors drew response surface plots. Plots were generated using the same software.

Factorial ANOVA test for comparisons of several average values was applied for determining differences amongst chemical characteristics of different samples.

RESULTS AND DISCUSSION

Chemical composition of milk, permeate and hydrolysed permeate is shown in Table 1. Characteristics of milk permeate are in accordance with literature data (Paterson, 2011; Ilić-Udovičić *et al.*, 2013b).

The effect of time of hydrolysis of permeate on lactose content in samples hydrolysed by different concentrations of β-galactosidase at 30°C is presented in Figure 2. After 60 minutes of lactose hydrolysis in milk permeate the content of lactose was 0.27 g/100g (the degree of hydrolyses was 95.15%). Complete hydrolyses of lactose was achieved after 20 min when 0.3% β-galactosidase was added, but in the case of addition of 0.5% enzyme it happened after 10 min.

The results of statistical analyses for glucose and galactose content of hydrolysed permeate samples treated with β-galactosidase during 60 min are presented in Table 2. The coefficients in Table 2 are related to actual variables. On the basis of the results it is evident that the time of hydrolyses has greater effect on content of glucose and galactose content of all samples is significant.

The ANOVA results for selected responses are reported in Table 3. Relatively high values of coefficient of determination for glucose and galactose in samples (R^2=0.862 and R^2=0.875, respectively) obtained for all responses indicate good fit of experimental data to Eq.1.

As for significance of polynomial coefficient their p-values suggest that the most important linear factor is concentration of enzyme. The effect of time and concentration of enzyme on glucose and galactose content of hydrolysed permeate samples are shown in Figure 3.

Table 1. pH value and chemical characteristics of milk, permeate and hydrolysed permeate

Samples	pH	Total Solids (g/100g)	Lactose (g/100g)	Glucose (g/100g)	Galactose (g/100g)
Milk	6.75	12.67	5.47	0	-0
Permeate	6.50	5.55	5.57	0	0
Hydrolyzed permeate	6.43	5.07	0	2.12	2.11
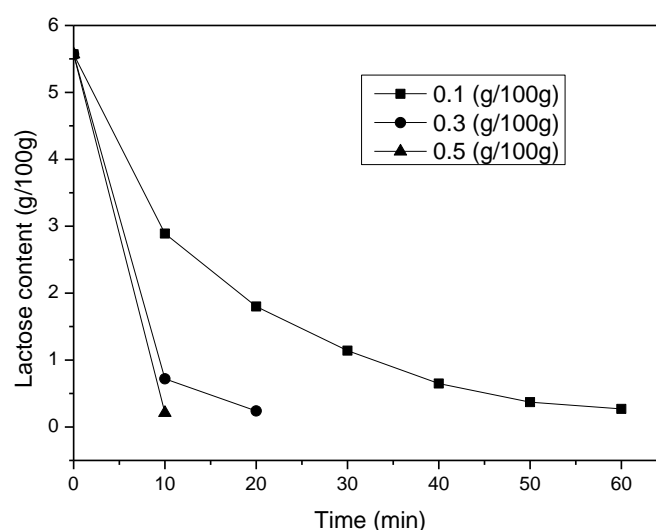
Figure 2. The effect of enzyme concentration on lactose content in permeate samples

Table 2. Regression equation for the response of hydrolysed permeate samples

Effects	Glucose		Galactose	
	Coefficient	p-value	Coefficient	p-value
Intercept				
b_0	-0,6206	0,273972	-0,65382	0,232233
Linear				
b_1	0,14257	0,000006	0,11681	0,000037
b_2	5,48393	0,138506	6,17946	0,086433
quadratic				
b_{11}	-0,00159	0,000088	-0,00139	0,000234
b_{22}	-5,19643	0,36131	-7,01786	0,20552
interaction				
b_{12}	-0,02446	0,454511	0,02188	0,485896

[a]Effects are statistically significant, p=0.0

Table 3. Analysis of variance (ANOVA) for response of hydrolysed permeate samples

Response	Source						F-value	p-value	R^2
	Residual			Model					
	DF	SS	MS	DF	SS	MS			
Glucose	15	3.411	0.227	6	142.640	23.773	104.54	0	0.878
Galactose	15	3.149	0.210	6	132.080	22.010	104.84	0	0.863

DF-degree of freedom, SS-sum of squares, MS-mean squares

a)
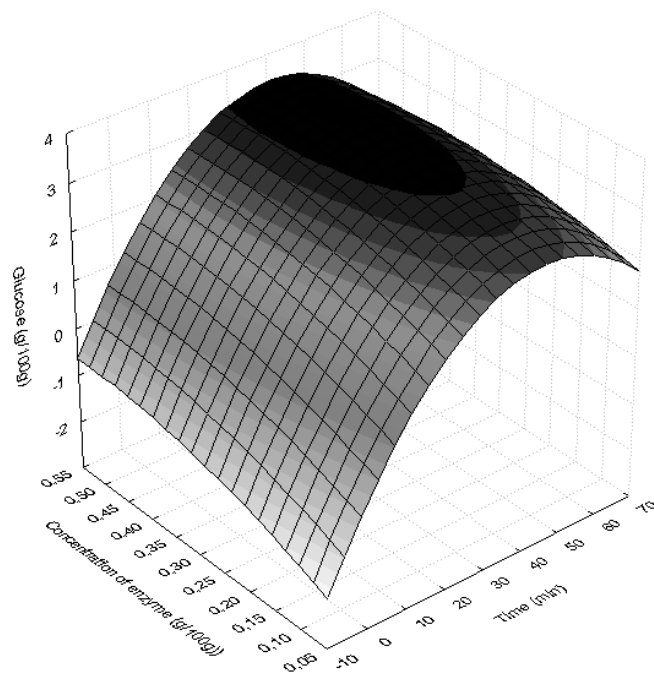
b)
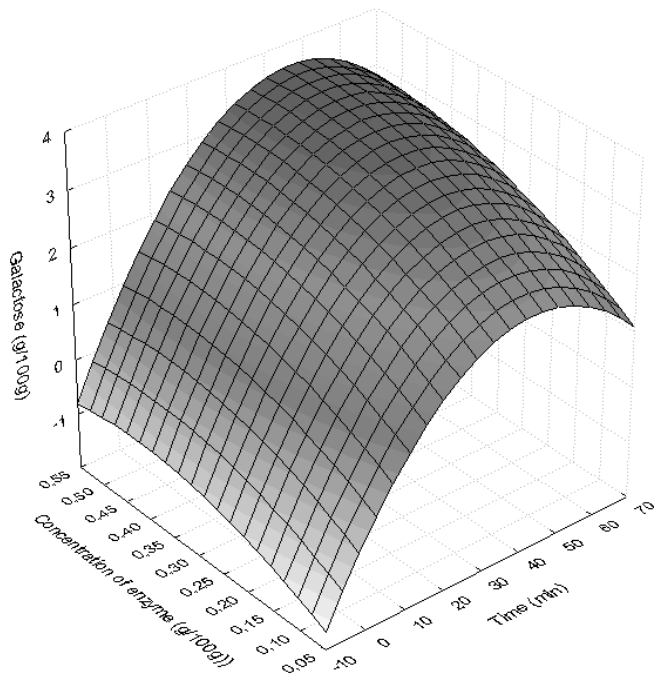
Figure 3. The effect of time and enzyme concentration on content of: a) glucose and b) galactose in hydrolysed permeate

CONCLUSIONS

The increase of β-galactosidase concentration (0.1-0.5g/100g) significantly influenced to the degree of lactose hydrolysis in milk permeate at 30°C during 60 minutes.The most efficient rate of hydrolysis was obtained by 0.5% enzyme concentration at 30°C after 60 minutes. The maximum yield of glucose and galactose was 3.02 and 3.52 g/100g, respectively. Relatively high values of coefficient of determination for glucose (R^2=0.8629) and galactose (R^2=0.875) in samples, obtained for all responses, indicate good fit of experimental model.

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ENTEROBACTERIACEAE PRESENCE DURING FERMENTATION OF TRADITIONAL DRY FERMENTED SAUSAGE - *PETROVSKÁ KLOBÁSA*

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ABSTRACT

Petrovská klobása is a traditional and autochthonous dry fermented pork meat product with a protected designation of its geographical origin and present a part of gastronomic heritage of Slovaks in Vojvodina. It is produced without use of nitrate/nitrite, glucono delta-lactone (GDL) and microbial starters and at the end of ripening *Petrovská klobása* is characterized by specific savoury taste, aromatic and spicy-hot flavour, dark red colour and hard consistency. In order to achieve a recognizable product of standardized supreme quality which will be continually produced in the controlled conditions and sold on the domestic and world markets, the aim of this study was to determine the mathematical model of *Enterobacteriaceae* presence and elimination during the production process of *Petrovská klobása*. The presence of *Enterobacteriaceae* was detected in all groups, the elimination in the case of all groups is fast and efficient, and is mainly implemented between 13 and 30 days. The function of eliminating is linear in all groups of sausages and the manner of packaging. Mathematical models for predicting a presence and elimination of *Enterobacteriaceae* during the production process of *Petrovská klobása* present the helpful tool in optimizing existing and developing new process.
Keywords: *Petrovská klobása, Enterobacteriaceae, aw, pH, mathematical models*

INTRODUCTION

Traditional foods are a significant element of the European cultural heritage, which production and sale provide a decisive economic input to many regions (European Commission, 2007). Special characteristics of foods from any region or area are connected to local ingredients and production techniques, which are deeply rooted in tradition and linked to the territory (Aquilanti *et. al.*, 2007). An important group among them are traditional dry-fermented meat products. Petrovská klobása is a traditional and autochthonous fermented pork meat product, which is a part of gastronomic heritage of Slovaks in Vojvodina (Northern Serbia) and which is produced in a traditional way in rural households in the Municipality of Bački Petrovac. Petrovská klobása is made by mixing partly cooled (cca 4h p.m.) or cold (cca 24h p.m.) medium chopped lean pork and fat (up to 10 mm) with addition of powdered red hot spicy paprika, salt, crushed garlic, caraway and sugar. A well-mixed filling, which is prepared within 15-30 minutes by using a unique technique of manual mixing with kneading and overturning, is stuffed into natural casings consisting of the rear part of pig intestines (rectum), forming units 35-45 cm long and 4.5-5.0 cm in diameter. After stuffing, the sausages are left to drain for a while and then they are smoked by a cold process for about 10-15 days with pauses, using specific kinds of wood (cherry wood in particular). When a smoking process is finished, the sausage is kept in a dry and well ventilated place to dry and ripen, until it achieves an optimum quality, which takes about 4 months (Petrović *et al.*, 2007; Ikonić *et al.*, 2010; Tasić, 2012). Due to the above said, and in order to achieve a recognizable product of standardized supreme quality which will be continually produced in the controlled conditions and sold on the domestic and world markets, the aim of this study was to determine the predicting model of *Enterobacteriaceae* presence and elimination during the production process of *Petrovská klobása.*

Enterobacteriaceae presents one of the parameters of typical "house flora" during the production process of *Petrovská klobása*, which is crucial because of the safety (pathogenic flora), acceptability (spoilage flora) and sensorial quality (technological flora) (Talon *et al.*, 2008; Leroy *et al.*, 2010; Janković *et al.*, 2013; Lakićević *et al.*, 2014).

MATERIAL AND METHODS

Sausages were produced during December and the processes of drying and ripening lasted 270 days. The prepared filling was stuffed into natural casings (sausages of A1 and B1 groups) and artificial collagen casings (sausages of A2 and B2 groups). Batches B1 (natural casing) and B2 (artificial casing) were left to be smoked, fermented and dried in the household B while batches B3 (natural casing) and B4 (artificial casing) were taken to Im „Kolbis" where smoking, further fermentation and drying were performed in controlled conditions. Samples from each batch (A1, A2, B1, B2, B3 and B4) (n=3) were transported to the laboratory under refrigeration (4°C) and analyzed on the same day. Isolation and identification of *Enterobacteriaceae* was done according to SRPS ISO 21528-2. All results were presented as mean values of three independent repeats ± standard deviation (Sd). Data were processed using the Microsoft Excel software package for Windows 2007 and the software package Statistica 9.1 for Windows, Stat Soft, Tulsa, Oklahoma, USA.

RESULTS AND DISCUSSION

The figure 1 shows the growth rate of enterobacteriaceae from the zero day until the 270th day (unpacked samples) for all six models of produced sausages (experimental batches A1, A2, B1, B2, B3 and B4).
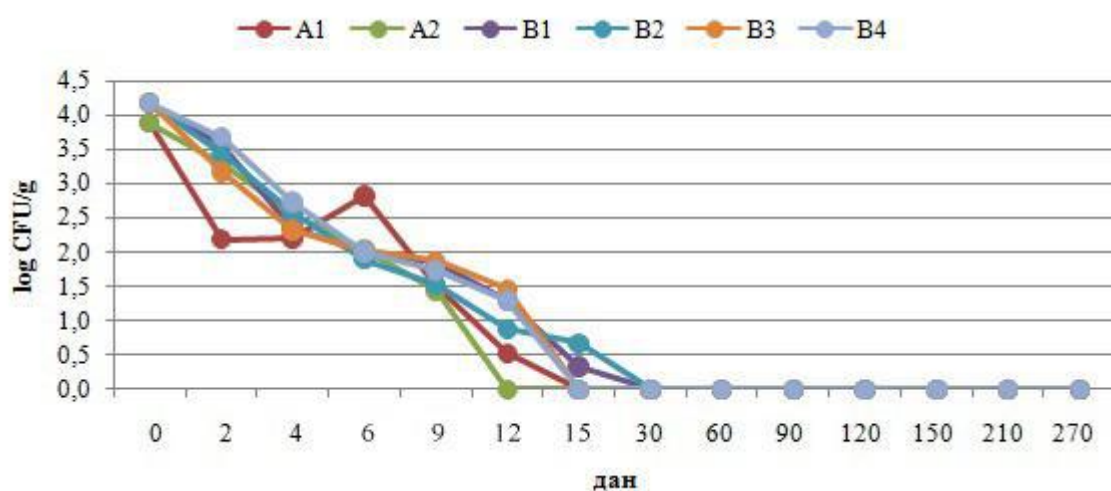
Figure 1. Growth rate of Enterobacteriaceae in 6 experimental batches of Petrovac sausage during the smoking, fermentation, drying and storage

As it can be seen on the figure 1, the presence of *Enterobacteriaceae* was detected in all batches but they completely disappeared after the 30th day. *Enterobacteriaceae* were most quickly disappearing from the sausages of A2 batch (samples traditionally produced from the warm meat and stuffed into artificial casings) starting from the 12th day, on the 15th day they were not detected in samples from the A1 batch (samples traditionally produced from the warm meat and stuffed into natural casings), B3 (samples produced from the cooled meat, smoked and dried in the meat processing facility – natural casing) and B4 (samples produced from the cooled meat, smoked and dried in the meat processing facility – artificial

casing) and as of the 30th day, they were not detected in samples from the B1 and B2 batches.

A1

A2

B1

B2

$y = -0.2255x + 3.7899$
$R^2 = 0.9399$

B3

$y = -0.2338x + 3.7583$
$R^2 = 0.9298$

B4

$y = -0.2099x + 3.6607$
$R^2 = 0.8451$
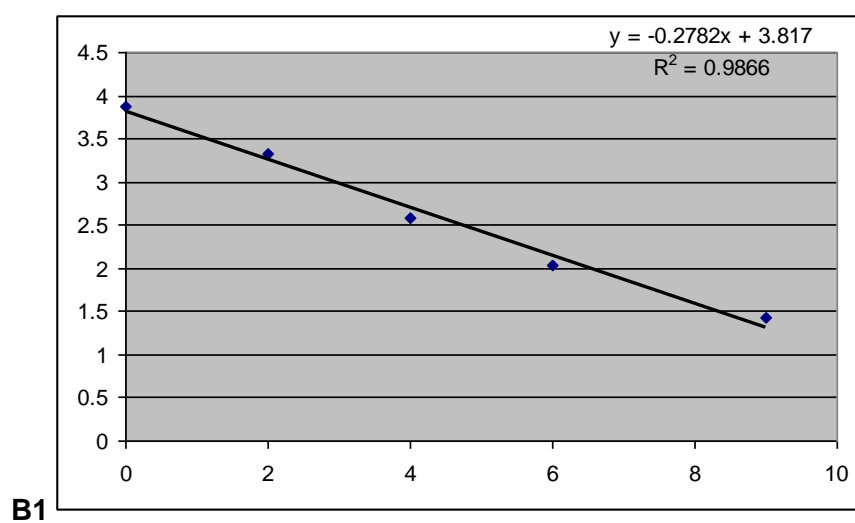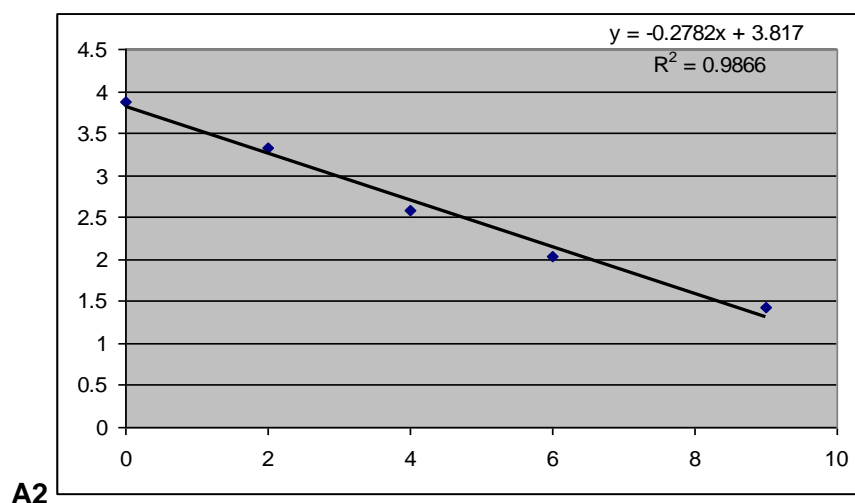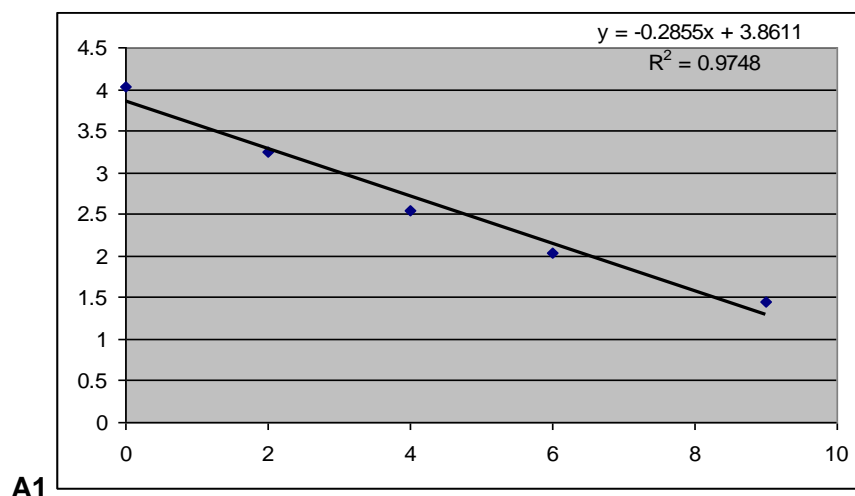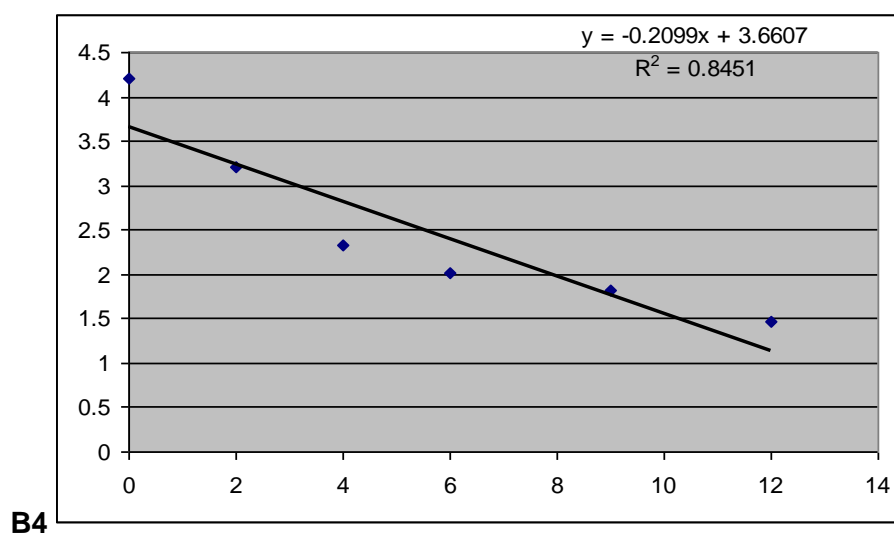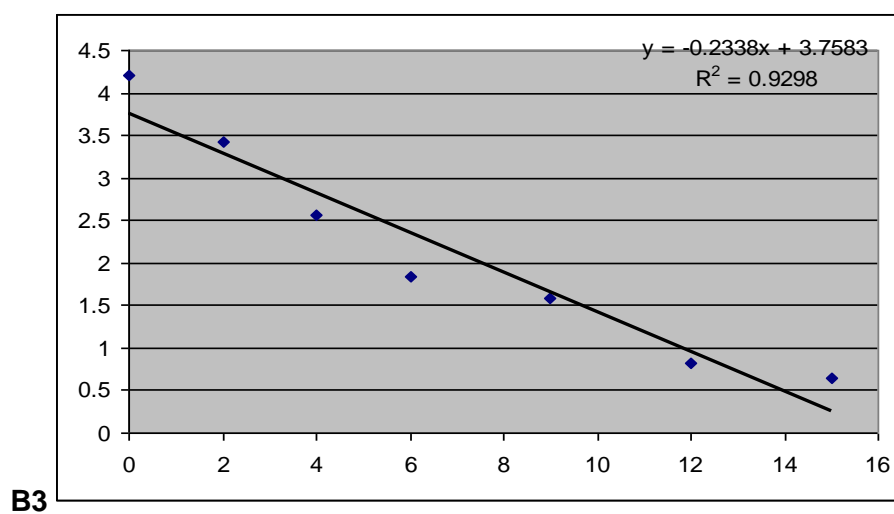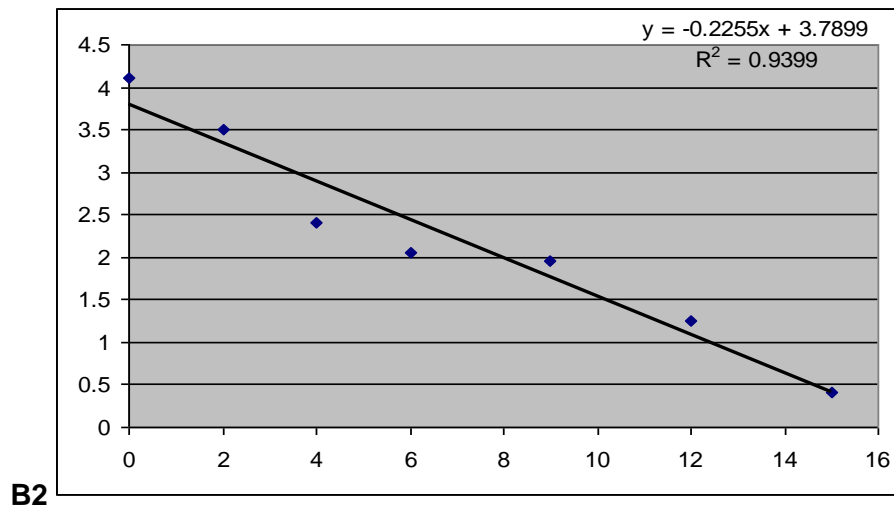
Figure 2. Linear functions of Enterobacteriaceae elimination with squares of the correlation coefficient of all sausage batches (respectively left figures for batches A1, A2, B1, B2, B3 and B4)

Elimination of *Enterobacteriaceae* in all batches is fast and efficient and mostly completed in 9 to 15 days. The function of this elimination is linear in all cases of produced batches of sausages (Figure 2). The application of a mathematical model capable of providing

expressions suitable for analysis of the population changes of *Enterobacteriaceae* during the production process of *Petrovsk*á *klob*ása is proposed to facilitate better control of the safety parameters.

Similar results concerning *Enterobacteriaceae* were obtained after examining Spanish fermented sausages Salpicão and Chouriça when the increased number of *Enterobacteriaceae* was detected (> 4 $\log_{10}CFU/cm^2$) and *E. coli* (Salgado *et al.*, 2006). Zdolec *et al.* (2007) detected the presence of *Enterobacteriaceae* in the filling of fermented sausages until the 60th day, Rantsiou *et al.* (2006) with the maximum on the 10th day of 3,20±0,42 and the total elimination on the 90th day. Similar results were obtained by other authors (Drosinos *et al.*, 2005, Kozacinski *et al.*, 2006), which showed that, due to a faster and more intensive acidification, the elimination of *Enterobacteriaceae* started earlier.

CONCLUSIONS

Based on the analysis of the growth rate from the zero until the 270th day (unpacked samples), for all six models of the *Petrovsk*á *klob*ása (experimental batches A1, A2, B1, B2, B3 and B4), it was detected the presence of *Enterobacteriaceae* in all samples of all batches but they completely disappeared after the 30th day. The mathematical model provides critical points that are crucial for safety control: the beginning of the exponential phase of inhibition, the time during which the rate of inhibition is at a maximum, and the phase in which the rate of decline of the population of microorganisms decreases until they virtually disappear from the sausage. In our research work, the function of elimination of *Enterobacteriaceae* in all cases of produced batches of sausages was linear.

ACKNOWLEDGEMENTS

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DEVELOPMENT OF HPLC-DAD METHOD FOR DETERMINATION OF THIAMETHOXAM IN HONEY FROM AUTONOMOUS PROVINCE OF VOJVODINA

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ABSTRACT

After the EU ban on the use of the neonicotinoids in flowering crops that honeybee might visit, there has been an increased interest on determination of the neonicotinoids residues in honeybee products such as honey. The objective of this study was to develop HPLC-DAD analytical method with QuEChERS sample preparation procedure for the analysis of neonicotinoid thiamethoxam in honey samples. The liquid chromatographic conditions were optimized by Response surface methodology with *Box-Behnken* design and the global *Derringer´s* desirability. The optimized method was validated to fulfill the requirements of SANCO/12495/2011 for the sample pretreatment procedure providing results for accuracy (R, 70-120%), repeatability and within-laboratory reproducibility (RSD, <20%), limit of detection (LOD, 2.5 µg kg^{-1}) and quantification (LOQ, 7.5 µg kg^{-1}). Matrix effects were compensated by the use of matrix-matched calibration. For the first time, sunflower honey samples collected from all 7 counties of Autonomous Province of Vojvodina were analysed discovering the presence of thiamethoxam, therefore implicating the necessity of ongoing control of this type of food.
Keywords: *Neonicotinoids, thiamethoxam, QuEChERS, honey, HPLC-DAD*

INTRODUCTION

In less than 20 years, neonicotinoids became the most widely used class of insecticides and their presence now accounts for at least one quarter of the world insecticide market (Agropages, 2013). Like nicotine, they are nicotinic acetylcholine receptor agonists. (Decourtye & Devillers, 2010; Tomizawa & Casida, 2005). Nowadays, they are authorized in more than 120 countries for more than 1000 uses for the treatment of a wide range of plants including sunflower, corn, potatoes, rice, sugar beets, oil rapeseed, soy, fruits etc. (Biever *et al.*, 2003). Neonicotinoids are systemic insecticides translocated to the whole plant (flowers, pollen and nectar) (Van der Sluijs *et al.*, 2013), revealing ways by which some honey bees and other beneficial pollinators can be exposed to these compounds. Different studies in Europe and USA have demonstrated that sub lethal amounts of neonicotinoids cause disorientation, reduced communication, impaired learning and memory, reduced longevity and disruption of honeybee brood cycles (Farooqui, 2013). Moreover, residues of these insecticides may finally be found in bee product such as honey. The European Commission has banned the use of imidacloprid, thiamethoxam and clothianidin in crops attractive to pollinators in next two years emphasizing the awareness of potential harmful impact of the neonicotinoids on honeybees and their products (Commission, 2013; EFSA, 2013; Gross, 2013). Therefore, monitoring and determination of trace levels of the neonicotinoids in honey are necessary and demand highly efficient, selective and sensitive analytical techniques. Neonicotinoids are usually determined by liquid chromatography (LC) coupled to different detectors such as diode array detector (DAD) (Vichapong *et al.*, 2013), ultraviolet detector (Rahman *et al.*, 2013), fluorescence (García *et al.*, 2007), mass spectrometer (Jovanov *et al.*, 2013), electrochemical detector, post-column photochemical reactor (Rancan *et al.*, 2006), or even detectors based on thermal lens spectrometry (Franko, 2008). Preparation of honey samples for the liquid chromatographic analysis presents an analytical challenge due to high amounts of different compounds found in honey. For these reasons we decided, due to the

good results achieved, to employ extraction methodology based on QuEChERS (Jovanov *et al.*, 2014) protocol as a sample pretreatment procedure in neonicotinoids analysis of honey. After the selection of the appropriate sample pretreatment procedure it was mandatory to acquire the optimal chromatographic conditions using Response-surface methodology (RSM) (Wang *et al.*, 2011; Zhou *et al.*, 2009) for the determination of the selected thiamethoxam neonicotinoid. Box-Behnken designs (BBDs) combined with Derringer's desirability function, were chosen used for simultaneous optimization of different chromatographic parameters.

The objective of the present work was to develop a rapid, sensitive, optimized and accurate analytical method based on HPLC-DAD combined with selected extraction procedure for determination of thiamethoxam neonicotinoid insecticides along with other potentially found neonicotinoids in honey samples such as dinotefuran, nitenpyram, clothianidin, imidacloprid, acetamiprid and thiacloprid. Finally, the proposed procedure was validated and real honey samples collected from the Autonomous Province of Vojvodina were analyzed on the presence of the selected neonicotinoids.

MATERIAL AND METHODS

Standards of neonicotinoids (certified purity > 99%) and formic acid (purity 98%, w/w) were obtained from Sigma-Aldrich (Steinheim, Germany), while acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany). The purified water used was produced by a Simplicity UV system from Millipore (Bedford, MA, USA). Stock solutions of neonicotinoid standards (100.0 mg L^{-1}) were prepared in water and stored in a freezer at -10 °C, and were stable over a period of at least three months. Multicomponent standard solution (100.0 µg L^{-1}) was prepared by mixing and properly diluting the calculated amounts of each standard stock solution with water. The obtained multicomponent solution was used for spiking honey samples, matrix-matched calibration (MMC), and solvent based calibration (SC). The MMC standards were prepared by spiking of blank honey samples with multicomponent stock solution at the final reconstitution step, over the range from the limit of quantification (LOQ) to 100.0 µg kg^{-1} for all analyzed neonicotinoids. Kits for QuEChERS sample preparation (buffered extraction kits; part no ECQUEU750CT and general fruits and vegetables sample cleanup kits; part no ECMPS15CT) were purchased as ready to use from United Chemical Technologies (UCT inc., Bristol, USA). The 51 honey samples of sunflower floral origin were collected from different locations in 7 counties of the Autonomous Province of Vojvodina, Republic of Serbia.

The QuEChERS procedure was performed as follows: honey samples (15.0 mL of 50.0 g L^{-1} spiked honey solution corresponding to 10.0 g of honey) were measured in 50 mL extraction vials and mixed with buffering salts (4.0 g of magnesium sulphate, 1.0 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1.0 g of sodium citrate tribasic dehydrate) from the separate pre-packaged pouches and acetonitrile (10.0 mL). The mixture was vortexed for 1 minute using a vortex mixer (BOECO, Germany) and centrifuged at 3000 rpm (Tehtnica, Yugoslavia) for 10 minutes. The acetonitrile extract layer (6.0 mL) was transferred into the sample cleanup vials (containing 0.9 g of magnesium sulphate and 0.15 g of primary-secondary amine (PSA)). The procedure was followed by vortexing of the cleanup vials for 1 minute and centrifuging at 3000 rpm for 10 minutes. The extract (1.5 mL) was transferred into 2 mL vials and solvent was evaporated under the stream of nitrogen. Dry residues were reconstituted in 1.0 mL (1.5 mL for spiked honey samples) of the mobile phase prior to the analysis with HPLC-DAD.

Experimental design was performed by use of Design-Expert 7.0.0. (Stat-Ease, Minneapolis, USA). Selection of factors (eluents composition and flow rate) for optimization was based on preliminary experiments. The procedure followed in this work optimization of retention time of the chromatographic peak and resolution of the peaks is a modification of the method developed by Derringer and Suich (Sivakumar *et al.*, 2008). The optimized factors were incorporated in HPLC–DAD method.

An Agilent 1200 Series HPLC system (Agilent Technologies Inc., USA) consisting of a solvent degassing unit, a quaternary pump, an autosampler and a thermostated column compartment was used in the HPLC-DAD system. Separation of the analytes was achieved on a ZORBAX Eclipse XDB-C18 column (50 mm × 4.6 mm i.d., 1.8 µm) with a column temperature of 30 °C. The mobile phase consisted of two eluents, ACN (A) and ultrapure water with 0.2% formic acid (B), delivered at a flow rate of 0.7 mL min^{-1}. The selected neonicotinoids were separated, in order to determine thiamethoxam, with the following gradient program: 90% B at start followed by a linear gradient reaching 60% B after 6 min and then switch back to 90% B at the end of the run at 10 min. Thiamethoxam was identified according to the retention times and quantification was based on peak areas with DAD monitoring wavelengths set at 244 nm.

The proposed HPLC-DAD method was evaluated to fulfill all the necessary requirements of method validation and quality control procedures for pesticides residue analysis in food and feed (SANCO/12495/2011). The calibration curves in pure solvent (LOQ to 100.0 µg L^{-1}) and in honey matrix (LOQ to 100.0 µg kg^{-1}) were obtained by plotting the peak areas against the concentrations of the corresponding calibration standards at six levels. The linearity of calibration curves was expressed by the square correlation coefficient (r^2). The limit of detection (LOD) and limit of quantification (LOQ) were estimated by injecting decreasing concentrations of matrix-matched standards and measuring the response at a signal-to-noise ratio (S/N) of ≥ 3 and ≥ 10 for the LOD and LOQ, respectively. The accuracy of the method was determined with the percentage recovery (R, %) using spiked blank honey samples (at 3 concentration levels) prior to analysis and matrix-matched calibration curves by comparing the mean measured concentration with the spiked concentration of the analyzed neonicotinoids. The precision of the method was expressed in terms of repeatability (the analysis was performed on same day (n=5) with the same instrument and the same operator) and within-laboratory reproducibility (three different days with the same instrument and by the different operators) as relative standard deviation (RSD). Precision values below 20% were targeted.

RESULTS AND DISCUSSION

The validation of the chromatographic method for determination of thiamethoxam under adopted optimized conditions was carried out. Under these conditions retention times (t_r) were constant with the RSD never exceeding 0.1% (Table 1). The calculated LODs and LOQs using matrix-matched calibration curves are also shown in Table 1. The reached LOQs were lower than the European Commission established MRLs for the neonicotinoids in honey.

Table 1. Retention time (t_r), limits of detection and quantification of thiamethoxam with maximum residue levels (MRLs) for honey regulated in European Union (EU)

Neonicotinoid	mean t_r (n=15) (min)	RSD (%) of t_r	EU MRLs (µg kg^{-1}) *	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
Thiamethoxam	3.9	0.01	10.0	2.5	7.5

* ("EU Pesticides database," 2013)

For the proposed method, the use of matrix-matched calibration (MMC) standards was done to compensate the matrix effects, expressed as the signals from the thiamethoxam in the honey matrix compared to the signals in the solvent. Matrix effects were calculated as signal suppression/enhancement (SSE), i.e. slope ratios for MMC and SC. Signal suppression/enhancement caused by the matrix effects was observed for thiamethoxam. Due to manifested matrix effects for precise quantification the use of matrix-matched standards is required. The thiamethoxam matrix-matched calibration curve obtained using

QuEChERS pretreatment procedures was linear over the range LOQ-100.0 µg kg^{-1}. Specificity was demonstrated by identifying the thiamethoxam based on the UV absorption spectra as well as the relative retention times (compared to the standards). The accuracy of the proposed method was expressed as the mean recovery (R, %). The R and RSD values were determined for spiked blank honey samples prior to analysis in 5 replicates at three concentration levels (10.0, 50.0 and 100.0 µg kg^{-1}) using the MMC curves. The recovery results confirmed that the optimal recovery (R, 70-120% with RSD of ≤ 20%) was obtained. Precision, expressed as the repeatability and within-laboratory reproducibility gave RSD values ≤ 20%, indicating a good precision of developed method.

The developed method was applied to the analysis of 51 sunflower honey samples collected from multiple locations in the Autonomous Province of Vojvodina, Republic of Serbia. The results indicate that thiamethoxam was detected in 5 wildflower honey samples (LOD<detectable<LOQ).

CONCLUSIONS

In this work, an HPLC-DAD analytical method based on QuEChERS sample pretreatment procedure was developed and optimized for the determination of thiamethoxam in honey. The developed method was validated to ensure quantification of low concentrations of selected neonicotinoid. Residues of thiamethoxam were found in wildflower honey samples, therefore implicating a potential usefulness of ongoing control of this type of food.

ACKNOWLEDGEMENTS

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THE INFLUENCE OF STARTER CULTURE TYPE ON RHEOLOGY AND TEXTURE OF FERMENTED MILK PRODUCTS

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ABSTRACT

Yoghurt and probiotic yoghurt have been proven as a healthy and nutritious food. The possibility of the application of kombucha as a non-conventional starter culture and its technological and nutritional aspects in dairy products has been described recently. The basic characteristics of yoghurt gel are the appropriate textural and rheological properties. The major structural changes during the milk fermentation are related to casein micelles, and they are due to the change of the pH value.

The aim of this study was to investigate the effect of the addition of three different starter cultures: yoghurt starter culture, probiotic starter culture and kombucha inoculum on fermentation time, rheological and textural properties of fermented milk products. During the fermentation process involving kombucha, yoghurt or probiotic strarter culture, samples were taken at the pH values of 5.4, 5.1, 4.8 and 4.6.

The viscosity of the samples was measured at 5°C using a viscometer HAAKE RheoStress 600HP (Karlsruhe, Germany) with a cone and-plate-sensor PP60Ti (gap 1 mm). Textural properties of produced samples were analyzed by Texture Analyser TA.HD[plus] (Stable Micro System, England).

The kombucha starter exhibited a similar influence on the casein networking pattern as the yoghurt and probiotic starter cultures. The applied starter cultures induced different gelation rates and final gel viscosity of the sample. The changes in the texture of all samples were pronounced between the pH 5.4 and 5.1, which is in correlation with the analyzed rheological properties.

The rheology and texture of the samples produced by kombucha followed the same patterns as noticed for the probiotic and yoghurt samples.

Keywords: yoghurt, kombucha, viscosity, textural properties

INTRODUCTION

Fermented milks have been an important component of nutrition and diet. Besides unique flavors and textures as exemplified in traditional fermented product such as yoghurt, many novel benefits, especially with respect to health, can be conveyed via fermentation technology (Hugenholtz, 2013). Milk fermentation by kombucha results in products similar to yoghurt or kefir and have been described recently (Malbaša *et al.*, 2009a, b). Kombucha is a mixture of acetic and lactic acid bacteria (Marsh *et al.*, 2014) and yeast, which are embedded within a cellulosic matrix that floats above the fermentate. The role of yeasts in the fermentation of kombucha is to convert sucrose to organic acids, carbon dioxide and ethanol, with the latter then used by acetic acid bacteria to form acetaldehyde and acetic acid. Acetic acid bacteria also used yeasts–derived glucose to synthesise bacterial cellulose and gluconic acid (Dufrense and Farnworth, 2000).

Milk fermentations generally involve the metabolism of lactose to lactic acid, a characteristic common to all fermented milks, by lactic acid bacteria, mostly *lactococci* and *lactobacilli*. During fermentation the casein particles aggregate as the pH approaches 4.6 as a result of lactic acid production. This, together with the denaturation of the whey protein and its association with caseins, results in increased gel firmness and viscosity (Lucey, 2002; van Vliet *et al.*, 2004). Yoghurt is a typical example of a weak viscoelastic and thixotropic (time–dependent) gel (Benezech and Maingonnat, 1994).

The aim of this study was to investigate the effect of the addition of three different starter cultures: yoghurt starter culture, probiotic starter culture and kombucha inoculum on

fermentation time, rheological and textural properties of fermented milk products. Textural characteristics: firmness and consistency, and rheological properties (hysteresis loop area) in fermented milk products were compared.

MATERIAL AND METHODS

The samples for investigation were produced from homogenized and pasteurized milk (AD Imlek, Division Novi Sad Dairy). During the fermentation process involving kombucha, yoghurt or probiotic strarter culture, samples were taken at the pH values of 5.4, 5.1, 4.8 and 4.6. The particular samples were designated by the capitals K (kombucha), P (probiotic) and Y (yoghurt), and the corresponding pH value. The kombucha was cultivated on black tea (*Camellia sinensis*– oxidized, 1.5 g/L) with the sucrose concentration of 70 g/L. The tea was cooled to room temperature, after which inoculum from the previous fermentation was added in an amount of 10%. The incubation was performed at 29°C for 7 days. The kombucha inoculum (30 mL) was added to the milk in an amount of 10% (Malbaša *et al.*, 2009b). The probiotic starter culture was ABT-7 – probiotic culture-Probio-Tek®, containing LA-5®, *Lactobacillus acidophilus*, BB-12®, *Bifidobacterium*, *Streptococcus thermophilus*, CHR Hansen, Denmark. The yoghurt starter culture (YF-L812) contained thermophilic starter culture Yo-Flex®, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. The commercial starters were added according to the manufacturer's specification – 0.005 g/100g. All the samples were produced in triplicate at 42°C.

Rheological properties of fermented milk samples were measured at 5°C using a viscometer HAAKE RheoStress 600HP (Karlsruhe, Germany) fitted with sensor PP60Ti (gap 1mm). For each sample, replicate measurements were done independently, and data processing was performed using a RheoWin Pro software package (Version 2.94, Thermo Haake, Karlsruhe, Germany). Thixotropy test was initially applied to characterize the flow behavior of the fermented milk samples. Shear stress was recorded at increasing shear rates from 0 to 40s (upward flow curve), followed by decreasing shear rate from 40 to 0 s within 50 s (downward flow curve). Other rheological parameters considered were the area under the upward flow curve, the area difference under upward and dawn ward flow curves (ΔA or hysteresis loop area) (Hassan *et al.*, 2003).

Textural properties: firmness and consistency of produced samples were analyzed by Texture Analyser TA.HD[plus] (Stable Micro System, England) through a single compression test, using a back extrusion cell (A/BE) disk (diameter 35 mm; distance 30 mm; speed 0.001 m/s) and an extension bar, applying a 5kg load cell. Using the option *Return to Start*, a trigger force of 10 g was applied.

RESULTS AND DISCUSSION

Physicochemical characteristics of fermented milk products manufactured using three different starter cultures: yoghurt starter culture, probiotic starter culture and kombucha inoculum are presented in Table 1. Fermentation process was two times faster in the probiotic and yoghurt samples than in the samples produced by kombucha. The shortest fermentation time (4.0 h) was noticed in fermented milk produced with probiotic starter and that is in accordance with the result reported by Iličić *et al.*, 2013.

Table 1. Physicochemical characteristics of fermented milk products manufactured using different starter cultures

Samples	pH value	Fermentation time (h)	Total solid (g/100g)	Fat (g/100g)
Y4.6	4.6 ± 0.1	4.5	11.12±0.17[a]	1.95±0.03[a]
P4.6	4.6 ± 0.1	4.0	10.81±0.21[a]	1.95±0.03[a]
K4.6	4.6 ± 0.1	8.2	10.34±0.18[b]	1.90±0.04[a]

abcd - Means in the same column with different letters are significantly different (p<0.05)

Flow curves of fermented milk products manufactured with three different starter cultures (kombucha, probiotic and yoghurt) are presented in Figure 1. It is evident that shear stress in all of samples increased with decrease of pH value of samples. The smallest differences in the shear stress were measured between pH 5.1 and 5.4. Kombucha fermented milk samples (Figure 1A) had higher yield stress compared to probiotic and traditional fermented milk samples (Figure 1B and 1C, respectively).

A)
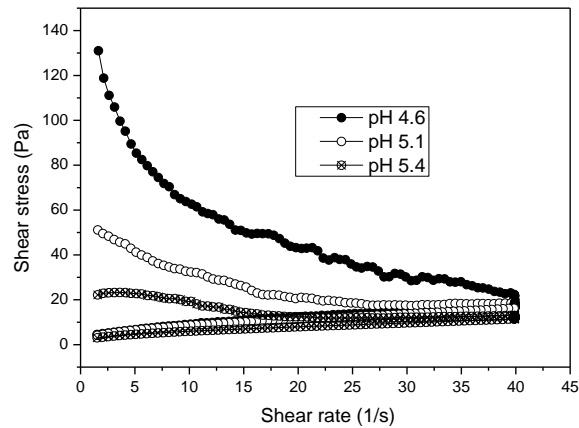
B)
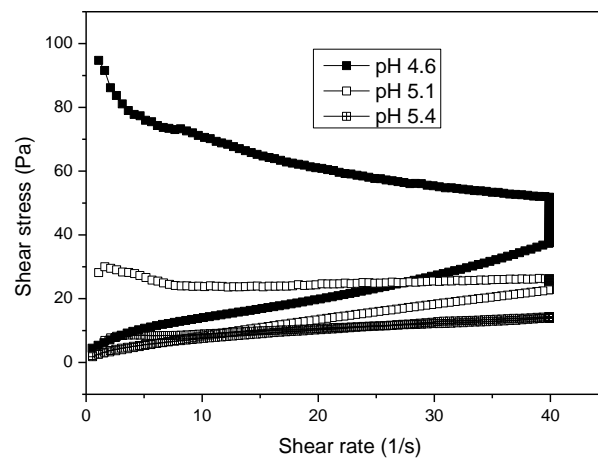
C)
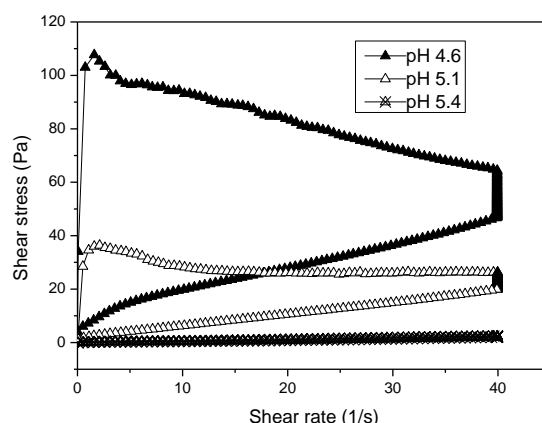
Figure 1. Flow curves of fermented milk samples produced with different starter cultures:
a) kombucha b) probiotic c) yoghurt

Differences in flow curves between samples affected the values of hysteresis loop area for samples produced with different starter cultures (Figure 2). The hysteresis loop area (ΔA) is the indicator of yoghurt structural breakdown and rebuilding (a degree of tyxotropy) during shearing (Hassan *et al.*, 2003; Paseephol *et al.*, 2008). The decrease of pH value in all samples caused the increase of the hysteresis loop area. Fermented milk sample with yoghurt starter (pH=4.6) showed the highest hysteresis loop area (2201 Pa/s), indicating that more structural breakdown and better structural reversibility took place during shearing. Kombucha fermented milk samples showed lower values of hysteresis loop area (1444 Pa/s) than hysteresis area of probiotic and yoghurt samples. Differences in hysteresis area between samples could be explained by the structuring effect of using starter cultures. Also, probiotic and yoghurt samples had higher total solids and more compact structure than kombucha fermented milk product.
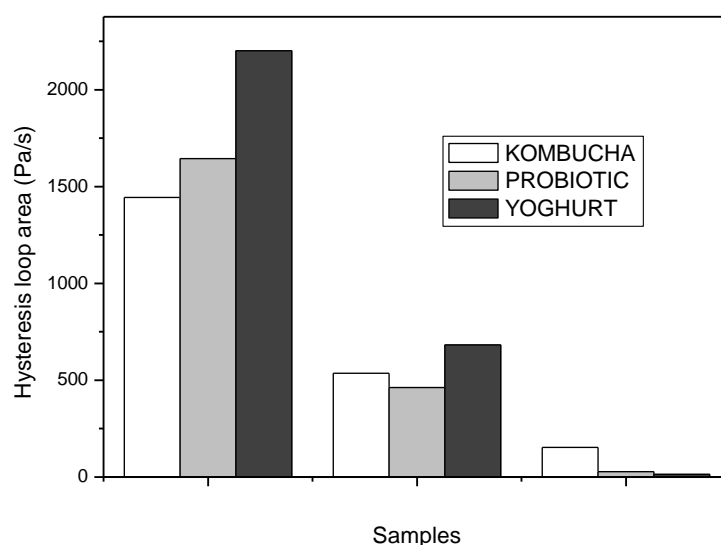
Figure 2. Hysteresis loop area of fermented milk samples

The textural properties: firmness and consistency of the samples are presented in Figure 3. Fermented milk produced with yoghurt starter had the highest firmness (204.01 g) and consistency (5377.62 gs), while fermented milk produced with probiotic starter had the lowest

firmness (123.7 g) and consistency (2706.3 gs). The most pronounced changes in the texture of all samples were observed between the pH 5.4 and 5.1, which is in correlation with the analyzed rheological properties.
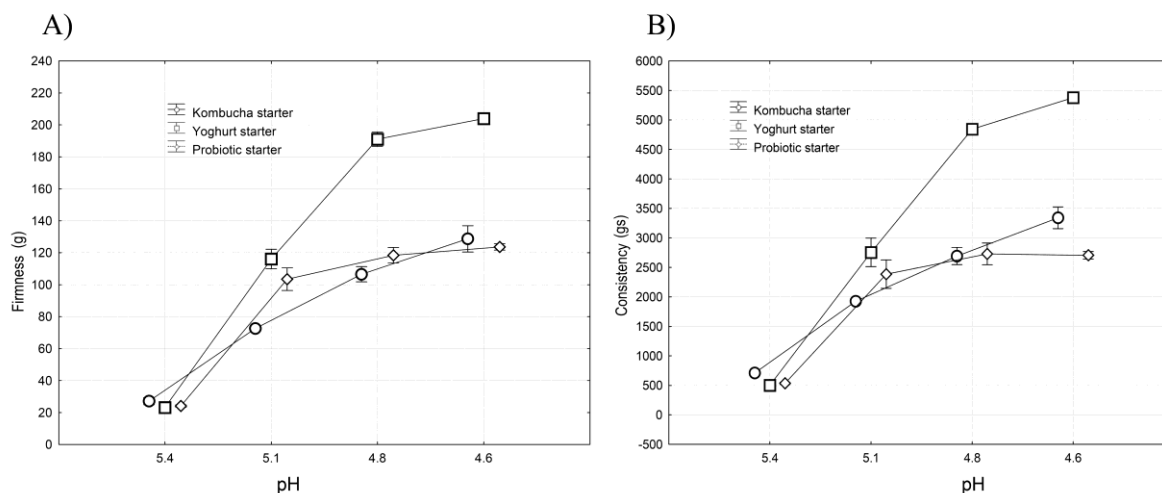
Figure 3. Changes of firmness (A) and consistency (B) of fermented milk products manufactured using different starter cultures during fermentation

CONCLUSIONS

Type of starter cultures usage for manufacturing fermented milk products had great impact on fermentation time. Fermentation process was two times faster in the probiotic and yoghurt samples than in the samples produced by kombucha.
Differences in flow curves between samples affected the values of hysteresis loop area. Fermented milk sample with yoghurt starter showed the highest hysteresis loop area, indicating more structural breakdown and better structural reversibility during shearing.
The most pronounced changes in the texture of all samples were observed between the pH 5.4 and 5.1, which is in correlation with the analyzed rheological properties.

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ELISA AND LC-MS/MS DETERMINATION OF AFLATOXIN M1 IN MILK SAMPLES

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ABSTRACT

Aflatoxin M1 (AFM1) is derivate of aflatoxin B1 (AFB1) which is formed in liver and excreted into the milk in the mammary gland of lactating animals that have been fed with AFB1 contaminated diet.
Considering that AFM1 was included in the first group by carcinogenicity and milk and its derivates are consumed daily, there is a need for fast, sensitive, reliable and accurate analytical method for its determination.
The objective of this study was to compare performances of Enzyme Linked Immunosorbent Assay (ELISA) and liquid chromatography with triple quadrupole mass spectrometry (LC-MS/MS) methods for determination of AFM1 in naturally contaminated milk samples. For LC-MS/MS analysis, the samples were prepared using immunoaffinity columns while for ELISA milk samples were analyzed without sample preparation. Both methods were validated according to the European Union Regulative using certified reference material (CRM). The obtained validation parameters indicate that ELISA and LC-MS/MS are suitable for determination of AFM1 in milk samples. Additionally, the validated methods were applied for analysis of ten naturally contaminated milk samples. Samples were selected with the aim to cover two different ranges of concentrations, around 0.05 and 0.50 µg/kg. Analysis showed that ELISA method did not give false positive results since presence of AFM1 determined using ELISA was confirmed with LC-MS/MS. Furthermore, ELISA gave slightly higher values of AFM1 in comparison to LC-MS/MS.
Keywords: aflatoxin M1, ELISA, LC-MS/MS, milk

INTRODUCTION

Aflatoxins (AFs) are one of the most known and investigated group of mycotoxins which can be found as contaminants in different types of food and feed. AFs are mainly produced by *Aspergillus* species in agricultural products from tropical and subtropical regions (Ardic *et al.*, 2007). Among approximately 18 identified AFS, aflatoxin B1 (AFB1) is the most common as well as highly toxic, mutagenic, teratogenic and carcinogenic compound (IARC, 1993). Aflatoxin M1 (AFM1) is the 4-hydroxy derivative of AFB1, formed in liver and excreted into the milk in the mammary glands of lactating animals that have been fed with AFB1 contaminated diet (Fallah *et al.*, 2009; Gurbay *et al.*, 2010). AFM1 is included in the first group by International Agency for Research of Cancer (2002) due to its demonstrated carcinogenic effects.
Milk has the greatest demonstrated potential for AFs introducing into the human diet since it represents one of the main foodstuffs in human nutrition. Furthermore, infants and young children eat and drink more relative to their size than adults, and due to high intake of milk, children are the most susceptible population to the effects of AFM1 (Erkekoglu *et al.*, 2008). Furthermore, AFM1 is stable during production processes of pasteurization or ultra-high temperature treatments, and because of that it is of great importance to provide the most effective control of milk and dairy products in accordance with the defined maximum residue levels set by the regulations. Maximum residue levels (MRL) of AFM1 in milk varies from 0.05 µg/kg in European Union (European Commission, 2006b) to 0.5 µg/kg established in United States (FDA, 2011). Regulation for MRL of AFM1 in milk in Serbia (Serbian Regulation, 2011) was recently harmonized and adopted with European Union Regulation (European Commission, 2006b). However, presence of AFM1 in milk during January and February 2013 (Kos *et al.*, 2014) resulted in Regulation changes. During March 2013, Serbian Government changed previously MRL of AFM1 from 0.05 µg/kg to 0.50 µg/kg

(Serbian Regulation, 2013). However, at the beginning of July 2014 MRL was returned to 0.05 µg/kg (Serbian Regulation, 2014a), and after only then days MRL was changed to 0.25 µg/kg (Serbian Regulation, 2014b).

Since AFM1 had proven toxic effects at very low concentrations there is a need for sensitive, reliable and accurate analytical method for its determination (Turner *et al.*, 2009). A number of analytical methods for the determination of AFM1 are available in the literature. Numerous studies in the recent years highlighted Enzyme Linked Immunosorbent Assay (ELISA) as the most frequently used technique for that purpose, followed by High Performance Liquid Chromatography with fluorescence detector (HPLC-FLD) and Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) (Shephard *et al.*, 2012; Shephard *et al.*, 2013).

ELISA is defined as routine screening method which may be performed with a great number of commercially available test kits (Neogen Veratox®, Lansing, USA; Tecna S. r. l., Trieste, Italy; Ridascreen, R-Biopharm, Darmstad, Germany; Immunolab GmbH, Kassel, Germany; etc.). The major advantages of ELISA method are minimal sample clean-up and preparation, simple procedure and low cost. However, the major disadvantage of ELISA method is possible cross-reactivity to similar compounds. Therefore, to avoid risk of obtaining false-positive results confirmation by liquid chromatography based procedure is required (Anklam *et al.*, 2002).

On the other hand, LC-MS/MS method for analysis of AFM1 needs a cleanup process, usually using immunoaffinity columns (IAC) before detection. This step is mainly multistage, expensive and time-consuming. Furthermore, LC-MS/MS method has to be operated by highly trained analysts and require the use of expensive analytical instruments (Wang *et al.*, 2011). In general, LC-MS/MS represent one of the most widespread analytical techniques for quantitative purpose and also offer significant advantages over other techniques since they provide good sensitivity and detection of trace level of toxins (Manetta *et al.*, 2005; Wang, 2009). Whichever sample preparation procedure and technique are selected for determination of AFM1 the whole procedure must be validated according to European Regulation (European Commission, 2002) and Technical Report from European Committee for Standardization (CEN/TR 16059, 2010).

The aim of this study was to validate and check performance of ELISA and LC-MS/MS methods and to apply them for AFM1 analysis in naturally contaminated milk samples.

MATERIAL AND METHODS

Samples
The heat-treated skimmed milk samples were collected from supermarkets in Novi Sad (Serbia) during September 2013. Examined samples were produced in one out of six largest dairy producers in Serbia. Immediately after collection, the samples were transported to the laboratory and analyzed. Before analysis the whole amount of samples in original packing material were mixed on horizontal shaker (Benchmark Scientific, Orbi Shaker, Edison, USA) in order to ensure homogeneity of the samples.

Out of twenty analyzed milk samples using ELISA technique, then were selected with the aim to cover two different ranges of concentrations. The range of contamination were selected with the aim to cover concentration near MRL according European Union (0.05 µg/kg) and Serbian Regulation (0.50 µg/kg) which was valid at the time of examination.

Reagents
Determination of AFM1 by ELISA has been done using I 'screen AFLAM1 test kit (Tecna S. r. l., Trieste, Italy). For LC-MS/MS analysis methanol, acetonitrile and formic acid were obtained from J. T. Baker (Deventer, The Netherlands). Ammonium formate was order from Fluka Analytical (Sigma-Aldrich, Steinheim, USA). Sample preparation for LC-MS/MS analysis was done using AflaStarTM M1 R-Immunoaffinity Columns (IAC) (Romer Labs Inc., Union, MO, USA). Ultra-pure water was produced by Milli-Q purification system (Milli-Q from Millipore, USA).

AFLA M1 standard with certificated concentration of 10 μg/ml was purchased from Sigma Aldrich (Prague, Czech Republic). Standard stock solutions were prepared in acetonitrile and stored at – 10°C.

Determination of AFM1 by ELISA

Milk samples were prepared according to the manufacturer´s instructions. Samples were centrifuged at 3000g for 10 minutes (Tehtnica, Yugoslavia). The upper creamy layer was removed by Pasteur pipette and 100 μl from the lower phase was used for the analysis. Analyses were performed according to the test kits instructions.

Sample preparation for LC-MS/MS

Fifty ml of warm milk (30-35 °C) was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK) and applied to the IAC. Flow rate of the milk was approximately 1-3 ml/min. After the milk completely passed, IAC was rinsed with 20 ml of ultra-pure water. The AFM1 was eluted with 2 ml of methanol. Eluate was collected and evaporated to dryness under gentle stream of nitrogen and reconstituted with 400 μl of initial mobile phase.

Determination of AFM1 by LC-MS/MS

For LC-MS/MS analysis LC system (Agilent 1200,Agilent Technologies Inc., USA) was coupled to the mass spectrometer Agilent 6410 Triple Quad LC/MS (Agilent Technologies Inc., USA).

The mobile phase consisted of eluent A containing methanol/formic acid (99:1, v/v) and eluent B consisting of ultra pure water/formic acid (99:1, v/v). Both eluents contained 5 mM ammonium formate. The linear gradient program was applied from the beginning until 3.5 min with a decrease of B from 70% to 20%. Further increase of B up to 70% was achieved in following 6 min with holding time of 2 min. The retention time of AFM1 was 4.85 min. Typical chromatograms of AFM1 standard and naturally contaminated milk sample are shown in Figure 1.
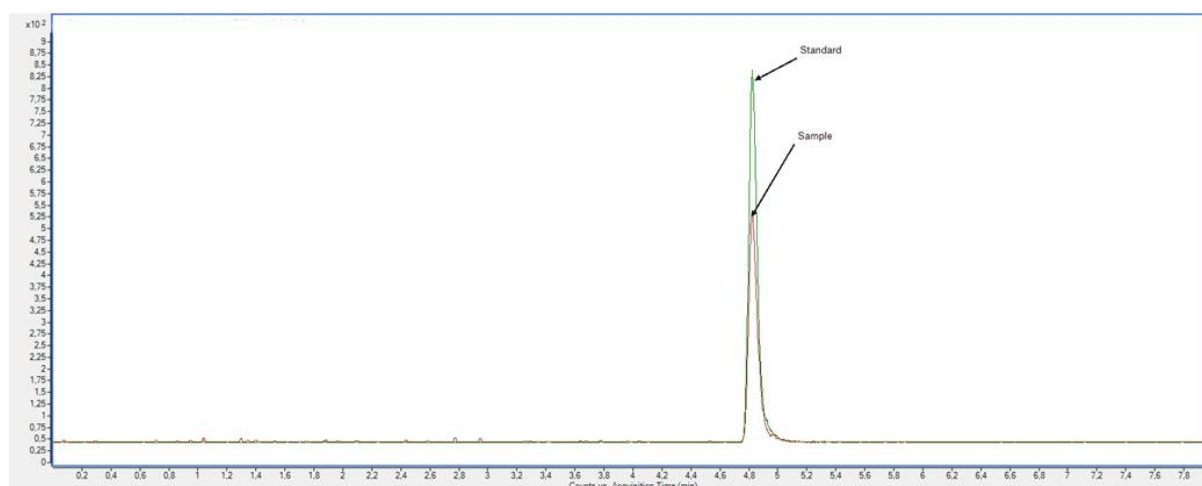
Figure 1. LC-MS/MS chromatogram of AFM1 standard and natural contaminated milk sample

Quality control

The analytical quality of the ELISA and LC-MS/MS methods was assured by the use of certified reference material (CRM). Partially defatted raw lyophilized milk sample with certified AFM1 content of 0.053 μg/kg (MI1142-1/CM, Progetto Trieste, Test Veritas, Padova, Italy).

Statistical analysis

Statistical analysis of variance was carried out by Duncan's multiple comparison tests using STATISTICA software version 11 (StatSoft Inc., Tulsa, USA). P values < 0.05 were regarded as significant.

RESULTS AND DISCUSSION

The validation parameters for ELISA and LC-MS/MS methods (Table 1) were calculated and expressed using European Official Decision procedure (European Commission, 2002). The proposed methods were validated with the respect to limit of detection (LOD), limit of quantification (LOQ), recovery and precision under repeatability and reproducibility conditions.

Table 1. Validation parameters for ELISA and LC-MS/MS methods

Parameters	Method	
	ELISA	LC-MS/MS
LOD	1.50	1.20
LOQ	5.00	4.00
RSD$_r$	3.54	8.22
Recovery	108	71.9

LOD: limit of detection (ng/kg).
LOQ: limit of quantification (ng/kg).
RSDr: relative standard deviation calculated under repeatability conditions (%).
RSDR: relative standard deviation calculated under reproducibility conditions (%).

As can be seen from the Table 1, validation parameters were in accordance with recommendations given in Regulation of European Union (European Commission, 2006a; CEN/TR 16059/2010).

Quantification of AFM1 by ELISA method was achieved using logarithmic dependence. This dependence showed good correlation (0.9908) between absorbance and AFM1 concentration. Under the optimal experimental conditions, the linearity of LC-MS/MS methods was calculated from standard calibration curves (SC) in two concentration ranges. Obtained coefficient of determination indicates good linear correlations between AFM1 detector response and its concentration. Furthermore, quantification of AFM1 by LC-MS/MS method requires existence of one more calibration, matrix-matched calibration (MMC). Matrix effects were calculated as signal suppression/enhancement (SSE), i.e. slope ratio for MMC and SC, which equals 90.3% and 94.5% for 0.5-20 ng/ml and 20-100 ng/ml ranges, respectively. The obtained matrix effects are in the range of ±20% which was considered as tolerable (Frenich et. al, 2011). Characteristics of the calibration curves for ELISA and LC-MS/MS methods were shown in Table 2.

Table 2. Characteristics of the calibration curves

Methods	Range (ng/ml)	Solvent based calibration		Matrix-matched calibration		
		Dependence	r^2	Dependence	r^2	SSE
ELISA	0.005-0.25	y= -0.421ln(x)+2.524	0.9908	-	-	-
LC-MS/MS	0.5-20	y=44.762x+11.883	0.9910	y=40.44x+11.30	0.9993	90.3
	20-100	y=33.623x+199.07	0.9982	y=32.05+120.4	0.9938	94.5

r^2: coefficient of determination (%).
SSE: signal suppression/enhancement, i.e. slope ratio for matrix-matched and solvent based calibration.
-not determined.

After validation, ELISA and LC-MS/MS methods were applied for analysis of naturally contaminated milk samples. With the aim to compare results obtained with ELISA and LC-MS/MS methods Table 3 shown results noncorrected and corrected for recovery.

Table 3. Content of AFM1 determined by ELISA and LC-MS/MS techniques

Samples number	Results MV (µg/kg)		Results corrected for recovery MV (µg/kg)	
	ELISA	LC-MS/MS	ELISA	LC-MS/MS
1	0.043[b]	0.028[a]	0.040[a]	0.039[a]
2	0.047[b]	0.022[a]	0.044[a]	0.030[a]
3	0.048[a]	0.041[a]	0.045[a]	0.056[a]
4	0.052[a]	0.044[a]	0.049[a]	0.061[a]
5	0.054[b]	0.024[a]	0.051[a]	0.033[a]
6	0.505[a]	0.439[a]	0.469[a]	0.610[b]
7	0.451[a]	0.418[a]	0.419[a]	0.581[a]
8	0.570[a]	0.414[a]	0.529[a]	0.576[a]
9	0.569[a]	0.441[a]	0.528[a]	0.613[a]
10	0.627[b]	0.422[a]	0.583[a]	0.586[a]

Different letters in the same row indicate significant differences (P < 0.05) between results obtained using three applied according to the Duncan´s multiple range test
MV±STD (µg/kg): Mean value

Regarding the results it can be seen that ELISA method did not give false positive results since presence of AFM1 determined using ELISA method were confirmed with LC-MS/MS method. Furthermore, ELISA gave slightly higher values of AFM1 concentration in comparison to chromatographic methods which is in accordance with some previously reported studies (Colak *et al.*, 2006).

The statistical analysis of variance between methods for the results noncorrected for recovery showed that significant differences were noted for the results of samples 1, 2, 5 and 10. Furthermore, among then results corrected for recovery only result of sample 6 obtained using LC-MS/MS was significantly different in comparison to result obtained using ELISA method. According to obtained results it could be observed that better correlation between results obtained with different methods were noted after correction of results with recovery.

The obtained results in this study could not be completely compared to the literature data since, to our knowledge, none of the previously reported studies have focused on the comparison ELISA and LC-MS/MS methods for AFM1 determination.

CONCLUSIONS

Obtained validation parameters as well as results for real samples indicate that ELISA and LC-MS/MS methods were sufficiently efficient and suitable for AFM1 analysis. Which methods will be selected mostly depends on availability of the equipment and highly trained analysts. In comparison to long-lasting and expensive chromatographic techniques, this study indicates that ELISA method for AFM1 determination offers many advantages including shorter analysis time, absence of complicated sample preparation step and simplicity of the analytical procedure.

ACKNOWLEDGEMENTS

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SURVIVAL AND GENETIC CHARACTERIZATION OF *LISTERIA* SPP. DURING THE PRODUCTION OF *PETROVSKÁ KLOBÁSA*

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ABSTRACT

Listeria monocytogenes is a psychrotrophic, opportunistic Gram-positive bacteria, that has become one of the biggest problems in the food industry because of its ability to grow at very low temperatures and to survive long periods of time under adverse environmental conditions. The aim of this study was to analyze survival of *Listeria* spp. during the preparation of *Petrovská klobása* and to genetically characterize the isolates by amplification of the *hlyA* gene. Results obtained by conventional microbiological and molecular methods showed that during production of *Petrovská klobása*, red hot paprika powder, meat batters and sausages were positive for the presence of *Listeria* spp. This group of bacteria was present in sausages from 2nd, 6th, 9th, 15th days of production.
Keywords: Petrovská klobása, Listeria spp., hlyA gene, traditional production

INTRODUCTION

Listeria monocytogenes, opportunistic Gram – positive bacteria is widespread in a variety of habitats such as soil (Weis and Seeliger, 1975), water (Watkins and Sleath, 1981), vegetation (Weis and Seeliger, 1975; Welshimer and Donker - Voet, 1971), feed (Caro *et al.*, 1990), industrial plants (Destro *et al.*, 1996) and farms (Dijkstra, 1975; Kimura, 2006). It can also be readily isolated from humans, domestic animals, raw agricultural and fishery products, food processing environments and home. It is microaerophilic and psychrophilic bacteria.

L. monocytogenes became one of the biggest problems in the food industry because of its ability to grow at very low temperatures (0°C to 7°C) and to survive long periods of time under adverse environmental conditions, although *Listeria* is non – spore and non – capsule forming bacteria (Watkins and Sleath, 1981). Various studies have indicated that certain strains of *L. monocytogenes* survive well within the food processing environment (Kathariou, 2002; Tompkin, 2002; Pan *et al.*, 2006) and the persistence of such strains is of concern as they have the potential to act as a continual source of contamination (Pan *et al.*, 2006). Moreover, the detection of nonpathogenic *Listeria* spp. can be considered as a useful indicator of a deterioration in hygiene or process conditions during food production (PHLS, 2000), leading to an increased risk of contamination with pathogenic *Listeria* spp. Therefore, the detection of all *Listeria* spp. is necessary when testing food and environmental samples.

The aim of the present study was to analyze survival of *Listeria* spp. during the preparation of *Petrovská klobása* and to genetically characterize the isolates by amplification of the *hlyA* gene.

MATERIALS AND METHODS

Bacterial strain, media and growth conditions
The strain *Listeria monocytogenes* 4b ATCC 19115 was obtained from the American Type Culture Collection (ATCC; Manassas, Va., USA). Standard strain was grown under aerobic conditions on brain-heart agar (BHA) (Merck, GmbH Darmstadt, Germany) and buffered peptone water (Merck) at 37°C.

Sausage preparation
The sausages were manufactured from a mixture of lean minced pork and pig back fat in a ratio 80:20. The spices were added in the following percentages: 2.5 % red hot paprika powder, 1.80 % salt, 0.20 % crushed garlic, 0.20 % caraway and 0.15 % sugar. Half of obtained meat/fat mixture was inoculated with 0,015% of commercial starter culture (Quick Starter, Lay Gewirze OHG, Germany), whose composition (*Staphylococcus carnosus* 25 %, *Staphylococcus xylosus* 25 %, *Lactobacillus sakei* 25 %, *Pediococcus pentosaceus* 25 %) is most similar to the identified profile of indigenous microflora in traditional production (Petrovic *et al.*, 2007). The seasoned batter was immediately stuffed in collagen casings (500 mm long and Ø = 55 mm) and raw sausages, with and without starter culture, were subjected to industrial controlled drying conditions and to uncontrolled traditional conditions by a cold process for about 10 -15 days with pauses, using specific kinds of wood (cherry wood).

Microbiological analysis
Sampling was carried out prior to stuffing (at day 0) and on 2nd, 6th, 9th, 15th, 30th, 60th, 90th, 120th. The samples were placed in sterile plastic bags with lateral filter and taken to the laboratory in a cooled container to 4°C, where they were analyzed within four hours from sampling.
The portions of 25 g were used for microbiological analyses and the isolation and identification of *Listeria* was performed following the SRPS EN ISO 11290-1: 2010.
The identity of suspected *Listeria* spp. colonies was confirmed by Real Time PCR method.

DNA extraction and Real Time PCR
Genomic DNA from isolates was extracted by using the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, USA).
Real Time PCR was performed in a final volume of 25 µl containing Maxima® Probe/ Rox qPCR Master Mix (Maxima® Hot Start DNK polymerase, Maxima® qPCR buffer, dNTPs, ROX passive reference dye) (Fermentas, UAB, Lithuania), 0.3 µM of each primer (hlyQF: 5 -CATGGCACCACCAGCATCT - 3, hlyQR: 5-ATCCGCGTGTTTCTTTTCGA - 3), 0.2 µM of probe (hlyQP: 5 - FAM - CGCCTGCAAGTCCTAAGACGCCA -TAMRA -3) and 0.1-1 µg of DNA sample (Table 1). Samples were amplified in a Stratagene Mx3005 QPCR system (Agilent Technologies, USA), for 10 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 63°C.

Table 1. The sequences of primers and probe used in this study

	Name	Sequences (5′ to 3′)	Target gene	PCR product (bp)
1.	hlyQF	5′- CATGGCACCACCAGCATCT -3′	*hly*A	64 bp
2.	hlyQR	5′- ATCCGCGTGTTTCTTTTCGA -3′		
3.	hlyQP	5′- FAM - CGCCTGCAAGTCCTAAGACGCCA -TAMRA -3′		

RESULTS AND DISCUSSION

Results obtained by conventional microbiological and molecular methods showed that during production of of *Petrovská klobása*, red hot paprika powder, meat batters and sausages were positive for the presence of *Listeria* spp. Also, this study reveals a contamination of this group of bacteria on 2nd, 6th, 9th and 15th days of production. The microbiological identification results show that pathogenic *L. monocytogenes* isolates were obtained from 5 samples of sausages. These isolates were positive by Real Time PCR using specific primers *hly*QF, *hly*QR and *hly*QP probe (Figure 1). Also, non-pathogenic species, *L. innocua* was isolated from red hot paprika powder and *L. grayi* from meat batters.
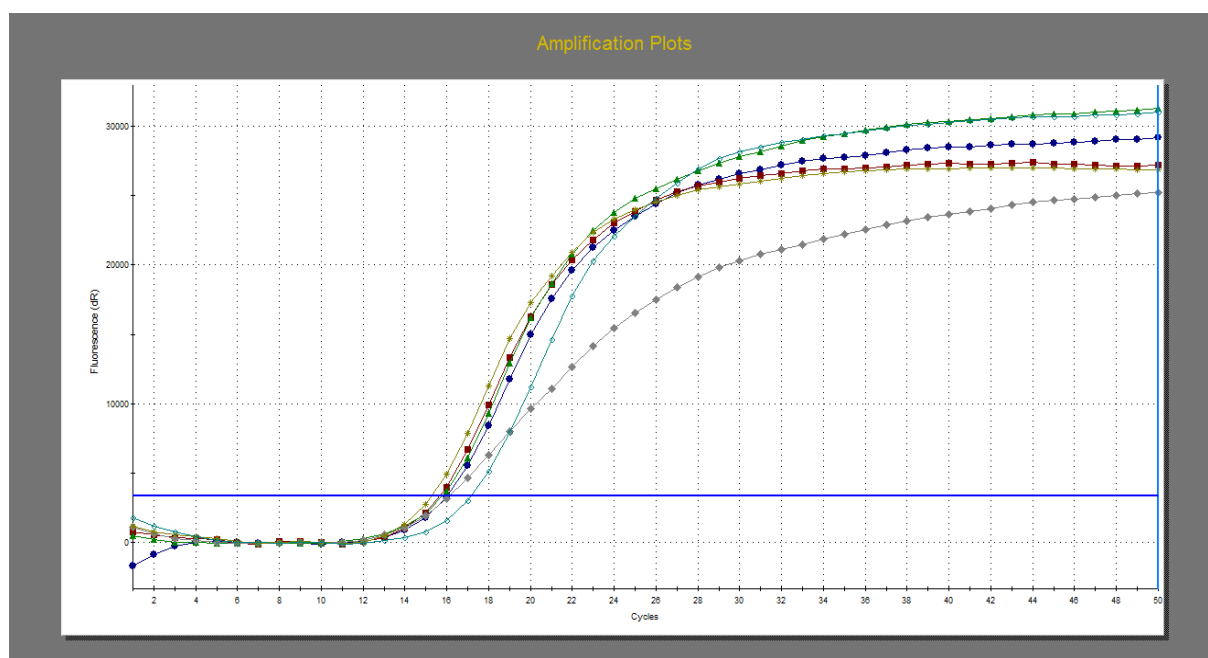
Figure 1. The positive results of specific detection of L. monocytogenes using primers and probe for hlyA gene

Survival and growth of *L. monocytogenes* in fermented sausages largely depend on the sausage type, conditions during fermentation (micro- and macroclimate), starter cultures used and adaptability of the pathogen to meat substrate (Encinas *et al.*, 1999; Thévenot *et al.*, 2005a, 2005b; Zdolec *et al.*, 2005). Within the concept of protective technologies in the production of fermented sausage, the use of protective bacteriocinogenic cultures has shown to be an additional protective factor against *L. monocytogenes*.

Previous studies of different traditional fermented meat sausages produced in Northern Portugal (i.e. *Alheira, Salpicão de Vinhais, Chouriça de Vinhais*) showed that these products are often contaminated with *L. monocytogenes*. Frequent contamination as well as high counts of *L. monocytogenes* have been reported in *Alheira* finished products *(*Felício *et al.*, 2007; Ferreira *et al.,* 2007), and in the production stages of *Salpicão de Vinhais* and *Chouriça de Vinhais*, but not in finished products. These differences are probably due to differences in processing, including the fact that *Salpicão de Vinhais* and *Chouriça de Vinhais* products include a longer smoking process (3-4 weeks) and less handling and manual labor as compared to *Alheira* (Ferreira *et al.*, 2009). In their study, Degenhardt and Ernani have examined survival of *L. monocytogenes* in low acid Italian sausage produced under Brazilian conditions. They concluded that naturally contaminated sausages presented a small increase in the counts of *L. monocytogenes* in the first days of the process, followed by a gradual decrease until the end of the process. The main source of *L. monocytogenes* is usually contaminated raw meat, and its prevalence is more pronounced in sausages maintained for short ripening periods (e.g. German Mettwurst) (Leroy *et al.*, 2005). Presence of *L. monocytogenes* in raw ingredients emphasizes the importance of adequate cooking (thermal processing) to destroy the organisms. Furthermore, *L. monocytogenes* can also be introduced into the processing area from or by employees, equipment, environmental reservoirs or ingredients. Also, *L. monocytogenes* has the tendency to form biofilms when resident populations become established in niches in the plant. These resident populations and the biofilms they form to enhance their survival are not easily eliminated by general-purpose cleaners or sanitizers and normal sanitation procedures.

CONCLUSION

Listeria monocytogenes survives extremely well in the relatively closed environments such as processing plant environment. It may be introduced into processing plants through a variety of routes, including raw materials, employees' shoes or clothes and equipment (boxes, crates, carts). Many factors influence the ability of *L. monocytogenes* to survive in fermented matrices, e.g. the initial number of pathogen, pH, salt and water content, microclimate conditions of ripening competitiveness of natural microbial flora. It is evident that development and use of the HACCP concept is urgently needed for all processing plant.

ACKNOWLEDGEMENT

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VALUE CHAIN OF IMPORTANT COMMERCIAL SPICIES OF NON-WOOD FOREST PRODUCTS WITHIN THE STATISTICAL REGION OF VOJVODINA

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ABSTRACT

Non-wood forest products (NWTPs) exceeding the local use with growing market significance, and assuming an increasing importance of the market, particularly in the expansion of organic production. The research was conducted within the statistical region of Vojvodina. For the purpose of the research twenty-two enterprises were interviewed. All of them are engaged in purchasing, processing and selling of NWFPs. This research was conducted with the purpose of gaining insight into the commercialized quantities of the most important commercial species NWFPs in Vojvodina. The **aim** of this study was to identify the flows of the purchase of raw NWFPs and the sale of final products, within the scope of the study, in the area of Vojvodina. The subjects of the study are the enterprises dealing with NWFPs purchase, processing and sale, purchased quantities of raw products, and the contingents of final products realized on the domestic and foreign markets. The purpose of the research is to identify trends in the purchase and sale of NWFPs and to create the future predictions. The primary method used is modelling, followed with the statistical methods of trend, regression and correlation analysis. To verify the obtained regression models trend correlation coefficient *(R)*, *t*-statistics derived estimates of parameters and *F*-statistics (to assess the significance of the correlation coefficient) were used. For all tests, statistical significance was α = 0.05.
Keywords: *NWFPs, Vojvodina, enterprises, trend, value chain*

INTRODUCTION

Under the light of social and economic developments, forest functions other than timber production have gained international importance and recognition (Janse and Ottitsch 2005). The reasons for this are, among others, their commercial importance and the growing demand for the products of natural origin. Although previously called secondary forest products, in most cases, these products are not minor or incidental and many of them have a long history of human use as forest products (Chamberlain *et al.*, 1998). In particular, under the influence of organic production, the commercial importance of NWFPs becomes more emphasized. In the past, NWFPs played a crucial role in the daily subsistence livelihood of rural households. NWFPs exploitation by local communities serves as source of food, fodder, fuel, medicine, construction materials, small wood for tools and handcrafts, income and employment, fruits, nuts, vegetables, fish, ranges of plants barks, mushrooms, roots, honey, etc. (Ayanwuyi, 2013).

However, only in the last decades NWFPs have gained more attention in world trade (Mousavi, 2012). Forests are the ecosystems containing a large number of medicinal plants of outstanding properties, valued on the market and frequently used by pharmaceutical and cosmetic industries (Keča *et al.*, 2012/a). Due to the variety of plant species and convenient natural characteristics, the area of Vojvodina has been identified as highly suitable for the development of NWFPs based sector and organic production. In Vojvodina, small family enterprises with limited capacity for processing and placement of NWFPs dominate. However "…more intensive use of non-wood forest products and services opens number of possibilities for development of micro, small and medium entrepreneurships which can foster economical development in rural areas" (Vuletić *et al.*, 2009).

MATERIAL AND METHODS

For the purpose of the research twenty-two enterprises were interviewed. According to unofficial data, number of enterprises that deal with NWFPs in Vojvodina in 2011 was app. 40. All of surveyed enterprises are engaged in purchasing, processing and selling of NWFPs. Their business primarily is based on the purchase and processing of medicinal plants and reeds, the purchase and sale of snails, and also on the production of honey and mushrooms. They are, simultaneously their most significant final products, both on domestic and on foreign markets.

The data analyzed in this paper were collected through surveys, direct communication with entrepreneurs, who are engaged in purchasing, processing, and sale of NWFPs (Keča *et al.*, 2012/b). The questionnaire used in the survey included questions on quantities purchased and placed NWFP, as well as price of these products.

The primary method used is modeling, followed with the statistical methods including trend, regression and correlation analysis. To verify the obtained regression models trend correlation coefficient (*R*), *t* - statistics derived estimates of parameters and *F - statistics* (to assess the significance of the correlation coefficient) were used. For all tests statistical significance was α = 0.05. The research covered the period from 2004 - 2010.

RESULTS AND DISCUSSION

The main products of surveyed enterprises are: medicinal plants, reeds, snails, honey and mushrooms.
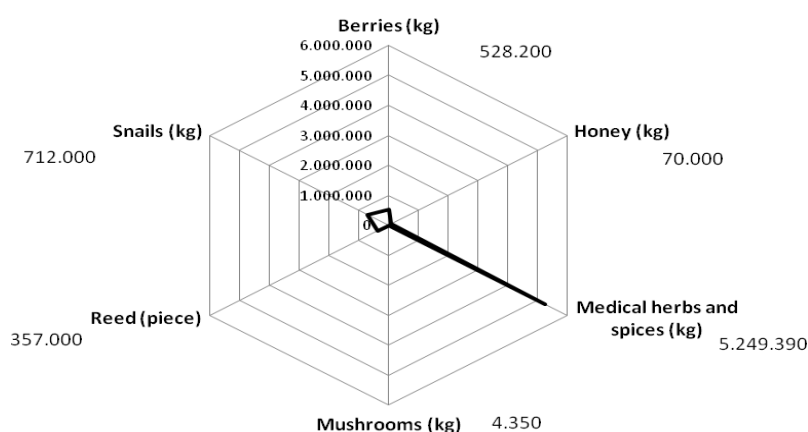
Figure 1. Purchase of row NWFPs

In total purchased quantities of NWFPs in surveyed enterprises medicinal and aromatic herbs dominate (Figure 1). The most common types of herbs are: mint, chamomile, elder, marshmallow ears and nettles. NWFPs differ significantlly in ease of collection, required technology and skills for processing, strength of demand, etc. (te Velde *et al.* 2006). Products covered by the survey include the traditional NWFPs and for them there is a constant demand both in the domestic and international market.
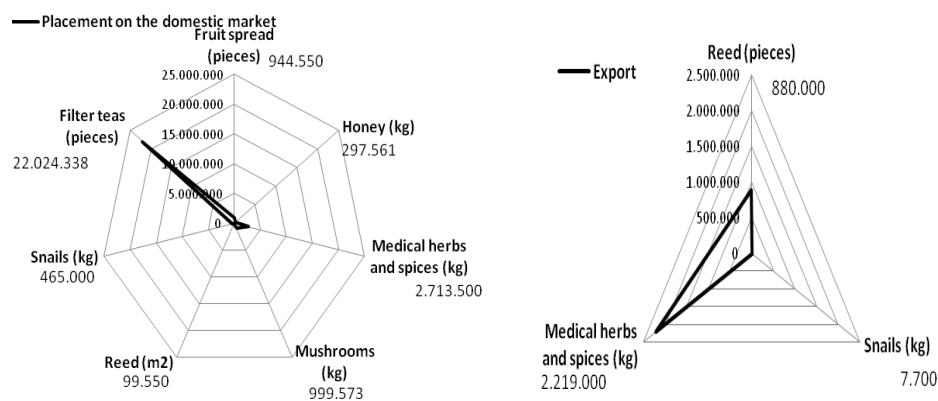
Figure 2 and 3. Selling on the domestic market and export

Regarding the placement of the domestic market and exports, the largest share has medicinal and aromatic herbs. Marketing of NWFPs used as raw materials in industry is usually carried out in two main phases: the marketing of raw materials or the collection until it comes to industrial processors, and marketing of final or semi-finished industrial and consumer products (FAO, 2011). In analogy to conducted research in Central Serbia (Nonić *et al.,* 2013), the surveyed enterprises mostly sell raw or partially processed products, as well. At the domestic market the most common placement have filter teas, while the foreign market the most common placement have herbs in bulk (Figure 2 and 3). The most important export markets for berries are: Austria, Germany, Switzerland and Belgium. For medical and aromatic herbs these are: Bosnia and Herzegovina and Croatia. Mushrooms are mostly exported to the markets of Germany, Italy, Switzerland and Spain. Export of NWFPs from Serbia in the past has been accompanied by numerous barriers, primarily due to complicated customs and administrative procedures, which make export procedures slower and more expensive (Keča *et al.*, 2012/c). Average purchase price of certain types of NWFPs are: for berries 0.8 €·kg⁻¹, honey 2.07 €·kg⁻¹, medical herbs and spices 2.09 €·kg⁻¹, mushrooms 2.5 €·kg⁻¹, bundles of reed 0.4 and for snails 0.5 €·kg⁻¹. Average sales prices for certain types of NWFPs are: for fruit spread 1.38 €·kg⁻¹, honey 3.5 €·kg⁻¹, medical herbs and spices 13.5 €·kg⁻¹, mushrooms 1.5 €·kg⁻¹, reed product 5.9 €·kg⁻¹, snails 1 €·kg⁻¹ and filter teas 3 €·kg⁻¹. If extraction and cultivation eventually occur at the same time, prices will fall (Stanley *et al.,* 2012). However, for NWFP is characteristic that the market price is largely conditioned by natural factors and yields in a given year.
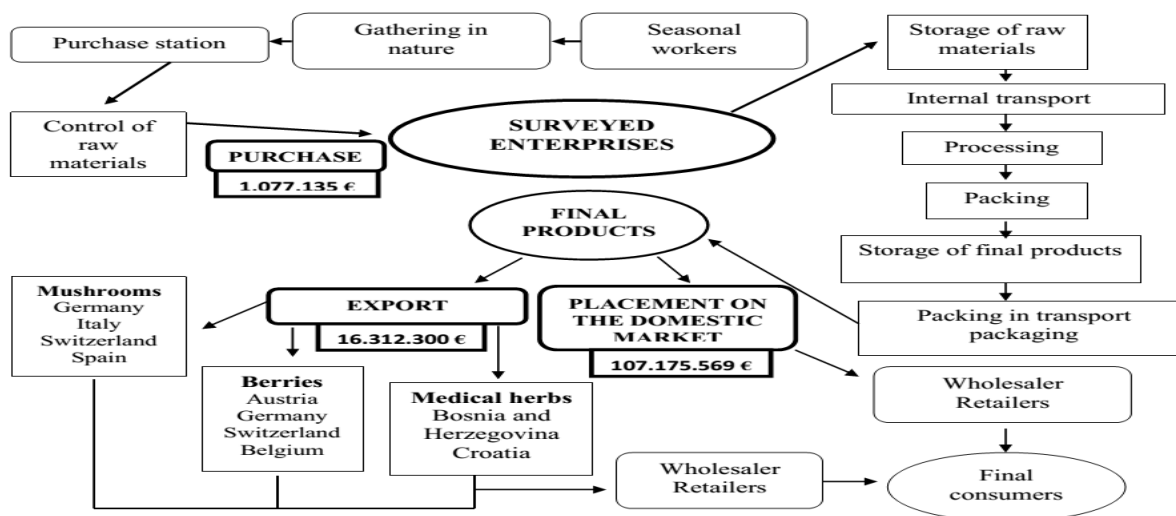
Figure 4. Value chain of NWFPs according to surveyed enterprises

Figure 4 shows the value chain and flow of products from raw materials to the final consumer. The first phase is collecting by the local collectors in the nature who sell raw products to the nearest collection points. The NWFPs sector in Serbia is a "supply driven chain" that is characterized by a horizontal based structure driven by local firms and enterprises which are dependent mainly on private and commercial capital (Keča *et al.* 2013). Enterprises, such as surveyed, purchase products from collection points and further perform their logistical handling within their capacities. These are stages such as storage, internal transportation, referral to treatment, storage of finished products and preparation for transport. Products are implemented, both in the domestic and international market. Distribution is done through retail or wholesale trade facilities in the country and abroad (Figure 4). The main final products sold to domestic and international markets are: fruit spreads, honey, bee glue, pollen, wax, dry extracts, filter teas, mix of medical herbs, spices, snails, reed products (reed plates, pressed reed, etc.). Expenditures that were earmarked by surveyed enterprises for the purchase of raw materials (for the period 2004 - 2010 years) are app. 1,077,135 €. Revenue from domestic sales for the same period is app. 107,175,569 €. By exports approximate revenues of 16,312,300 € are achieved. Many traditional products of the NWFPa, which were once associated with low-income people, are today considered as a natural product or as a medical specialties in the food industry, and represent a significant source of income (Meadley,1989).
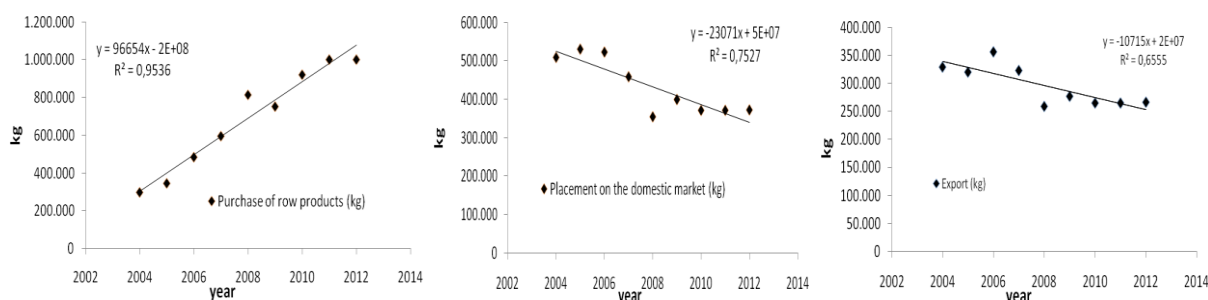
Figure 5, 6 and 7. Trends of purchase, placement on the domestic market and export of NWFPs

Based on the collected data a positive trend in the purchase of raw NWFP is determined (Figure 5). The contrary, a negative trend of placement on the domestic market and exports is determined (Figure 6 and 7). Such a negative trend can be attributed to, among other things, to the bankruptcy of enterprise whose share in the purchase was significant.

Table 1. Basic elements of regression analysis

Purchase of row NWFPs Y = 96654x - 2E+08					
Parameter		**t**		**R** = 0.9765	**F** = 143.88
a = -193390206.9	**b** = 96653.5	**t(a)** = -11.9526	**t(b)** = 11.99529	average exponential growth rate = 15.9%	
Placement on the domestic market Y = -23071x + 5E+07					
Parameter		**t**		**R** = 0.8675	**F** = 21.30
a = 46758160.38	**b** = -23070.8	**t(a)** = 4.658415	**t(b)** = -4.61538	average exponential growth rate = -5.2%	
Export Y = -10715x + 2E+07					
Parameter		**t**		**R** = 0.8096	**F** = 13.31
a = 21811320	**b** = -10715	**t(a)** = 3.69950	**t(b)** = -3.649368	average exponential growth rate = -3.6%	

For purchase a strong correlation is determined (0.976) and the correlation coefficient is statistically significant, as indicated by the error of about 0.0006% (*Significance F*). Parameters are significant (the error level of α = 0.05%) because the absolute value > 2, and the corresponding *P-value* indicates an error about 0.0006%. Exponential growth rate of purchase is 15.91% (Table 1).

Regarding the placement at the domestic market strong correlation is determined (0.867) and the correlation coefficient is statistically significant, as indicated by an error of about 0.2% (*Significance F*). Parameters are significant (the error level of α = 0.05%) because the absolute value > 2, and the corresponding *P-value* indicates an error about 0.2%. Exponential growth rate of placement on the domestic market is -5.2% (Table 1).

By analyzing export a strong correlation is determined (0.810) and the correlation coefficient is statistically significant, as indicated by the error of about 0.82% (*Significance F*). Parameters are significant (the error level of α = 0.05%), because the absolute value > 2, and the corresponding *P-value* indicates an error about 0.7%. Exponential growth rate of export is -3.6% (Table 1).

The general conclusion is that in Vojvodina the most developed market is market of medicinal herbs and honey. This is indicated by dominance of enterprises engaged in the purchase and processing of these products, compared to other enterprises in the field of NWFPs in Vojvodina. In the present study that indicates the quantity of purchased and placed medical herbs and honey to the domestic and foreign markets, as well. Export was not largely represented, and in the period of 2004-2010 significantly less income from exports than from sale in domestic market was achieved. Only in the purchase of raw materials a positive growth rate is achieved, while the placement at the domestic market and the export had a negative growth rate. Options for improving of the NWFPs sector are to close the entire production process, from production, through processing enterprises, to the final product. Additionally, by investing in processing capacities (due to higher product finalization) and intense promotional activity, enterprises could achieve significant economic results.

CONCLUSIONS

The main products of surveyed enterprises are: medicinal plants, reeds, snails, honey and mushrooms. In total purchased quantities dominate medicinal and aromatic herbs. The most common types of herbs are: mint, chamomile, elder, marshmallow ears and nettles. Based on the collected data a positive trend is determined in the purchase of raw NWFPs, and contrary a negative trend of placement on the domestic market and export is determined. Using the regression and correlation analysis in all three cases (purchase, sales in the domestic market and export) a strong relationship between the parameters and statistical significance of the parameters and the correlation coefficient is determined, based on which the results can be trusted. Exponential growth rate of purchase is 15.91%. Exponential growth rate of placement on the domestic market is -5.2% and of export is -3.6%. On the domestic market the most common placement have filter teas, while the foreign market the most common placement have herbs in bulk. The most important export markets are: for berries Austria, Germany, Switzerland and Belgium, for medical and aromatic herbs Bosnia and Herzegovina and Croatia and for mushrooms Germany, Italy, Switzerland and Spain. Average purchase price of certain types of NWFPs are: for berries 0.8 $€·kg^{-1}$, honey 2.07 $€·kg^{-1}$, medical herbs and spices 2.09 $€·kg^{-1}$, mushrooms 2.5 $€·kg^{-1}$, bundles of reed 0.4 and for snails 0.5 $€·kg^{-1}$. Average sales prices for certain types of NWFPs are: for fruit spread 1.38 $€·kg^{-1}$, honey 3.5 $€·kg^{-1}$, medical herbs and spices 13.5 $€·kg^{-1}$, mushrooms 1.5 $€·kg^{-1}$, reed product 5.9 $€·kg^{-1}$, snails 1 $€·kg^{-1}$ and filter teas 3 $€·kg^{-1}$. Expenditures that were earmarked by surveyed enterprises for the purchase of raw materials (for the period 2004-2010 years) are around 1,077,135 €. Revenue from domestic sales for the same period is around 107,175,569 €. By exports it approximate revenues of 16,312.300 € are achieved.

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VALUE CHAIN ANALYSIS OF FOREST MUSHROOMS IN SERBIA

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ABSTRACT

Although non - wood forest products (NWFPs) are traditionally linked to local communities, they are becoming increasingly involved in the international trade. The increasing of global demand has enabled the companies from Serbia, in accordance with its capabilities, more oriented towards exports of final products of mushrooms. The objective of this study was to identify and analyze the production-consumption continuum of mushroom value chains in Serbia. Data were collected using a combination of techniques: literature review, focus group discussions and individual interviews in three major mushroom growing regions in Serbia. In order to gain insight into the developmental tendencies of local companies involved in the purchase, processing and marketing of mushrooms, a survey has been conducted in order to define trends in their placement. The purpose of the research has been to determine trends in the exports of certain types of raw and processed mushrooms in Serbia. The main subjects of the research are the quantities of placed NWFPs at the foreign market. The applied methodology is based on the trend analysis of time series which defined the trends of quantities realized at the foreign markets and enabled the projections of exports in the future.

Keywords: mushrooms, value chain, Serbia, enterprises

INTRODUCTION

Mushrooms grow within different habitats and on different substrates (the surface or medium on which mushrooms grow) in all climate regions. Serbia is known for a rich spectrum and a wide distribution of mushrooms (country is located in a moderate northern zone with rich deciduous and coniferous forests). Large number of plant species, diverse soil types and climate are appropriate for growing of commercially important species such as: *Boletus edulis, Chantherellus cibarius, Craterellus cornucopioddes, Lactarius deliciosus, Marasmius oreades, Tuber aestivum and Tuber magnatum*.

The overall mushroom production and the mushrooms from wild population are subjected only to the necessary treatments, which include drying, freezing, pickling etc. The mushrooms value chain (VC) plays a significant role in the Serbian agribusiness sector. Nearly all products are sold on exports markets and at the same time provide income or additional income for significant number of households in the rural areas of the country. The collection of mushrooms is seasonal, starting in the early spring, and finishing in the late autumn. Only the winter months are not covered with any collection activity.

Estimates of total (seasonal) employment in this VC vary greatly. There is consensus that thousands families are engaged in collection in Serbia-some estimates even go up to 120,000–150,000 individuals across all commercially valuable items, that include other harvested herbs and spices in addition to mushrooms. In 2007, Serbia exported slightly above USD 10 million worth of fresh (chilled) forest mushrooms to the EU 25. The dried forest mushrooms were the second item regarding exported quantities with around USD 8.5 million, followed by preserved forest mushrooms with USD 3.6 million.

The Regulatory framework is a major driving force behind the mushrooms VC. The collection and export of mushrooms is a highly regulated activity. The government establishes a quota for the VC, limiting the total quantity of exports. This quota is allocated among corporate applicants (te Velde *et al.*, 2006) on the basis of their capacity and experience. Mushroom exporters are interested in processing (packaging) of wild mushrooms collected in neighbouring countries to take advantage of Serbia's established markets, encounter other constraints. Mushroom exporters understand opportunities for adding value-such as

branding, retail packaging for niche items (Marčeta and Keča, 2014), introduction of end-user specialty products. But they perceive considerable risk in competing in target markets with their own buyers.

There are 30 companies in Serbia exporting mushroom, but leading 10 companies, account over 80% of exported quantities. While Italy is the biggest importer of fresh and semi processed mushrooms, most of Serbia's exports are sold to bulk importers, who then reprocess them and often, re-export them.

For drying of wild mushrooms there are in general two different processes. The mushrooms drying varies from open (simple, traditional) sun drying, to using of different modern machines for drying of the mushrooms in different phases, with different capacities and with different energy sources used. The dried wild mushrooms are considered as the highest value product at the market and they reach the highest price. Most of the quantities of *Boletus* and *Cantharellus* are sold as dried mushrooms.

MATERIAL AND METHODS

To understand the implications of promoting mushrooms commercialization, it is necessary to understand who and what is involved in the production-to-consumption system (Belcher *et al.* 1998) or VC. Intention of VC is to describe the full range of activities which are required to bring a product or service from conception (Kaplinsky and Morris 2001). There were 46 registered small and medium enterprises (SME's) dealing with mushrooms in Serbia in 2013 (Internal documentation of Ministry, 2013). For this research 30 of them have been interviewed with applying of cognitive interview (Campanelli, 1997), mainly those that are focused more on export of the final products.

It is important to mention that mushrooms pickers in Serbia sell products to the "purchase stations", which are usually located near the SME's (Figure 1) (Keča *et al.*, 2013) or directly sell the products to the companies, which organised several truck collecting lines and immediately pay in cash for product.

The VC will be presented through the diagram of operations in processing operations of mushrooms and growing rate of export of the main mushrooms from each region will be described.
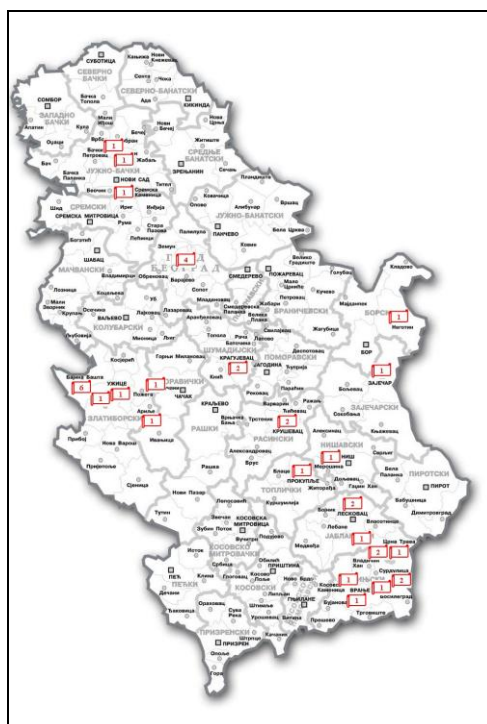
Figure 1. Map - Distribution of interviewed entrepreneurs

RESULTS AND DISCUSSION

Under the Harmonization System (HS) for classifying trade statistics, four groups apply to mushrooms. Within each group, the HS classification distinguishes between "Genus Agaricus"—basically button mushrooms—and other mushrooms and truffles. For our purposes, the first category is of little interest, since it deals with effectively cultivated mushrooms. Thus, the trade statistics, imports into the EU 25, of interest for looking at the market for collected mushrooms refer to the "other than Genus Agaricus". The HS classification includes the following breakdowns: HS 070951—fresh/ chilled mushrooms (chanterelles, flap mushrooms, truffles); HS 071159—mushrooms preserved, not for immediate consumption (for example, brined); HS 071239—mushrooms dried, cut, sliced, etc.; HS 200390—mushrooms, other than Genus Agaricus prepared or preserved.

The major suppliers of fresh mushrooms to the EU 25 include Romania, Russia, and Belarus, in addition to the countries of the former Yugoslavia. For C*hanterelles*, Serbia's price comes out to USD 9.07/kg. For comparison, the corresponding prices for Romania, Russia and Belarus (the top exporters to the EU 25) are USD 7.18, USD 5.77 and USD 5.90 per kg.

Table 1. Main species of exported mushrooms from Serbia in 2013

Species	Fresh (kg)	Dry (kg)	Brined (kg)	Export
Boletus edulis	1,353,200	347,786	180,172	Italy, Germany, Slovenia, Hungary, B&H, Switzerland, Bulgaria, Romania, Cyprus, France, Nederland, Austria, USA.
Cantharellus cibarius	299,500	13,640	37,030	
Craterellus cornucopioides	10,000	36	/	
Lactarius deliciosus	64,400	/	28,600	
Marasmius oreades	2,000	/	/	
Tuber aestivum	40	80	/	
Tuber magnatum	30	/	/	

Main species of exported mushrooms are: *Boletus, Cantharellus, Craterellus, Lactarius and Tuber*. The main export destinations are: Italy, Germany, Slovenia, and Austria.
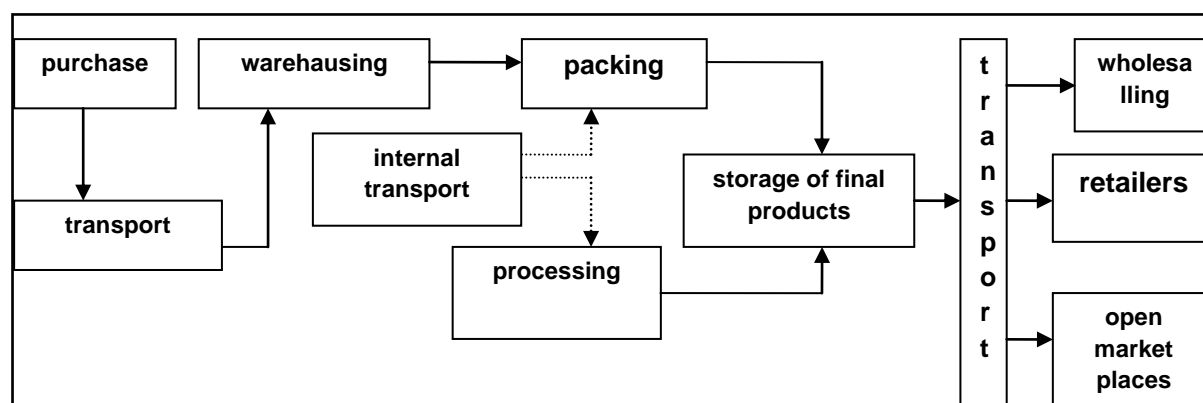
Figure 1. Distribution channel for mushrooms

For successful business in the NWFPs it is crucial that enterprises have high quality physical distribution (stock management, warehousing, transport, and stock control), particularly with mushrooms which are very perishable good (Keča *et. al.*, 2013).

Serbia's forest mushroom VC comprises all economic activities related to collecting, assembling, processing, packaging, and especially exporting mushrooms grown in the wild in Serbia's forests. The VC may also include processing of wild mushrooms collected in other countries, and exporting them to the final markets. Finally, the VC also involves related

economic activities, in particular the production of packaging material and the provision of transport services (Figure 1).

The highest positive growth rates of mushrooms have dry *Boletus* in Belgrade statistical region and dry *Chanterelles* in Šumadija and Western Serbia (Table 2).

Table 2. Growth rate of the main mushrooms species exported from Serbia in the period (2009-2013)

Statistical region	Product	Growth rate
Belgrade	Dry *Boletus*	+16.4
Southt and Eastern Serbia	Fresh *Boletus*	+4.9
Šumadija and Western Serbia	Dry *Chanterelles*	+6.57
Vojvodina	*Agaricus* sp.	+0.44
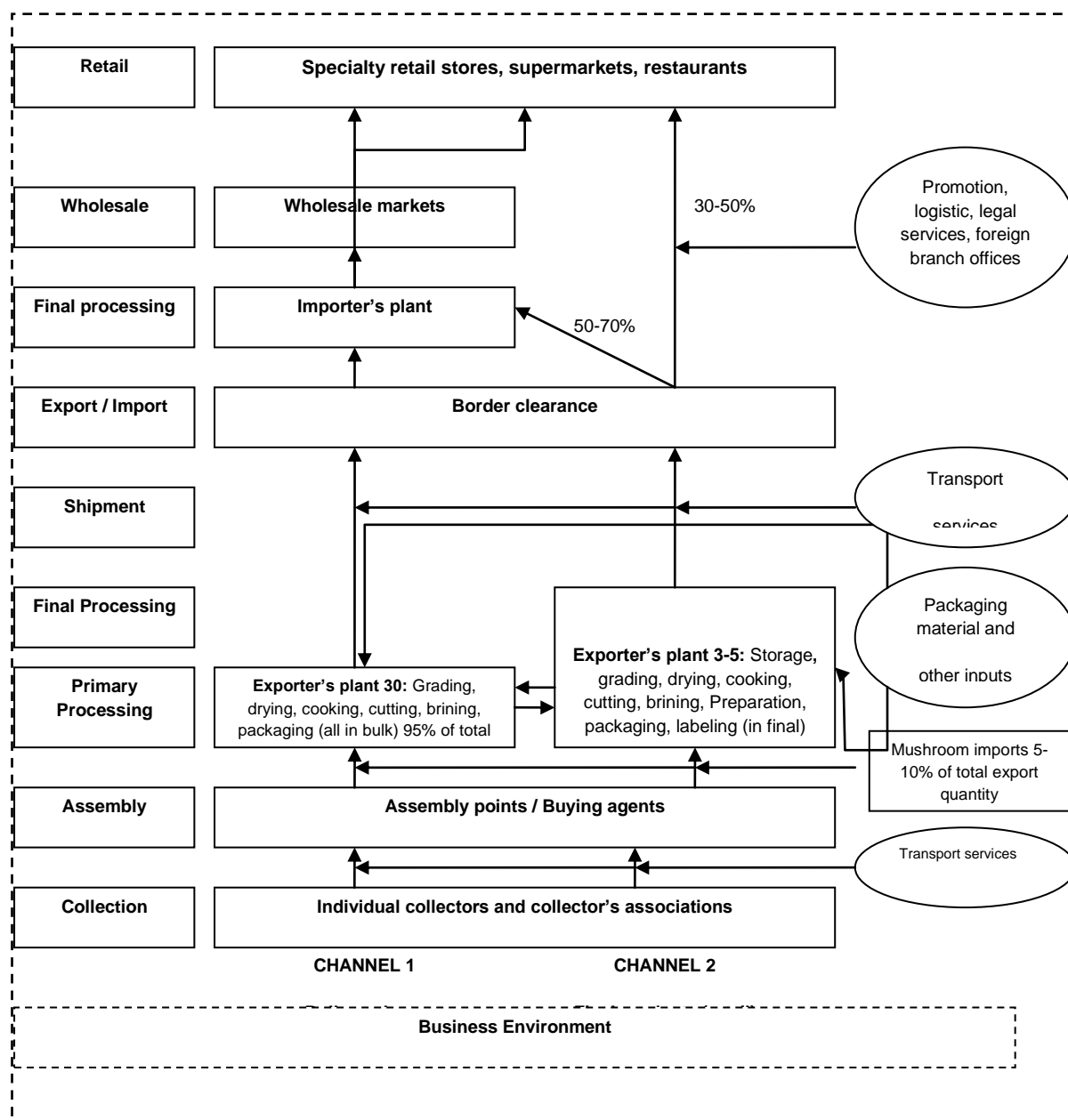
Figure 2. Value chain for forest mushrooms in Serbia

VC analysis starts with collectors (individual collectors or associations). In mushrooms VC there are 2 channels: bulk products and final products (figure 2). The collected mushrooms are placed in assembly points and direct to primary processing. After these phases follows final processing, and distribution to retailers and wholesalers.

CONCLUSIONS

At present, the forest mushroom collecting, processing and marketing is a stable business in Serbia. The collected mushrooms are either exported fresh to the EU (with some going to the US), or semi-processed (cooked and brined, or dried). There are 30 companies in Serbia exporting mushroom, but leading 10 companies, account over 80% of exported quantities. Main species of exported mushrooms are: *Boletus, Cantharellus, Craterellus, Lactarius and Tuber*. The main export destinations are: Italy, Germany, Slovenia, and Austria. The highest positive growth rates of mushrooms have dry *Boletus* in Belgrade statistical region (+16.4) and dry *Chanterelles* in Šumadija and Western Serbia (+6.57). VC analysis starts with collectors (individual collectors or associations). In mushrooms VC there are 2 channels: bulk products and final products (figure 2). The collected mushrooms are placed in assembly points and direct to primary processing. After these phases follows final processing, and distribution to retailers and wholesalers.

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THE INFLUENCE OF WATER HYGIENE IN DAIRY INDUSTRY ON MICROBIOLOGICAL QUALITY OF BUTTER

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ABSTRACT

This paper presents investigation results of the influence of water quality used in dairy industry on the quality and microbiological safety of butter. Determination of physico-chemical composition and microbiological status of water that is used in butter production was analyzed along with the investigation of hygienic production conditions. The aim of this paper was to determine the water quality as well as its suitability in butter production from the hygienic and technological aspect in order to obtain safe products. Microbiological examinations were carried out and interpreted according to current legislation as well as with the elements of self-control prescribed in HACCP plans.

Based on investigation results, it can be concluded that microbiological safety and quality of water used in butter production (areas for cleaning, manufacturing and packaging of butter) meet the criteria set in current legislations. The results of microbiological investigations showed that measured count of aerobic mesophilic bacteria (10 cfu/mL) was lower than prescribed limits. The presence of pathogenic bacteria was not determined in water samples analyzed in this study.

Based on the data on microbiological safety of butter samples in certain phases of churning and production, it is evident that the microbiological quality of the butter produced was satisfactory. The presence of pathogenic bacteria was not found in the analyzed samples, which indicates that the product is safe and the hygiene of the production process is adequate.

During the production process, however, special attention has to be paid to microbiological safety and quality of raw materials, as well as to hygienic and sanitary conditions during the production process (washing and disinfection of equipment, temperature pasteurization regimes, etc.).

Keywords: butter, chemical composition, water, microbiological quality

INTRODUCTION

In most cases, microorganisms that cause the spoiling of milk and dairy products (Magan *et al.*, 2001), to some extent diminish their quality which results in significant economic loss (Randolph, 2006). Diminished quality is the result of different chemical and biochemical bonds which cause the change in the look, smell, texture, taste and the aroma of the products. The appearance of those bonds is conditioned by the metabolic degradation of some components of the product, by the microorganism which cause the spoilage or by their enzymes (Sorhaung and Stepainak, 1997; Walstra *et al.,* 1999).

Although water does not represent the most favorable environment for growth of microorganisms, many species can remain viable for a long time and even multiply. The water used in the food industry must be of drinking water quality as prescribed, according to the Regulations on hygienic quality of drinking water (Off. Gazette of FRY No. 42/1998, hereinafter Regulation 4).

As contaminants in the water may appear different microorganisms from the air, the soil, the plants, the animals and humans. Among the microorganisms from the air dominant are saprophytic microorganisms that contaminate food. From soil it can get into the water, beside the saprophytes forms of pathogens such as *Clostridium tetani* and *C. botulinum*. However, the most significant contaminants of water are of human origin. In drinking water they arrive with feces and urine through the canal water, septic tanks but also through surface water during flooding, large showers and other disasters. With this in mind, it is necessary the

mandatory control of drinking water and water used for washing, cleaning and other operations in the dairy industry (Škrinjar, 2001).

Requirements in relation to the quality of drinking water used in the dairy as fluid, are regulated by national and international standards. In regulation 852 (EC) "Drinking water" means water that meets the minimum requirements introduced by Council Directive 98/83 / EC (amended by Regulation (EC) No 1882/2003, quote Vranjes *et al.*, 2007).

AD Mlekara - Subotica for process of production consumes large amounts of water, and for the supply of process water plant uses water from their own wells, which is then prepared by special technical and technological solutions and processed to the quality that matches purposes.

Upon leaving the springs after the filtration process the water is treated to be disinfected by sodium hypochlorite based on continuous measurement of residual chlorine from the plant, and is distributed in the hydrant, where it is further distributed to the installed new equipment for water treatment, which involves a series of procedures to treat water to make it fully matched standards. During the new technological process water passes through the sand filter process which is used to absorb any impurities from the water. The next stage is the iron removal from crude well water, whereby iron in contact with oxygen enters the ferric form and is separated in the form muddy precipitate of ferric hydroxide. In addition to iron arsenic and manganese are also separated. Thereafter, the water passes through an additional filtering process, through activated carbon. As a final process before the water is stored in the receiving tanks, it is applied disinfection of water flow by the UV lamp. Otherwise, water treatment plant is automated with minimal involvement of human labor.

The safety of butter and its micro flora directly depends on: the count and the kind of microorganisms present in the raw milk, micro flora of the pasteurized sour cream (an intermediate from which butter is produced), hygienic status of the unit it is produced in (churning equipment) etc. Besides the mentioned, internal features of the butter like a_w, pH, content of salt, homogenic distribution of humidity and the size of droplet, additionally affect its microbiological stability.

Butter is produced from sweet cream in a machine for continued production in the dairy. Technically, the construction of the machine is suitable for all the processes which lead to the making of the butter, in a continued closed system (churning, rinsing and kneading). Considering the demanding nature of the raw material and the complexity of the production process, only the continuous control of the production process can guarantee the making of high quality and safe product.

Considering all mentioned above, the purpose of this paper is to determine the influence of the quality of water used in the washing process and the production process, on the quality and safety of butter, as finished product.

MATERIALS AND METHODS

In the manufacturing part of the plant where a business is churning and part of the plant where it butter packaging is performed, sampling of water from the tap is done, which is used in the production process and washing, in order to test its microbiological status in accordance with the Regulations on hygienic quality of drinking water (Off. Gazette of FRY no. 42/1998). Water sampling was done by the specialist and the patterns on the physical-chemical and microbiological tests were done in the laboratories of the Institute of Public Health in Subotica. The survey was done in three replicates. For dairy production Mlekara consumes large amounts of water, and for the supply of process units uses water from their own water wells, which is prepared and processed by special technical and technological solutions to the quality matched purposes.

Microbiological examination, were carried out to assess hygiene in the production process and to assess the making of a safe products. Types of examination and interpretation of the results was performed in accordance with the Regulations of general and special conditions of food hygiene at any stage of production, processing and trade ("Off. Gazette of RS", no. 72/2010) as well as with the elements of self-control prescribed in HACCP plans.

In addition, the following are other microbiological testing relevant for assessing the microbiological safety of butter to a "microbiological control of food" (Škrinjar, 2001), and determining the number of aerobic spore-forming bacteria in 1 ml, determine the number of thermophilic aerobic spore-forming bacteria in 1 ml, determining the total number of yeasts and molds in a 1 ml, the determination of proteolytic bacteria.

The presence of *Enterobacteriaceae* in samples of butter was determined by the EN ISO 21528-1 (2004) method, and the isolation and the identification of *Listeria* spp. by EN ISO 11290-1 (2004) method with the application of the biochemical systems API List (BioMeriex, France) for final identification. Analyses were repeated three times and the test results show the middle average value (x).

RESULTS AND DISCUSSION

Based on the results of laboratory analysis that are displayed in Table 1 and 2, chemical and microbiological quality of water used in the manufacturing part of the plant for washing, manufacturing and packaging of butter meets the requirements of Regulations 4 Regarding the microbiological quality of the water, it is present a small number of aerobic bacteria. However, this number is not significant since it is allowed by the Regulations 4 to the number of microorganisms in the water may range up to 10 in 1 ml.

Table 1 Microbiological and chemical indicators of water quality for the washing, production and packaging of butter

Microorganism	Results[*]
The number of aerobic mesophilic bacteria in 1 ml	5
Sulphate-reducing clostridia in 100 ml	ø
Proteus in 100 ml	ø
Number of coliform bacteria in 100 ml	ø
Origin of fecal coliform bacteria	ø
Pseudomonas aeruginosa u 100 ml / in 100 ml	ø
Faecal streptococci in 100 ml	ø

ø – not determined the presence
[*] Shown results are mean value of three repetitions

In every process of production of butter in the tested water samples showed no presence of pathogenic bacteria, whereby the physical-chemical water quality was correct (Table 2).

Table 2. Physical and chemical indicators of water quality for the washing, production and packaging of butter

Physical and chemical indicators	Results[+]	The reference value
Free residual chlorine, mg/l	0.2	0.5
Turbidity, NTU	0.87	5.0
pH	7.40	6.8-8.5
Ammonia, mg/l	0.83	1
Chlorides, mg/l	6.57	200
Nitrites, mg/l	<0.001	0.03
Nitrates, mg/l	0.20	50
Iron, mg/l	0.13	0.3
Arsenic, mg/l	0.007	0.01
Manganese, mg/l	0.016	0.05

[*] Shown results are mean value of three repetitions

Based on the data about microbiological correctness of samples of butter by certain phases of churning and production, it is clear that the microbiological quality of the butter produced

was correct (Table 3). The presence of pathogenic bacteria in the analyzed samples was not found which indicates that the product is safe and the hygiene of the process is satisfactory.

Table 3: Microbiological indicators of hygiene samples at different stages of churning butter

Microorganisms	Samples[a]			
	1	2	3	4
Proteolytic bacteria in 1 ml	60	0	0	0
Lipolytic bacteria in 1 ml	0	0	0	0
Salmonella spp. in 25 ml	0	0	0	0
Escherichia coli in 0,1 ml	0	0	0	0
Sulphite reducing clostridia in 0,1 ml	0	0	0	0
Listeria spp. in 25 ml	0	0	0	0
Coagulase positive staphylococci in 0,1 ml	0	0	0	0
The total number of *Enterobacteriaceae* in 1 ml	0	0	0	0
yeast in 1 ml	0	0	0	0
molds in 1 ml	0	0	0	0

[a] 1- Butter from the mixer; 2 – Butter from the spiral part; 3 – Butter at the start of regular production; 4 – Butter regular production in the middle;

Water used for washing butter as a source of microorganisms in butter may be of great importance, if the microbiological safety of the water used in the washing process of equipment and technological process is not controlled.

After churning cream, when separated from the buttermilk, butter is rinsed with cold water, so buttermilk, which still remained between lumps of fat, opens and with help of water separates from the butter. This procedure is repeated several times, until it starts to go off completely clean and clear water. Out of the water on this occasion turn into butter most common type of bacteria Pseudomonas, Proteus, coli-aerogenes (Kornacki *et al.*, 2001). Besides these, the contaminating water can enter directly into butter and in addition to present a non-pathogenic form, there could be some pathogen agents of various alimentary contaminations in humans (Kornacki *et al.*, 2001). Otherwise, as it is well known, a milk fat is an essential ingredient of butter. In doing so, the water is finely dispersed in the fat into tiny droplets of micron-sized, which are separated from each other, which makes the butter much less favorable environment for the cultured microorganisms, compared to milk and cream, in which water forms a continuous phase in which are located other ingredients. Water should be dispersed so that the butter looks dry, and the fingerprint section of the water droplets should be so small that they are practically not observed (Stojanovic and Katic, 1998). The capacity and the ability of microorganisms to grow in an emulsion depend in part on the volume of water droplets contained therein. Micro-organisms cannot grow in emulsions with droplet size of less than 10µm, approximately.

According to the data of some authors (Delamarre and Batt, 1999; Kornacki *et al.*, 2001; ICMSF, 2005) somewhere between 10 to 18 billion droplets of water is dispersed in 1 g of emulsion water in oil in a product such as the butter. Given the high level of microbes which is expected in pasteurized sweet cream which serves as the starting material for the production of butter (less than 20,000 cfu/mL) (Jay, 2000), most of the water droplets therein is sterile. This greatly depends on the droplet size and the degree of their dispersion.

Butter being as an emulsion "water in oil", due to the different stages in the process of production results in having different microstructures (Van Dalen, 2002). An example of the microstructure of butter seen by the technique called confocal microscopy is shown in Figure 1. When the water content of the butter is distributed in small water droplets which are well dispersed throughout the product, formation and movement of cultured microorganisms is limited and they are dying during storage (Delamarre and Batt, 1999; ICMSF, 2005).

Well-made butter contains the vast majority of small uniform droplet. In the production of butter the goal is that these droplets are sized 3-4µm. However, in most cases, the majority of the droplet size is between 3 and 10µm. The cells of L. monocytogenes are typically the size of 2µm in length and 0.5µm in width. Therefore, if the water droplets in a well-made

butter are contaminated with Listeria monocytogenes cells, they will be confined in space and available nutrients (ICMSF, 2005). Not well made butter (or butter made worse due to lack of the control) has a broader, larger and more heterogeneous distributed water droplet. The larger water droplets (> 10μm in width) will be able to provide easier growth of L. monocytogenes and other microorganisms that have that size (Voysey *et al.*, 2007).
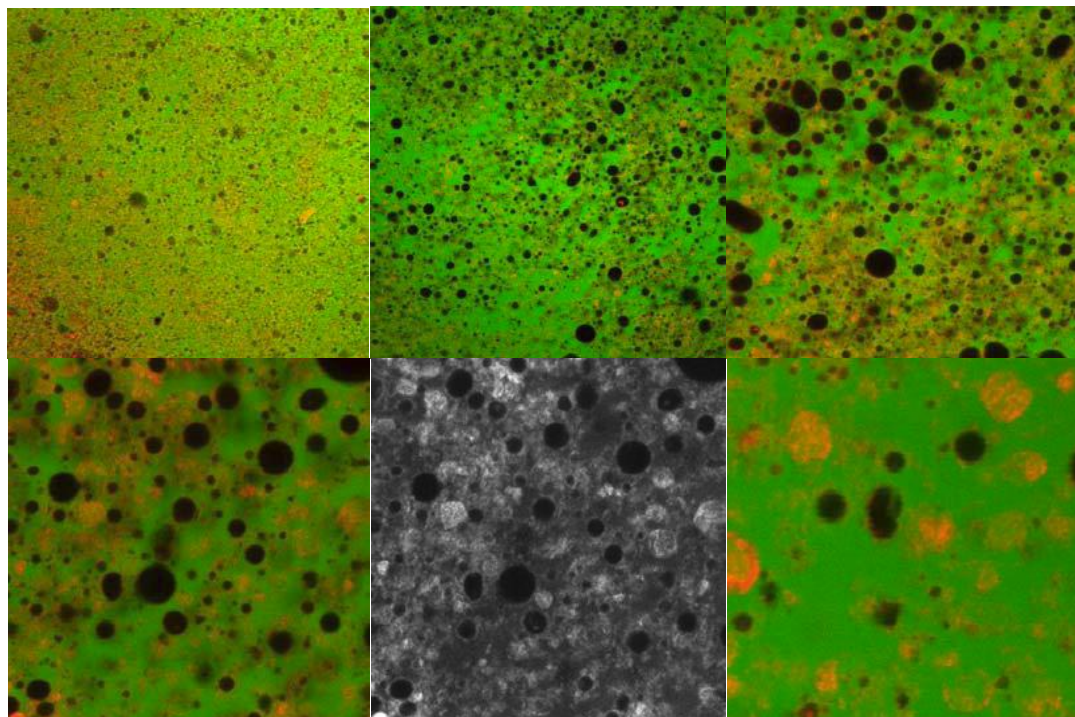
Black area = water droplets; Green zone = fat; red / orange zone = proteins.

Figure 1. Microstructure of butter (Voysey et al., 2007)

Bearing in mind that the butter is commonly used for food or as a food supplement without prior thermal treatment or any other means of destroying microorganisms, we must be careful regarding hygiene and microflora of water for washing butter. With this in mind, it is necessary to have a mandatory regular control of water used in food processing plants.

CONCLUSIONS

Based on the results obtained during these tests, which relate to the determination of the influence of the quality of water used in the washing process as well as the production process on the quality and microbiological safety of butter, following conclusions could be drawn:
 - Chemical and microbiological quality of water used in the manufacturing part of the facility for washing, manufacturing and packaging of butter on the basis of laboratory tests of individual parameters match the requirements of the Regulations 4.
- The results of microbiological samples of butter at certain stages of churning and production indicate that the microbiological quality of the produced butter was at an high level. In the analyzed samples of butter there were not detected pathogenic bacteria.
 - Based on the results of these studies it can be concluded, since the butter is food article, which is very perishable, the beginning of which primarily occurs during the process of production, there is a need to pay special attention to quality of starting materials, hygienic-sanitary conditions in processes of cleaning and disinfection of equipment, as well as the pasteurization temperature conditions to obtain high quality and microbiologically safe product must be met.

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DEGRANULATION OF SKIN MAST CELLS IN JUVENILE RATS CAUSED BY DIETARY ACRYLAMIDE

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ABSTRACT

Acrylamide (AA) is a novel substance discovered in food, processed primarily by frying and grilling. Since food commodities containing AA, from coffee and potato chips to baby food, are widely and extensively used by the human population, we aimed to investigate AA effect on skin, the largest organ in the body focusing on one of its immune cells, the mast cells. In order to assess the possible impact of AA, the histological estimation and stereological quantification methods were used. The 23 day old male Wistar rats were given AA *per os* in a dose of 25 mg/kg body weight, 5 days a week, followed by a two day pause, for three weeks. After the experiment had finished, the hair was carefully removed from the region of interest, and the skin samples dissected out. The samples were, thereafter, subjected to standard procedure for paraffin embedding, sectioned into 5 μm thin sections and stained with toluidine blue. In the upper dermis of the skin, predominantly degranulated mast cells were found, as indicated by morphological criteria. However, neither of the stereologically investigated parameters: the volume and the numerical densities of the total, intact and degranulated mast cells were significantly different between the control group and the treated group. Since the skin has an abundance of different immune cells and nerve endings producing biologically relevant mediators for immune reactions, the degranulation of mast cells i.e. releasing their mediators has undoubtedly the important role in these reactions, including under the AA influence.
Keywords: *acrylamide, skin, mast cells, degranulation, stereology*

INTRODUCTION

Acrylamide is formed during heating process of carbohydrate-rich food at temperatures above 120°C. The mechanism is known as the Millard reaction, identified as a reaction between reducing sugars and free amino acids at high temperatures (Mottram *et al.*, 2002; Stadler *et al.*, 2002). The major sources of acrylamide are potato chips, fried potato, bread, pastry, sweet biscuits, coffee, etc. (Friedman, 2003).

Upon ingestion, acrylamide is absorbed rapidly by the digestive system and further metabolized in liver. It is predominantly conjugated with reduced glutathione or, alternatively, it is oxidized to its metabolite glycidamide. Acrylamide is excreted via urine (Fuhr *et al.*, 2006), but it was found in milk in humans too (Sorgel *et al.*, 2002).

Taking all of the above-mentioned data, the scientific community has launched a number of studies in order to investigate whether the food-borne acrylamide or its epoxy metabolite glycidamide could produce biologically detectable effect. The first documented effect of acrylamide is neurotoxicity in laboratory animals, as evidenced by a progressive loss of nerve function and cell death (Ko *et al.*, 2002). Decrease in a set of hormones, including free and total testosterone, triiodotironine, tiroxin and corticosterone, was found in rats orally treated with acrylamide in 5, 10, or 15 mg/kg bw (body weight) dose continuously for 8 weeks (Hamdy *et al.*, 2012). *Per os* administration of acrylamide in a 50 or 100 mg/kg bw dose for 21 days to male rats showed muscle weakness, further, degenerative alterations of the liver parenchyma as the major histopathologic finding compared to the control group, and the decreased total proteins in a peripheral blood, but also the down regulation of iRNA for CYP2E1 (El-Bohi *et al.*, 2011). Study on apoptotic proteins in the central nervous system in rats receiving either 20 or 40 mg of acrylamide/kg bw three days a week during eight weeks,

the expression of caspase-3 increased in the spinal cord, while it was not affected in the cerebral cortex (Li *et al.*, 2006). The authors of the study related these findings with the acrylamide-induced degeneration of peripheral axons (Li *et al.*, 2006).

Further study of acrylamide biological effects pointed to its ability to cause DNA damage (Nixon *et al.*, 2012), cell proliferation (Camacho *et al.*, 2012), carcinogenicity to various organs (skin, thyroid, ovary, mammary) (Beland *et al.*, 2013), expression of embyonic gene through paternal exposure (Brevik *et al.*, 2011), etc. Studies have demonstrated that the effects concerning the mechanism of acrylamide genotoxicity is caused by its conversion to glycidamide, which bounds to DNA (Xu *et al.*, 2014).

The aim of this study was to investigate the sensitivity of skin mast cells in young rats administered with acrylamide *per os* for three weeks. Mast cells were chosen as an object of study because of their well-known importance as immune cells in the skin general defense system.

MATERIAL AND METHODS

The experiment was carried out on male Wistar rats aged 23 postnatal days at the beginning of the study. They were kept under constant laboratory conditions with $22\pm2°C$ temperature, 12 hours day with 12 hours light cycle and had access to food and water *ad libitum*. Acrylamide (99% purity, Sigma Chemicals Co., St. Louis, MO, USA) was dissolved in distilled water and administered *per os* at the dose of 25 mg/kg bw five days a week for three weeks (n=5). The control group (n=5) received the distilled water only. The study was approved by the Ethical Committee on Animal Experiments of the University of Novi Sad (No I-2011-03).

After the experiment had finished and the animals decapitated, all the organs of interest were harvested, including the skin. From a small area of skin in the interscapular region the hair was carefully removed and approximately 3x3 mm of skin dissected out. It was, thereafter, subjected to standard procedure for paraffin embedding, cut into 5 μm thin sections and stained with toluidine blue. This histological dye enables the staining and the visualization of mast cells among plethora of connective tissue cells in the skin.

The special interest of our investigation was the cutaneous upper dermis, because it's the site of the most intense immune-immune and immune-nerve interactions during the effect of ingested xenobiotic, but it is also extensively exposed to the external environment for the possible contact with these substances. Hence, the upper dermis was histologically evaluated and stereologically analyzed. Stereology was chosen as a quantification approach and the mast cells were analyzed using the M42 grid on 50 consecutive fields of vision per animal under the total magnification of the light microscope of x400. For the purpose of quantification analysis, mast cells were divided into two groups, based on morphological criteria: 1) intact mast cells, characterized by oval to round cell shape, deep violet-blue color and often with masked nucleus; and 2) degranulated cells, recognized by a wide variety of cell shape and size with week staining and often demasked nucleus. Regarding stereological analysis, the volume and numerical density of total, intact and degranulated cells were determined. The differences between the control and the acrylamide-treated group for each of the stereological parameter tested were determined with the Mann-Whitney test with the *p* value set at 0.05.

RESULTS AND DISCUSSION

Histological analysis of the control skin sections and the sections of skin obtained from the treated rats clearly demonstrate differences between the two groups. The upper dermis of the control rats was populated with predominantly intact mast cells with characteristic cell morphology and intense staining (Figure 1). Conversely, the same cutaneous area in the treated rats was occupied with altered cell morphology and prominently decreased staining of the mast cells, both indicators of cell degranulation (Figure 1).
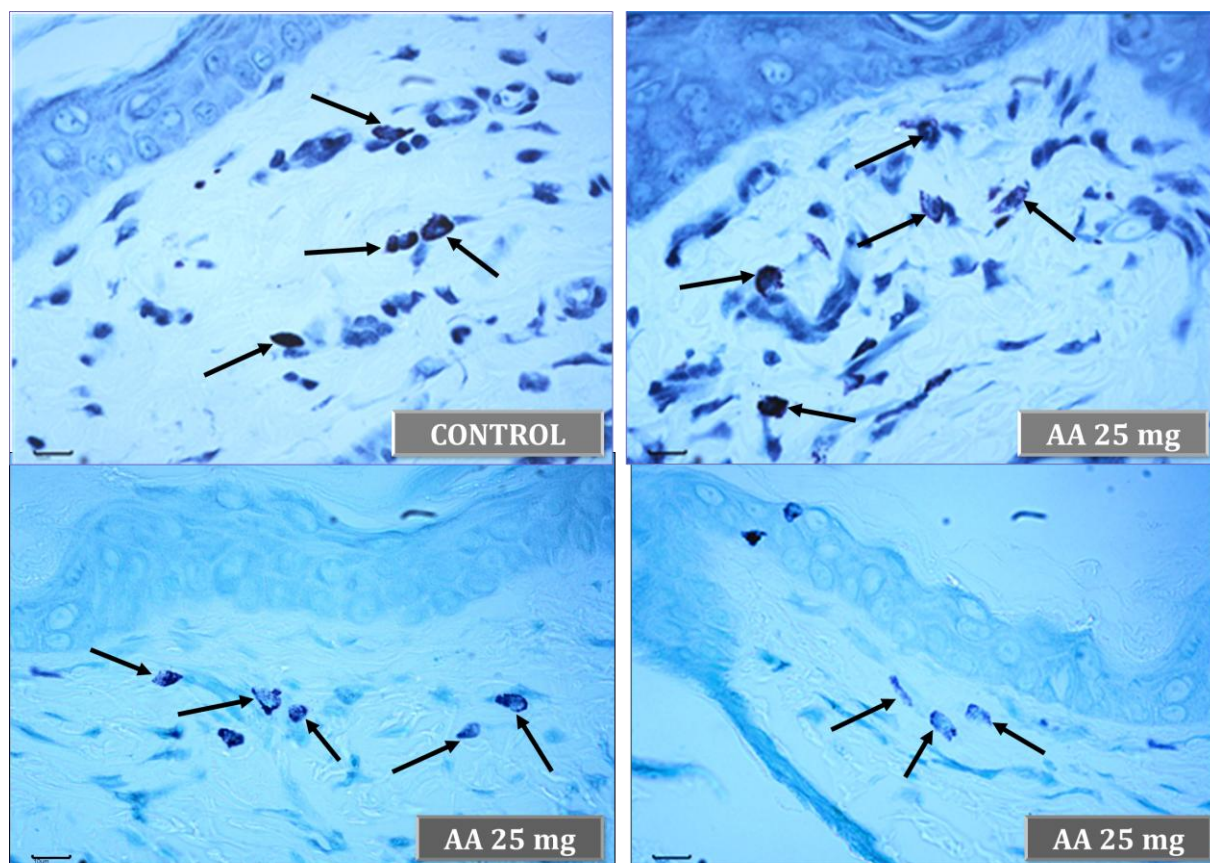
Figure 1. Photomicrographs of the upper dermis in skin sections stained with toluidine blue in the control group and the group exposed to 25 mg/kg bw of acrylamide. Scale bars indicate 10 μm. The deep blue stained cells indicated by an arrow in the upper two photomicrographs represent intact mast cells, while all the cells labeled by an arrow in the two lower photomicrographs indicate the degranulated cells. Note the difference in the intensity of staining of the cells in the upper part of the photomicrograph plate and the lower one, as well as the altered morphology of the degranulated cells due to the release of the mediators stored in the cell cytoplasm.

Quantification analysis, performed using the stereological methods, included six measured parameters which enabled a detailed insight into the morphological status of the investigated mast cells in the control group and the group exposed to dietary acrylamide. The stereological findings pointed to the increase in numerical density of total and degranulated mast cells, while the intact mast cells decreased (Table 1). All three parameters related to the volume densities of mast cells showed no marked alterations among groups (Table 1). The statistical analysis demonstrated that all stereological parameters tested were statistically insignificant (p>0.05) between the control group and the group receiving acrylamide in a 25 mg/kg bw dose. However, it is noteworthy that the numerical density of degranulated mast cells in the latter group increased by 65% compared to the control, although this difference did not reach statistical significance. Despite of such results of the statistical analysis, the severe degranulation of a number of cutaneous mast cells seen in acrylamide-treated group has its biological importance in the light of interaction between mast cells and other cutaneous immune cells as well as the interaction between mast cells and nerve endings in the skin.

Table 1. Median values with the lower and upper quartiles (in brackets) of all investigated stereological parameters for mast cells in the skin of control and acrylamide-exposed animals are given. Vv represent the volume density and Nv the numerical density of: total mast cells (Vvm, Nvm), intact cells (Vvm$_{INT}$, Nvm$_{INT}$) and degranulated cells (Vvm$_{DEG}$, Nvm$_{DEG}$)

Stereological parameter	CONTROL	ACRYLAMIDE 25 mg/kg bw	*p* value
Nvm (mm^{-3})	16213 (16156) (18012)	19493 (13713) (20518)	0.35
Nvm$_{INT}$ (mm^{-3})	3475 (3284) (7616)	3900 (3060) (4271)	0.60
Nvm$_{DEG}$ (mm^{-3})	9027 (8980) (14191)	14872 (10680) (17935)	0.12
Vvm (mm^{0})	0.0043 (0.0033) (0.0043)	0.0057 (0.0033) (0.0062)	0.34
Vvm$_{INT}$ (mm^{0})	0.0019 (0.0014) (0.0019)	0.0009 (0.0009) (0.0024)	0.34
Vvm$_{DEG}$ (mm^{0})	0.0024 (0.0024) (0.0029)	0.0038 (0.0024) (0.0048)	0.40

A possible mechanism of such response of mast cells with a widespread degranulation in upper dermis of the skin could involve an indirect effect of acrylamide on mast cells through mediators derived from cutaneous nerve endings. Mast cells frequently reside adjacent to nerve endings, the later abundantly identified in the skin. Subsequently, one of the health outcome would involve acrylamide-induced neuropathy. As stated above, this condition is an obsolete finding in laboratory animal experimentation, with a novel data now of a possibility of mast cells involved in this pathology, requiring necessary continuation of a study by histological and molecular biology methods in order to elaborate this hypothesis.

CONCLUSIONS

Acrylamide was able to produce alterations in cutaneous mast cells in young rats detectable by histological estimation. This finding might have health implications particularly if a certain pathology of the skin already exists, but also in pathologies of other organs to which mast cells could add to a certain extent, implied yet to be investigated.

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DETERMINATION OF VIABLE BIOFILM CELLS IN MICROTITER PLATES

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ABSTRACT

The ability of bacteria to attach to the food contact surface and to form biofilm is one of the important hazards in production of safe food. Given the tremendous clinical importance of biofilms, it is somewhat surprising that there is no standard method for investigating the cells in bacterial biofilms. For bacteria, a common method is to quantitate the mass of biofilms by crystal violet or safranin staining, followed by extraction of bound dye with a solvent and measurement of absorption. Simple and rapid methods which provide information about viability are required for an effective control and operation of the biofilm systems. Tetrazolium salts have become some of the most widely used tools in cell biology for measuring the metabolic activity of microorganisms and depend on the reduction produces of dye in living cells, that can be used for quantitative redox assays. The goal was to optimize several spectrophotometric methods to broaden their applicability for biofilm quantification. We tested tetrazolium dyes MTT, XTT, TTC and INT as indicators of cell metabolism and their application in biofilm quantification. We included also a microdilution method based on ATP bioluminescence measurement and resazurin fluorescence measurement which were previously found to be useful, rapid techniques for determining antibacterial efficiency. Assays were optimised and compared as quantitative methods of measuring bacterial viable cells on polystyrene surface.
Keywords: biofilm quantification, *viable cells, Listeria monocytogenes, metabolic activity*

INTRODUCTION

The ability of bacteria to attach to the food contact surface and form biofilm is one of the important hazards in production of safe food (Cappitelli and Villa, 2014). Several methods are available to detect adhesion properties of pathogens. They are based on different principles. The results are influenced not only by each bacterial adhesion properties, or anti-adhesion activity of bioactive agents, but also by the method used (Peeters *et al.,* 2008; Van den Driessche *et al.,* 2014). Polemics remains if the staining of live cells, dead cells and matrix in crystal violet (CV) staining gives misleading information (Skogman *et al.* 2012). However, by including new methodologies we could overcome CV staining method limitations which appear when injured bacteria or viable but non-culturable (VBNC) bacteria are present (Katsikogianni and Missirlis, 2004). This could be the case also in *Listeria* (Besnard *et al.*, 2000).

In this study we aim to optimize several cell viability indicators using spectrophotometric methods to broaden their applicability for adhesion and biofilm quantification. First, we tested tetrazolium dyes (MTT, XTT, TTC and INT), ATP bioluminescence and resazurin determinations as indicators of cell metabolism and their application in biofilm quantification. For each of possible metabolism indicator we evaluated the influence of dye concentration, incubation time and wavelengths used for spectrophotometric measurement. Finally, assays were compared in testing the inhibition of viable biofilm cells on polystyrene after tetracycline addition.

MATERIAL AND METHODS

Bacterial strain: *Listeria monocytogenes ŽM*58 (Institute for Hygiene and Microbiology, Wuerzburg, Germany) was prepared as presented on Figure 1.
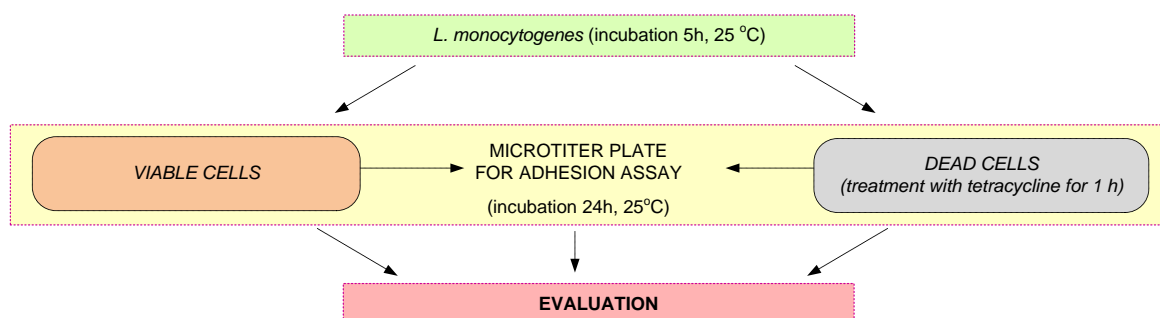
Figure 1. Preparation of L. monocytogenes viable and dead cells for evaluation on microtiter plate adhesion assay

Reagents:

- *Tetracycline* (Sigma-Aldrich, Steinheim, Germany) was diluted in NaOH / H_2O to obtain 5.125 mg/mL.
- *Tetrazolium dyes* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich); XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma-Aldrich); TTC (2,3,5-triphenyl-2H-tetrazolium chloride; Sigma-Aldrich); and INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium; Sigma-Aldrich) were diluted in TSB (Triptone Soy Broth; Oxoid, Hampshire, UK) to obtain appropriate concentrations.
- BacTiter-Glo^{TM} (Promega Corp., Madison, WI) commercial reagent.
- Resazurin (Promega) was diluted in TSB / H_2O, added to menadion (Sigma-Aldrich) to obtain 0.025 mg/mL.

Adhesion assay was performed in flat-bottomed polystyrene 96-well microtitre plates (Nunc, Denmark) after inoculation with 200 µL of bacterial inoculum (10^6 CFU/mL) that was previously treated or non/treated with tetracycline for 1 h (Figure1), and incubated at 25 °C for 24 h. We removed the supernatants that contained non-adhered cells from the attached cells and rinsed the plates 3 times with sterile ringer solution. Further we used different procedures according to dye used.

- *Tetrazolium dyes*: After washing we added 200 µL of tetrazolium dye in TSB (testing different concentrations), put the plate on sonificaton (room temperature, 5 min; 28 kHz; 300 W; Iskra Pio, Šentjernej, Slovenia), incubated (testing different time of incubation) and measured absorbance (at different wavelength for specific dye) (Figure 2).
- *ATP bioluminescence measurement*: After washing, we added BacTiter-Glo (testing different concentrations) (Figure 2), put on sonificaton (as described before), incubated for 5 min and measured bioluminescence.
- *Resazurin fluorescence measurement*: After washing we added resazurin (testing different concentrations), put on sonificaton (as described before), incubated (testing different time of incubation) (Figure 2) and measured fluorescence (560_{Ex} / 595_{Em}).

All experiments were independently repeated three times in 10-12 parallel samples and the mean values as well as the standard deviations were calculated.

RESULTS AND DISCUSSION

To select the best conditions for evaluating viable biofilm cells in microtiter plate we tested several metabolic indicators in assays using tetrazolium salts (Berridge *et al.,* 2005; Burton *et al.,* 2007), ATP determination and bioluminescence measurement or resazurin (Van den

Driessche *et al.,* 2014) and fluorescence measuring (Figure 2) which are known as rapid procedures for determining antibacterial efficiency (Klančnik *et al.,* 2010). In parallel to all experiments the number of *L. monocytogenes* strains was determined with plate count method (results not showed) in order to check the performance of metabolic indicators.

Figure 2. Flow chart of evaluated parameters in different procedures for quantification of viable biofilm cells

Tetrazolium salts

Tetrazolium salts have previously been used in the microdilution method to improve the detection of bacterial growth and determination of antimicrobial efficiency (Klančnik *et al.,* 2010). Reduction results in an easily identified colour change occurring in viable populations of aerobic bacteria, since they indicate the respiratory activity. Reduction occurs due to its function as an artificial terminal electron acceptor in respiration (Berridge *et al.,* 2005).
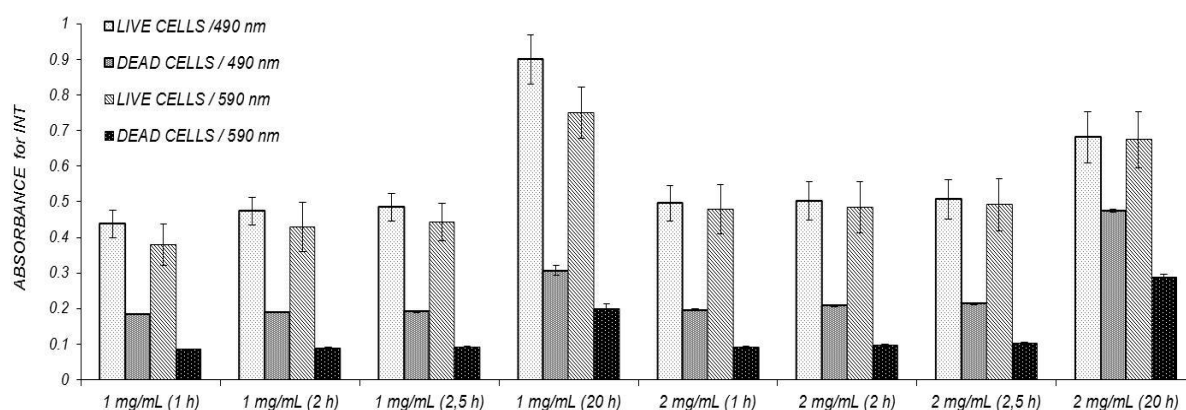
Figure 3. Evaluation of biofilm using INT dye. Data are means (n=36) ± standard deviation

Incubation of INT for 20 h and measurement of absorbance at 490 nm and 590 nm are less appropriate conditions for evaluation of using INT. Other protocols gave comparable results and must be further analysed (Figure 3).
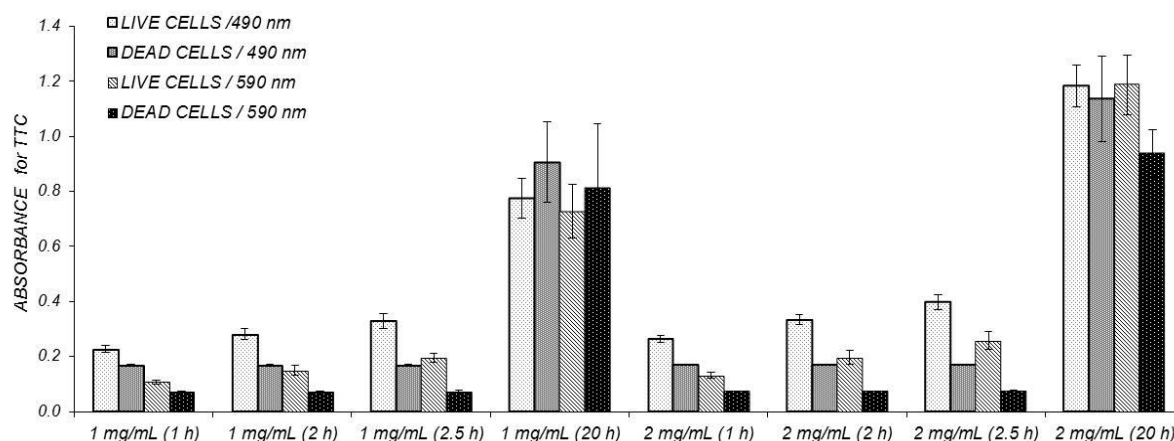
Figure 4. Evaluation of biofilm using TTC dye. Data are means (n=36) ± standard deviation

As visible from low absorbance values of viable cells, the TTC as indicator for metabolic activity is not suitable for evaluation of viable biofilm cells (Figure 4). This pattern was visible in all range of different conditions; therefore data obtained with TTC dye were not used in further analysis, since this assay did not allow differentiation between living and dead cells. Similar results were visible for measurements of absorbance at 490 nm and 590 nm (Figure 4).
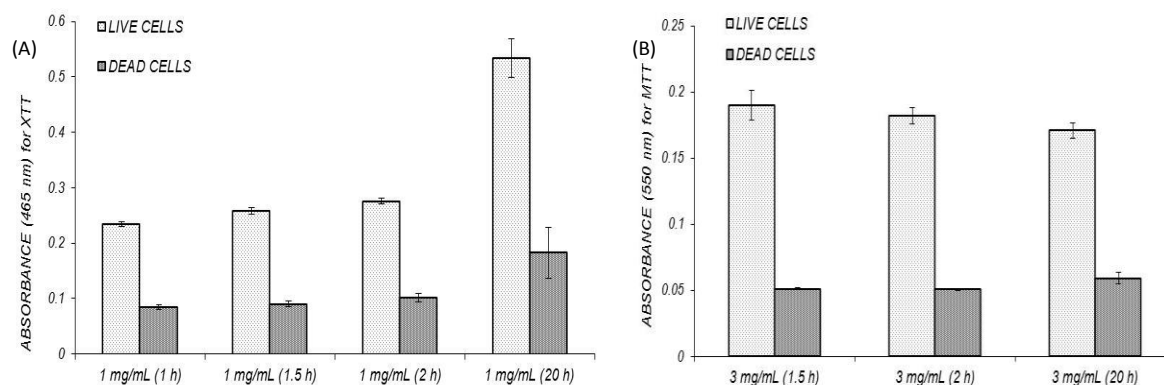
Figure 5. Evaluation of biofilm using XTT dye (A) and MTT dye (B). Data are means (n=36) ± standard deviation

Considering comparable absolute values of viable / dead biofilm cells in the range of 1 h to 2 h of incubation, based on evaluation with XTT, we eliminated long incubation (20 h) (Figure 5A) for further analysis of *L. monocytogenes* inhibition. Again, we have confirmed similar differentiation between viable and dead cells in the first 2 h of incubation with MTT as metabolic indicator (Figure 5B).

ATP bioluminescence measurement and resazurin fluorescence measurement

A lower tested concentration of reagent in ATP *bioluminescence measurement* was already sufficient to differentiate between live and dead biofilm cells adhered to polystyrene surface (Figure 6A). Regardless to the concentration used, the time incubation of biofilm with resazurin can influence the evaluation of viable cells in biofilm cells (Figure 6B).
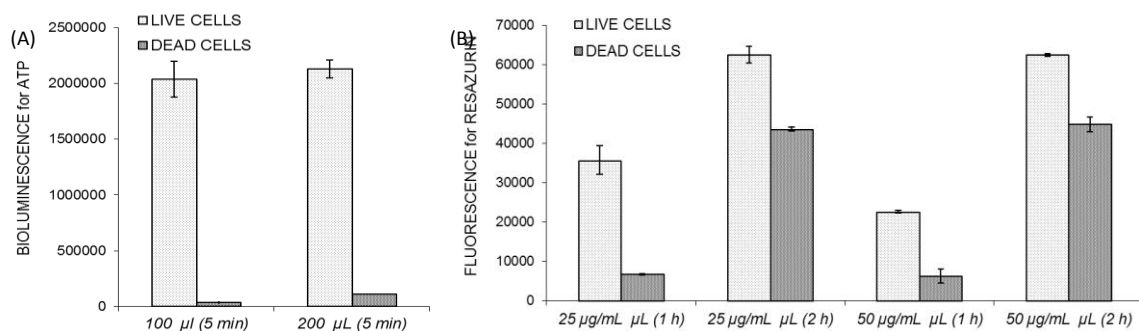
Figure 6. Evaluation of biofilm using ATP (A) and resazurin (B) measurement. Data are means (n=36) ± standard deviation

Comparison between viability indicators

To compare the procedures for each viability indicator, we calculated accuracy of inhibition measurement of *L. monocytogenes* biofilm cells (I) after treating the cells with tetracycline:

$$I = [(C - T) / C] \times 100 \quad [\%]$$

where C is the control cell concentration of *L. monocytogenes*, and T is the cell concentration of *L. monocytogenes* treated with tetracycline. Using inhibition (%) range we selected the most appropriate condition for each indicator of cell metabolism and thus we optimised biofilm quantification (data not shown). We further compared the inhibition in the assays using tetrazolim dyes, ATP and resazurin measurements for biofilm quantification on polystyrene surface. The evaluation of viable biofilm cells gave the best accuracy of inhibition measurement when ATP metabolic indicator was measured (Figure 7).
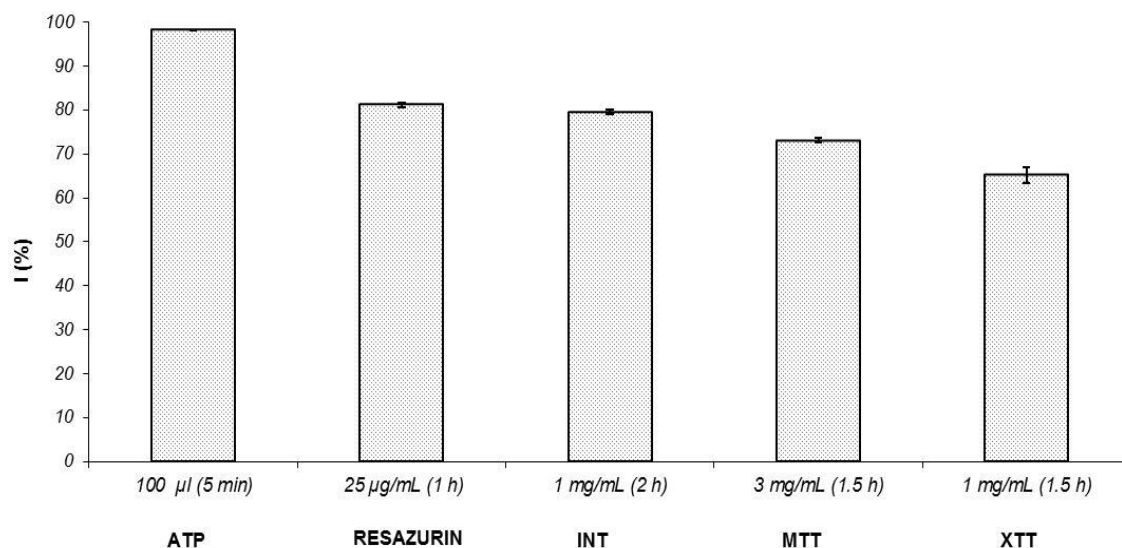
Figure 7. Comparison of the accuracy of inhibition measurement of L. monocytogenes biofilm cells [I (%)] evaluated by optimal procedure for each viability indicator. Data are means (n=36) ± standard deviation

When resazurin was used as a viability indicator it also gave results comparable to the best tetrazolium dye INT (Figure 7). Peeters *et al.* (2008) compared quantification of viable biofilm cells in a microtiter plate using CV, Syto9, the fluorescein diacetate (FDA), resazurin, XTT and dimethyl methylene blue (DMMB).Their results showed that CV and Syto9 could not differentiate between live and dead cells, and among others resazurin gave the most

repeatable results, with the procedure that was fast and suitable for high-throughput quantification of adhered cells.

CONCLUSIONS

The results suggest that ATP bioluminescence measurement offers the most suitable procedure for quantification of live biofilm cells. Unfortunately, an expensive commercial reagent is needed, but anti-adhesion test procedures should ideally be simple, rapid and cheap to maximize sample throughput, for example in screening of a larger number of anti-adhesion agents. For this we suggest a broad evaluation of the viable biofilm cells using INT tetrazolium dye for aerobic bacteria or evaluation using resazurin, which can be used for all bacteria, and then detailed quantification with ATP as viability indicator.

In this study, we have presented an optimised assay for control of pathogens by proper quantification of their adhesion as one of the first steps of biofilm formation. However, in testing anti-adhesion efficiency of different agents, like plant extracts, the assay should be further optimised.

ACKNOWLEDGEMENTS

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AN APPLICATION OF PORTABLE NIR INSTRUMENTS IN QUALITY CONTROL OF MILK

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ABSTRACT

Milk provides essential nutrients of great nutritional relevance for humans, particularly during childhood and elderly age. Its composition is not uniform and it is very important to know it in real time. This study seeks to test the potential of a novel, hand-held near infrared reflectance spectroscopy (NIRS) instrument based on MEMS technology (Micro-Electronical Mechanical Systems) for its application in the milk analysis as a fast, non-destructive and low-cost sensor for real time decision at farm level. The aim of this work was to setup and to assess the MEMS-NIRS implementation for *in-situ* physico-chemical analysis of milk.

Milk samples were scanned in diffuse reflectance and trans-reflectance modes over the NIR spectral range (1600–2400nm) using a hand-held instrument MEMS Phazir, with a window sampling area of 4 mm diameter and a liquid adapter employing a cuvette of 1 mm pathlength. The first step was the establishment of relevant specifications for collection of high milk quality spectra, including evaluation of sampling to build spectral libraries of milk for further development of NIRS chemometrics models for chemical characterization and labelling. The use of liquid adapter for raw milk showed signal saturation in the water absorption bands between 1900-2050 nm. The utilization of a 17 mm pathlength cuvette in static analysis mode, showed higher resolution spectra, similar to those obtained with *at-lab* NIRS instruments. The performance results showed variability of replicated measurements related with scattering effects of milk samples. The repeatability and reproducibility for both cuvettes in terms of root mean squared corrected for bias (RMSC) were better for 17 mm sample thickness. The results suggest that good performances on real samples obtaining high quality raw milk NIR spectra using a portable NIR spectrophotometer are useful for further *in-situ* NIR quantitative analysis, being of great economic importance for dairy farm management.

Keywords: NIR, hand-held instrument, milk analysis, in-situ analysis

INTRODUCTION

Milk and dairy products are a versatile source of nutrients in human diet and caught great interest due to its relevance, since it is widely consumed from childhood to elderly. Milk is an excellent source of macronutrients (proteins, lipids and carbohydrates) and micronutrients (vitamins, enzymes and minerals). Milk composition and its variation during lactation of cows can indicate imbalances in health or nutrition. Particularly changes of fat, protein, milk urea nitrogen content and concentration of ketone bodies provide suitable information on energy, protein and crude fibre supply and on metabolic imbalances in dairy cows. The instantaneous knowledge of dairy milk composition changes can be used both as an indicator of animal health and metabolic changes, and as additional information for dairy producers (Melfsen *et al.*, 2012). Due to these reasons a rapid and reliable measurement of composition underpins the value of milk.

An alternative for milk analysis can be the development of methodologies based on near-infrared spectroscopy (NIRS), because, there are numerous works applying *at-lab* NIRS technology to milk analysis (Holroyd, 2013). However, the possibility of analyzing milk during daily milking routine could prevent further complications (Melfsen *et al.*, 2012). The rapid measurement of milk quality during milking is an important element of the dairy farm and different alternatives have been investigated prior to the establishment of milk quality controls

on-farm. Saranwong *et al.,* (2008) developed a compact NIRS instrument composed of a diode array spectrophotometer and interactance mode, by adapting measuring head to perform transmission experiments on milk samples at 40ºC across 400-1100 nm and 600-1000 nm respectively. The results for fat, protein and total solids were satisfactory. More recently Aernouts *et al.* (2011) evaluated the potential of *on-line* NIR prediction of fat, crude protein and lactose during milking. These authors evaluated reflectance and transmission modes and different wavelength ranges.

The accuracy of NIR prediction for milk component is influenced by a number of factors. Water content in fresh milk is one of the major contributors to the variation in the NIR spectra due to the strong absorption bands of O-H groups in the NIR region, which can create a significant background problem in quantitative analysis. Sample preparation is often seen as a factor influencing the NIR spectra. Samples may require further processing to achieve uniform particle size.

These inconveniences related with sample complexity are increased when spectra are collected with versatile and mobile hand-held NIRS instruments, with small scanning window and narrow wavelength range. It makes necessary to develop the specific sampling procedures to collect high quality spectral data.

Taking into account all these considerations, the aim of the present work was to establish a sampling methodology for obtaining high quality milk NIR spectra using a hand-held NIR spectrophotometer, useful for further NIR quantitative analysis.

MATERIAL AND METHODS

Samples and pretreatment

A total of 156 fresh milk samples were collected from different cows of the experimental farm located in the Regional Institute for Research and Agro-Food Development (SERIDA), and from different farms located in the North of Spain (Asturias, Spain), as suppliers from commercial milks. All samples were homogenized by hand mixing during 20-30 sec and tempered at 40ºC in a shaking water bath before scanning.

Different sample pretreatments prior to NIR analysis were examined, all of them focused to obtain a robust spectral signal. Firstly was evaluated the effect of dilution with water on the liquid milk, and after that a filtration procedure preceding the analysis. Finally, the quality of NIR spectra on raw milk using two different cuvettes and instrumental conditions were tested.

NIR instruments and analysis methods

NIR analyses were carried out), using two analyzers with different characteristics:

- MicroPHAZIR TM from Thermo Scientific, with a scanning window of 4 mm diameter. All diffuse reflectance spectra were computed in a wavelength range between 1600 and 2400 nm, with a non-constant interval of around 8 nm (pixel resolution 8 nm, optical resolution 12 nm) using a hand-held micro-electro-mechanical system (MEMS) digital transform. This instrument was equipped with a liquid adapter accessory using a quartz cuvette of 1 mm pathlength. It was employed as *in-situ* instrument. In addition, spectra were collected using externally a liquid opaque cuvette, with a 17mm pathlength and an aluminum backside for trans-reflectance measurements (which combines reflectance and transmittance together into a single mode). All spectra data were recorded in reflectance mode (log 1/R).
- Foss NIRSytem 6500 monochromator working in a wavelength range between 400 and 2500 nm, equipped with transport module, was used as a reference instrument. Spectra were collected using a liquid opaque cuvette, with a 17mm pathlength and an aluminum backside (trans-reflectance measurements). The spectra data were recorded in reflectance mode (log 1/R) with ISI scan software (Infrasoft International

Inc). Each spectrum was the average of 32 scans performed on the liquid milk. It was used as reference *at-line* instrument.

The effect of the instrumental methodology on hand-held NIRS has been evaluated modifying the following parameters:
a) sample presentation - two cuvettes have been evaluated, the first one was a quartz cuvette of 1mm pathlength coupled to the hand-held NIR with an adapter module; and the second one was a 17 mm pathlength cuvette with an aluminum backside
b) number of scans to average for collecting one spectra - the range evaluated was between 5 to 80 scans/spectra
c) internal reference or external reference for scanning background.
In Table 1 are shown all the alternatives examined to optimize the collecting data procedure.

Table 1.Instrumental analysis methodologies evaluated with MicroPHAZIR NIR instrument to optimize spectral data collection

Number of scans/spectra	Reference Internal /external	Cuvette pathlength	Adapter
5	Internal	1 mm	Yes
	External		
	Internal	17 mm	No
10	Internal	1 mm	Yes
	External		
	Internal	17 mm	No
80	Internal	1 mm	Yes
	External		
	Internal	17 mm	No

The statistic root mean square error (RMS) was used to select and compare spectra in order to determine the differences in repeatability and reproducibility conditions. This statistical parameter is the averaged root mean square of differences corrected for the bias (RMSC) between two spectra (WinISI II, 2000), and the formula to calculate the RMSC is:

$$RMSC = 10^6 \times \sqrt{\frac{\sum_{i=1}^{n}(y_{im} - y_{ik})^2 - \frac{(\sum_{i=1}^{n}(y_{im}-y_{ik}))^2}{n}}{n-1}}$$

Where:

Y_{im} = log (1/R) value of m subsample at a wavelength i (λ_i).

$\overline{Y_{ik}}$ = log (1/R) value of k subsample at a wavelength i (λ_i).

n = number of wavelengths

Sample pretreatment and scanning modes giving spectra with the minimum value of RMS was selected for further development of calibration to predict quality parameters in milk.

RESULTS AND DISCUSSION

Milk is a very complex matrix for NIR analysis, consisting of proteins in colloidal dispersion, fat in emulsion and minerals in solution (Marinori, *et al.*, 2013). It is an opaque liquid with high water content that is highly light scattering caused by milk fat globules and casein micelles in suspension (Holroyd *et al.*, 2013). Water content in raw milk is one of the major contributors to the variation in the NIR spectra, due to the strong absorption bands of O-H groups in NIR region, with a basic characteristic region at 1940 nm (Shenk *et al.*, 1992) that could limit the detection of the analytes.
Figure 1 shows the raw spectra of milk recorded with reference instrument in the range between 400-2500nm. All spectra were characterised by the strong absorption bands related

to water that dominated the spectra at 1450 nm and 1940 nm, representing the O–H first overtone stretching and the O–H second overtone bending, respectively (Williams and Norris, 2001). The recognition of absorption bands attributed to the other components such as fat or crude protein also was possible related with 2310 and 2180 nm, respectively, although they were very weak in comparison with the O-H bands and were more difficult to see. After applying mathematical pretreatments to the spectral data improve their interpretation and emphasizes weak component absorbance bands.
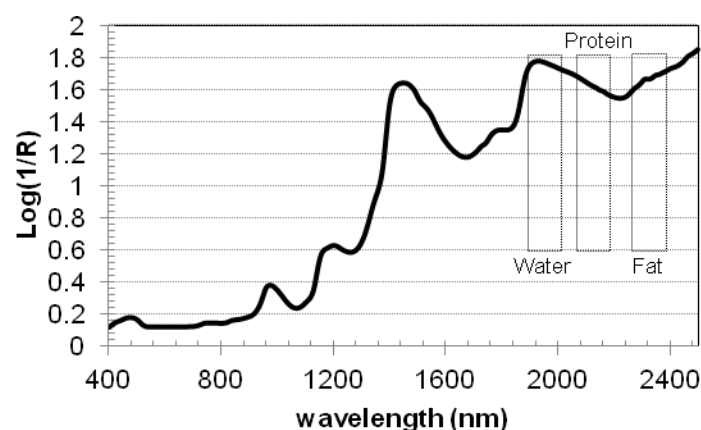
Figure 1. Averge of liquid milk spectra collected in reference instrument Foss NIR System 6500

All these specific wavelengths between 1600-2400 nm are included in the range of the hand-held instrument, however the wavelength range, the strong absorption of water and the small scanning window make difficult to obtain spectra like those obtained with the reference instrument (400-2500 nm).

In order to get a comparable spectra between reference and portable instruments, the sample pretreatments described in Material and Methods section were applied. Firstly, the 1mm pathlength quartz cuvette was employed. The results obtained trying to scan raw milk were distributed randomly, and approximately 90% of samples were imposible to scan, due to a power level of instrument, it was too low for recording the spectral data. To avoid this effect firstly samples were diluted between 25 and 300% and scanned (Figure 2a). As it can be seen in the Figure 2a, the strong NIR absorption bands attributed to water due to the hydrogen bonds have led a great saturation level for log (1/R) around 1940nm (water band). Small differences were observed between raw and diluted milk samples and the effect of power level of instrument, too low, was detected in 70% of samples and low repeatability and reproducibility were observed.

To minimize this effect, a filtration in conjunction with dilution pretreatment was examined for milk samples with different fat contents (Figure 2b). Tsenkova *et al.*, (2000) reported that milk fat content could change the baseline of milk spectra. Again, it not was possible to obtain good results and water band saturation was always observed.

The last step was to scan raw samples without pretreatment, recording 80 scans by spectra, using two different cuvettes to compare these spectra with the spectra recorded on reference instrument. In Figure 3 are shown the spectra obtained in hand-held instrument for the same milk samples with different cuvettes at 1 mm or 17 mm sample thickness with or without employing the specific adapter for scanning liquid samples, respectively.
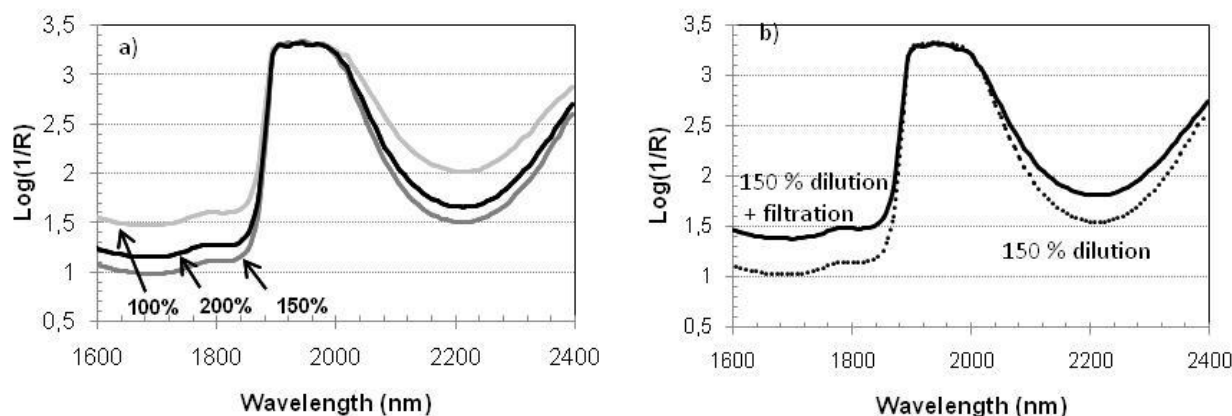
Figure 2.- Average liquid milk spectra collected in MicroPHAZIR after a) diluting sample b) filtering and diluting sample
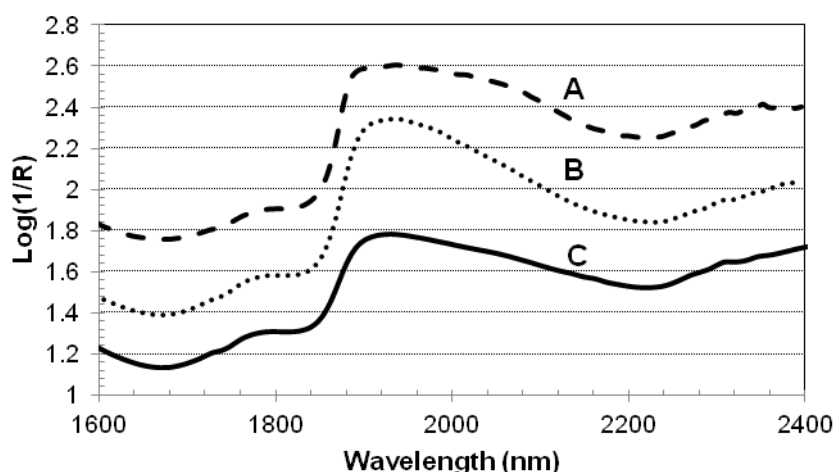
Figure 3. Average liquid milk spectra collected with different cuvettes: A) microPHAZIR cuvette with 1mm pathlength and adapter (microPHAZIR); B) cuvette with 17 mm pathlength (microPHAZIR); C) cuvette with 17 mm pathlength (Reference instrument)

From a spectroscopic point of view, it is necessary that each milk spectrum recorded in hand-held instrument was time-averaged from 80 scans, because when it was compared with the 5 or 10 average measurements the graph mean showed less spectral noise.

Related with cuvettes, best results were obtained using the17 mm pathlength cuvette with an aluminum backside. As saying before this cuvette does not employ the adapter for scanning milk samples on hand-held instrument, and the comparison of repeatability and reproducibility for both cuvettes results in terms of RMSC were better, as can be seen in Table 2. Related with the comparison between both instruments, RMSC values for reference instrument were better than hand-held device, although these results were included as hypothesis at the beginning of study due to the best instrumental characteristics of the reference spectrophotometer.

Table 2. Repeatability and reproducibility root mean square corrected for bias (RMSC) for milk spectra using different cuvette types

Intrument	Cuvette type	Repeatibilty RMSC	Reproducibility RMSC
microPHAZIR	1-mm + adapter	11190	45270
	17 mm	5309	4799
Foss NIR System 6500	17 mm	2568	3823

CONCLUSIONS

Among different methodologies evaluated and sample presentation modes using hand-held instrument working in a spectral range from 1600 to 2400 nm, measurements gave the best results using raw milk, 80 scans to average for collecting one spectra and 17 mm sample thickness in a cuvette with an aluminum backside, in spite of their complicate features and strong absorption of water.

Good performances on real samples could be exploited for a milk selection for its final purpose: obtaining high quality raw milk NIR spectra using a portable NIR spectrophotometer, useful for further *in-situ* NIR quantitative analysis.This may be of great economic importance for dairy farm management or field measurements.

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PREPARATION, CHARACTERIZATION AND *IN VITRO* DRUG RELEASE STUDIES OF DIFFERENT CURCUMIN FORMULATIONS

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ABSTRACT

In the present study the commercially available cellulose was used for curcumin encapsulation, which was performed by solvent displacement using simple dialysis method. The encapsulation efficiency was 99.03 % and the loading capacity was 49.52 % for encapsulated curcumin. In the second step the curcumin-cellulose mixture was further formulated with various carriers (glycerol tristearate and polyethylene glycol – PEG 1500) using supercritical fluid technology, namely Particles from Gas Saturated Solution technique (PGSS^{TM}). The weight ratio between the components in the formulation curcumin : cellulose : carrier was 1: 1: 8. The PGSS^{TM} formulation was carried out using supercritical carbon dioxide, which results in powderous product free of organic solvents. The efficiency of micronizations was 90 %. The best formulation results were obtained using PEG as carrier. This product was further used for studying the release of curcumin in simulated body fluids.
Keywords: curcumin, glycerol tristearate, PEG, cellulose, controlled release

INTRODUCTION

Curcumin , from turmeric is one of the most important food additives and it is used as spice and natural pigment, isolated from the rhizomes of *Curcuma longa L.* (Zhan et. al, 2011). It is a nutraceutical used worldwide for medicinal, as well as food purposes (Parvathy et. al, 2009). It has been reported to possess anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-proliferative and anti-angiogenic properties. Curcumin is an oil-soluble pigment, practically insoluble in water at acidic and neutral pH, and soluble at basic pH. Therefore it is stable at the pH conditions in the stomach but it dissolves in the small intestine. (Setthacheewakul *et al.*, 2010). The aim of this study was to formulate powdereous products containing curcumin and test the release of the drug in body fluids.

MATERIAL AND METHODS

Turmeric rhizomes were purchased from the Slovenian market. CO_2 (purity 2.5) was provided by Messer (Slovenia). Glycerol tristearate, cellulose and polyethylene glycol (PEG 1500) were purchased from Sigma Aldrich.

Formulation with PGSS^{TM}
Curcumin was first formulated with various carriers using supercritical fluid technology, namely Particles from Gas Saturated Solution technique (PGSS^{TM}). The melted fat (glycerol tristearate) was mixed with the emulsifier and the curcumin in different concentrations using a homogenizer. Then pure cellulose or pure starch was added to the mixture to improve the texture of the products. The autoclave was filled with the mixture and CO_2 was introduced by a high pressure pump to the desired pressure, depending on pre-expansion 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 gas evaporated instantly in the expanding chamber causing the micronization of the particles (Mandžuka *et al.*, 2010).
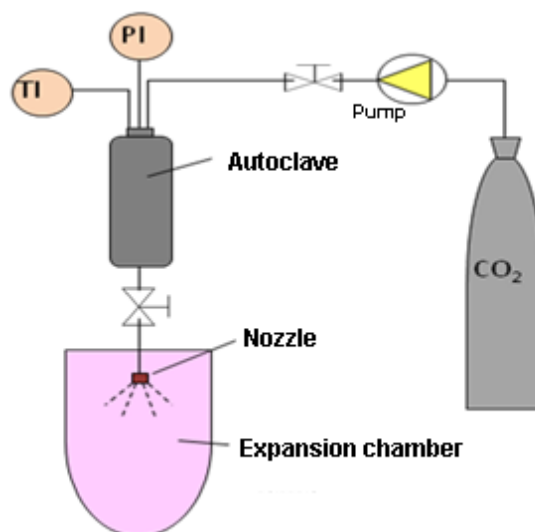
Figure 1. Scheme of PGSS^{TM} equipment (Knez et. al, 2012)

Nanoencapsulation

Nanoencapsulation of curcumin using cellulose was carried out by solvent displacement through dialysis. For encapsulation, curcumin:cellulose ratio 1:1 (w/w) were dissolved in ethanol to a final volume of 100 mL. The solution was then transferred into the dialysis tube (Membra –cel MD44 14 X 100 CLR) and dialyzed against distilled water (six changes of 1000 mL water each). The obtained aqueous suspension of curcumin-encapsulated nanoparticles was then freeze-dried. The amounts of curcumin incorporated into the polymeric particles and in the dialysate solution were determined using a Varian, Cary 50 Probe UV/VIS spectrophotometer at 420 nm. Dialysate-solution was subjected to UV/VIS absorption analysis directly, while the freeze-dried nanospheres were first dissolved in ethanol prior to analysis (Suwannateep et. al, 2011). The encapsulation efficiency (EE) and loading capacity were calculated as follows:

EE (%) = (weight of encapsulated curcumin / weight of curcumin used) · 100

Loading (%) = (weight of encapsulated curcumin / weight of curcumin loaded nanospheres) · 100

Drug dissolution test

The dissolution studies for curcumin were performed in a FARMATESTER-3 (Dema-Ilirska Bistrica) apparatus at a stirring rate of 50 rpm at 37 ± 0.5 °C using the conditions that simulated the stomach and intestinal environments. The apparatus consisted of a covered glass vessel surrounded by thermostated bath, a motor, a metallic stirrer and a cylindrical metallic basket. The weighed amount of sample was placed in the cylindrical basket, immersed in simulated gastric fluid (SGF; pH 1.2) or simulated intestinal fluid (KH_2PO_4; pH 6.8) and left under stirring for 24 h. Samples (2 mL) of the release medium were withdrawn and subjected to drug assaying by means of UV spectrophotometry at 420 nm (curcumin) using a Varian, Cary 50 Probe spectrophotometer. The removed dissolution medium was replaced by the same amount of fresh simulated gastric fluid or intestinal fluid.

RESULTS AND DISCUSSION

Results of micronization

Table 1 shows the composition of samples and the operating parameters applied for the formulations. The obtained curcuma products were homogenously colored fine free-flowing powders with colors from light yellow to orange.

Table 1. Results of formulation of curcumin extract by PGSS

Samples	Curcumin (%)	Emulsifier (%)	Glyc. Trist. (%)	Cellulose (%)	p (bar)	T (°C)	Yield (%)	Product
1	20	1	65	15	160	64	77.7	
2	50	1	45	5	160	70	90.6	
3	50	1	30	20	160	60	80.2	

The used operating parameters were about 160 bar and 70 °C in all experiments. By using cellulose the texture of obtained powder was better and drier in all cases.

The best formulation results were obtained using polyethylene glycol (PEG 1500) as carrier. The ratio between the components curcumin : PEG 1500 in the formulation was 1 : 19. The used operational parameters were about 160 bar and 70 °C. Figure 2 shows micronized curcuma powder product (sample 4), containing 5 % of curcuma and 95 % (PEG 1500).

Figure 2. Micronized curcuma powder product (sample 4)

Nanoencapsulation

EE of 99.03 % and a curcumin loading of 49.52 % were obtained with encapsulation by dialysis method, where curcumin-cellulose particles were prepared.

In vitro release of curcumin

The release of curcumin from cellulose-curcumin in the SGF and KH_2PO_4 was determined in vitro. The results indicated only a minimal release (less than 4 %) of the encapsulated curcumin after 24 h in both media. The release rate from curcumin-PEG 1500 was 100 % after 3 h in SGF solution and 97 % after 7 h in KH_2PO_4 solution. The release of curcumin from samples 1 – 3 was not observed.

CONCLUSIONS

In this work an environmentally friendly technology was applied for the formulation of curcuminoids from curcumin in powderous form. Curcumin was formulated using a high pressure process (PGSS[TM]) with CO_2 as processing media and glycerol tristearate fat mixed

with cellulose as carriers. The obtained products were homogenous fine powders with colours from light yellow to orange. The obtained products are interesting for the application in the food industry.

It can be concluded that the formulation of curcumin-PEG 1500 will not be stable in the pH range of the stomach; therefore, it is necessary to coat the particles with a material that withstand the pH of the stomach and allows degradation only in the small intestine. Our next goal is to find a suitable coating.

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INVESTIGATION OF THE EFFECT OF DIFFERENT ADDITIVES ON THE LEVEL AND DYNAMICS OF POLYETHYLENE FILM DEGRADATION

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ABSTRACT

The utilization of non-renewable packaging materials that cause emissions during their production and application, accumulation and disposal is a serious threat to the environment. One of the directions for ecological status improvement of packaging materials is the increased usage of biodegradable packaging materials. Polymer packaging materials could be natural biopolymers, which are biodegradable, or synthetic polymers that become biodegradable by addition of additives.

The aim of this study was to review the oxidative degradation and biodegradation mechanisms of synthetic polymers with additive addition. The methods for determination of the degree of decomposition and results of the decomposition level and dynamics in the chamber for accelerated aging of selected polyethylene films commonly used in the food industry are also presented. Polyethylene film samples of the same thickness with addition of various additives in the same concentrations were selected for testing. Before and during artificial aging in the chamber for accelerated aging, physico-chemical and structural properties were investigated by determining tensile properties, melt flow rate and structural properties of the polymer.

The obtained results for all tested samples showed the decrease in tensile properties below 5% of the initial value, the increase in the carbonyl index value, as well as higher melt flow rate value after treating samples in the chamber for accelerated aging, indicating the biodegradability of the tested films. The influence of additive types on the decomposition dynamics was proved. Moreover, the decomposition times, being in the range of 144-312 hours, were dependent on the type of additive used.

Keywords: *polymers, polyethylene, additive, oxo-biodegradation, properties*

INTRODUCTION

The use of plastics has increased in recent decades, mainly due to their low cost, good mechanical properties and light weight. However, this growth has brought many challenges related to their storage and their impact on the environment. It is possible to improve packaging ecological status by increased use of natural biopolymers and synthesis of biodegradable synthetic polymers (Quoden, 2008). In synthetic polymer group, the most abundant is polyethylene, which is used in the food packaging industry, as primary or secondary packaging, or as shrink foil for product wrapping. It can be extruded or co-extruded, casted, or used as sheet for thermoforming containers and cups, molded and blown products (cups, trays, bottles) (Lazic and Novaković, 2010; Milanović *et al.*, 2014).

Biopolymers are polymers derived from biomass (from renewable agricultural raw materials or animal origin, or derived from the seafood wastes, microbial sources, etc.). Additionally, materials which are biodegradable decompose to environmentally-friendly components: CO_2, water and quality compost, under the influence of natural microflora, oxygen and moisture. The second group consists of polymer materials which contain pro-oxidant (or pro-degradable) substances which are known as oxo-biodegradable polymers (Bonhomme *et al.*, 2003). Synthetic polymers are not subjected to biodegradation, without regard to favorable environmental conditions, mainly due to the structure and size of the molecules, as well as the chemical composition of the polymer. The molecular weight is the most important factor affecting the biodegradability of a synthetic polymer (Vujković, 1997). Additives make synthetic polymers become degradable. The influence of external factor (additive) significantly changes the chemical structure, which can be observed visually or by using

standard methods. The additive may be mixed with a variety of standard plastics, during the modeling phase. Transition metal ions, such as: iron, cobalt and manganese, are commonly used prodegradant additives.

Degradation process is composed of two stages: separation (depolymerization) and mineralization. Initial phase - depolymerization, is associated with a significant deterioration of physical characteristics, such as color, brittleness and fragmentation. In principle it is possible to classify the biodegradable polymers according to the mechanism of the first phase of degradation as a "hydro-biodegradable" when the first phase takes place by mechanisms of hydrolytic processes or mediation and as "oxo-biodegradable" when depolymerization occurs as thermal or photophysicaly inductive oxidation with or without extracellular enzyme reactions. At this stage there are significant changes in the mechanical properties of biodegradable polymers so the tests for biodegradability are based on monitoring changes in these properties (elongation at break, melt flow rate, etc...) (Lazić *et al.*, 2012).

The second phase - mineralization occurs when living microorganisms digest organic product under aerobic or anaerobic conditions, and convert them into common inherent product of digestion. Mineralization is the final conversion of the plastic fragments (after having been broken down into smaller molecules) to CO_2, H_2O and biomass, if the mineralization takes place under aerobic conditions, or CH_4, CO_2 and biomass in the case of anaerobic conditions (Krzan *et al.*, 2006).

Principles and mechanisms of plastics degradation

Apart from the way of material formation, equally is important the way in which material degrades (disintegrates). There are different mechanisms of plastics degradation, and some of the most important are:

• Photo-oxidative degradation,
• Thermal degradation,
• Hydrolytic degradation,
• Ozone-induced degradation,
• Mechano-chemical degradation.

Photo-oxidative degradation: Photodegradation mechanism involves the absorption of UV light, which leads to the formation of free radicals. Auto-oxidation process is further developed, which leads to final degradation (Ammala *et al.*, 2011). Agents are added to the polymer (photo-sensitive additives) to accelerate the natural tendency of the polymer photodegradation. These additives, when exposed to the sun's UV radiation, release free radicals, which randomly attack and tear polymer chains that are subjected to oxidation under aerobic conditions (Krzan *et al.*, 2006). Photodegradation changes the physical and optical properties of materials. During degradation polymer mechanical characteristics decreases, average molecular weights and molecular weight distribution changes. Polyethylene and polypropylene films exposed to solar UV radiation, easily lose extensibility, mechanical integrity and strength while average molecular weight has been reduced (Abadal *et al.*, 2006; Marek *et al.*, 2006). The rate of degradation increases with the UV radiation flux increment.

Thermal degradation: Under normal conditions, photochemical and thermal break-up are similar and classified as oxidative decay. The difference between these two methods is sequence of steps which leads to auto-oxidation cycle. Other differences include thermal reaction that occurs in the bulk of a polymer sample while photochemical reaction occurs only at the surface. Polymer thermal degradation occurs randomly and it is initiated by heat and UV light (Teare *et al.*, 2000).

Hydrolytic degradation: The process requires the presence of hydrolytic group, such as ester groups, ethers, anhydrides or amide group present in the starch, polyesters, polyanhydrides, polycarbonates, polyamides or polyurethanes. These components absorb

moisture from the surrounding environment, leading to a hydrolytic cleavage of the polymer chain, which can be controlled by chemical agents or enzymes (Krzan *et al.* 2006)

Ozone-induced degradation: The presence of ozone in the air significantly accelerates polymer material aging. This process, in case of saturated polymer, is accompanied by intensive formation of oxygen-containing components, altering the molecular weight and the mechanical and electrical properties of the sample. The reactions of ozone with organic polymers are occurring in the main chains which contain C=C double bond, an aromatic or saturated hydrocarbon rings connection. The reaction is continued by unstable intermediates formation, which may be degraded or izomerized, thereby causing decomposition of the macromolecule (Singh and Sharma, 2008).

Mechano-chemical degradation: This process involves the use of shear forces in order to decompose polymer. This is an important method for the polymer size reduction (Li *et al.* 2006). Splitting the molecular chains due to shear or mechanical force is often fueled by chemical reactions and it is known as mechano-chemical degradation.

Biodegradation - Biodegradation is defined as the ability of the material to be broken down into its component molecules by natural processes (often microbial digestion). It is expected that the metabolites liberated upon degradation are not toxic to the environment (Singh and Sharma, 2008). Abiotic hydrolysis, photo-oxidation and physical degradation of the polymer can enhance the biodegradation of the polymer by increasing its surface area for colonization by micro-organisms or reducing their molecular weights. During the stage of microbiological degradation, most of the microorganisms use abiotic oxidation products (low molecular weight components - average molecular weight should be less than 5000 g mol^{-1}). This process greatly affects the amount of available microorganisms and their microbiological activity (they are sensitive to environmental parameters such as temperature, humidity, pH, C/N ratio, and the amount of available oxygen).

This paper examines the first stage of decomposition (photo-oxidative degradation) of polyethylene samples in which are added various additives for degradation in same concentration.

MATERIAL AND METHODS

For the experiment purposes, polyethylene film samples of the same thickness with addition of various additives for the oxidative degradation and biodegradation in same concentrations were selected for testing and marked as: sample A, sample B, sample C and sample D due to the addition of various additives. Some additional explanation should be added to differentiate the samples (eg sample A (the type of additive)).

Samples were exposed to UV radiation in the device for accelerated aging, "Weathering Tester QUV/spray" according to SRPS G.S2.519:2011, SRPS G.S2.520:2011 and SRPS:G.S2.664:2011. Prepared samples were placed in the appropriate metal rack. The samples were exposed to the cycles: 20h UV radiation at a temperature of 50ºC and 4h condensation at a temperature 40ºC (cycle A, SRPS G.S2.520).

Tensile properties were examined before and after treatment in accelerated aging chamber and the end point of degradation were determined by tensile tests. Tests were carried out according to SRPS G.S2.737:2011 and SRPS G.Z2.010:2011. Tensile properties were tested on the "Instron 4301" (Model No 4301, Instron Engineering Corp., Canton, MA) testing device. The initial grip separation was set at 50 mm, and crosshead speed was set at 100 mm/min. Results are given as the % of elongation at break. Specimens for testing were prepared by cutting a minimum five measuring sample strips (15x100) mm, with a sample preparation device "Film-/Paper Strip Cutter Model". At the end point of degradation material reaches the point of brittleness. At this point, at least 75% of the samples had an elongation value lower than 5% of the initial elongation value.

Sample structural characteristics determination by infrared spectrophotometry (FTIR) were obtained according to standard SRPS G.S1.511:2011 and carbonyl index was determined before and during the treatment in the chamber for accelerated aging. Spectra were

determined by FTIR spectrophotometry by FTIR Spectrophotometer, model "Nicolet IS10". The carbonyl index (CI) was calculated as:

$$CI = \frac{A_{1713}}{A_{1463}}$$

where A_{1713} was absorbance read at 1713cm^{-1} and A_{1463} was absorbance read at 1463 cm^{-1}. As degardation evidence, it was necessary that value of the carbonyl index after the treatment in the accelerated aging device is higher than the initial value.

Determination of the melt flow rate (MFR) of the sample, before and after treatment in a chamber for the accelerated aging, was obtained according to standard SRPS G.S2.521:2011. MFR was determined by extrusion plastomer "Tinius Olsen MP600", according to the procedure A-190/2.16. The result was expressed as g/10min. The proof of degradation is increased value of MFR after the treatment in the accelerated aging chamber, which indicates a reduction in molecular weight and/or degree of crosslinking of the polymer.

RESULTS AND DISCUSSION

Results of tensile properties of samples A-D, in which were added various additives, and the determination of the end point of the degradation are shown in Figure 1.

Sample A

Sample B
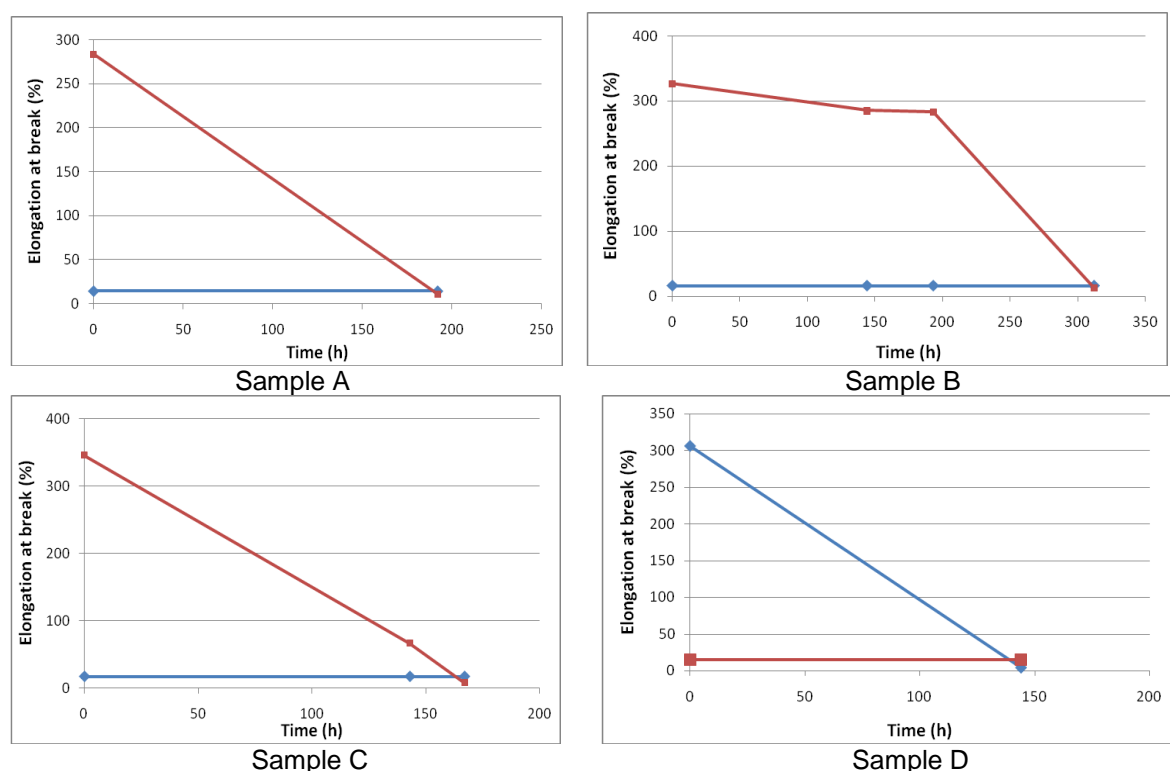
Sample C

Sample D

Figure 1. Elongation at break (%)

Figure 1 shows the average value of elongation at break, before and during the treatment in the chamber for accelerated aging for all samples of the films in which were added various additives. Line parallel to x-axis represents the brittlement point of each sample, i.e., a moment when degradation of the material occurs. The test specimens have reached the brittlement point (were degraded) at about 192h (sample A), 312h (sample B), about 167h (sample C) and about 144h (sample D).

The results of samples structural characteristics determination with the addition of various additives are shown in Table 1.

Table 1. Carbonyl index (CI) during treatment in the chamber for accelerated aging

	Sample A		Sample B		Sample C		Sample D	
Treatment time (h)	0	192	0	312	0	167	0	144
A_{1713}	0.059	0.065	0.072	0.095	0.069	0.098	0.003	0.005
A_{1463}	0.676	0.690	0.851	0.958	0.735	0.823	0.081	0.059
CI	**0.087**	**0.094**	**0.085**	**0.099**	**0.094**	**0.119**	**0.037**	**0.085**

Obtained results show an increase in the carbonyl index after treatment in the device for the accelerated aging for all samples, which is due to the decomposition of the samples.
Table 2 shows the results of melt flow rate (MFR) determination of the film samples with the addition of various additives.

Table 2. Melt flow rate (g/10 min)

	Sample A		Sample B		Sample C		Sample D	
	Before	After	Before	After	Before	After	Before	After
MFR	**0.181**	**0.297**	**0.192**	**0.516**	**0.207**	**2.223**	**0.213**	**4.331**

The obtained results show that melt flow rate increase after treatment in the device for accelerated aging, as a result of polymer molecular weight reduction, that is the evidence of degradation of the samples.

CONCLUSIONS

Polymer packaging materials play an extremely important role in the modern world, due to the favorable properties used in almost all fields of human activity. Due to good performance and economic feasibility, quickly became dominant in many areas, particularly as packaging materials for food industry. However, plastic products, especially packaging, are a major environmental problem of the modern world that causes a large amount of packaging waste, although the production, transport and application does not require a lot of energy. By additive addition, these synthetic materials can become biodegradable, which solves their biggest drawback. Under laboratory conditions it is possible to identify and track the process of degradation of polymer materials that would occur in nature, but accelerated several times. Based on the obtained results it can be concluded that all the tested samples are degradable. The process of degradation lasted from 144h (sample A) to 312h (sample B). The influence of added additive affects degradation duration.

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MODIFIED GUAR-XANTHAN MIXTURE IMPACT ON STARCH BASED EDIBLE FILM PROPERTIES

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ABSTRACT

Present paper examined guar-xanthan modified mixture effect on mechanical, barrier and structural characteristics of starch based edible films. Films were obtained from water solution that contained gelatinized modified starch, glycerol and guar-xanthan modified mixture in 3 different concentrations: 0.1%, 0.3% and 0.5%, by casting it on a Petri dish and evaporating at room temperature. Thickness, mechanical properties (tensile strength and elongation at break), barrier properties (water vapor permeability) and structural characteristics were examined. Obtained films had thickness in the range from 0.068 to 0.079 mm. Guar-xanthan modified mixture addition improved film flexibility and enabled better film folding and handling. Mechanical properties with guar xanthan modified mixture addition improved since tensile strength decreased from 0,049 N/15mm to 0,009 N/15mm and elongation at break increased from 2.90 to 3.45 % for samples with lowest and highest guar xanthan modified mixture addition, respectively. Regarding water vapor permeability, it was proved that guar-xanthan modified mixture addition negatively affected it. Water vapor permeability value increased from 205.84 g/m^2 24h, for sample with 0.1% guar-xanthan addition, over 214.33 g/m^2 24h for sample with 0.3% guar-xanthan addition to 236.35 g/m^2 24h for sample with 0.5% guar-xanthan addition. Structural properties were determined by analyzing spectra obtained by FT-IR Spectrometer in the spectral range of 4000–500 cm^{-1} with a 4.0 cm^{-1} resolution. Software Omnic 8.1. and TQ Analyst were used to operate the FTIR spectrometer, collect and present all the data. Results pointed to quantitative law dependency between added amount of guar xanthan modified mixture and spectra absorption values which was described by equation: y=0.385x+(-0.0295).
Keywords: *packaging, edible films, starch, guar-xanthan, characteristics*

INTRODUCTION

Interest in maintaining food quality while reducing packaging waste has encouraged the exploration of edible films. Edible films are thin layers of biopolymers that are used for food packing. Starch, as biopolymer that has ability to form films, is one of the most preferred green packaging material due to its rapid biodegradable nature, renewable sources and low cost production on large scale (Parra *et al.*, 2004; Majdzadeh-Ardakani *et al.*, 2010; Qiao *et al.*, 2010; Nejad *et al.*, 2011; Liu *et al.*,2011). Starch-based films application in food packaging is promising because of their environmental appeal, low cost, flexibility and transparency (Muller *et al.*, 2009; Bilbao-Sáinz *et al.*, 2010). Starch modifications, which include physical, chemical, enzymatic and combined modifications, can be used to alter the starch to be suitable for special goals and to have functional properties (Sandhu *et al.*, 2008; Liu *et al.*, 2012). Starch based edible films are tasteless, odorless and transparent, thus prevent a change of taste, flavor and appearance of food products (Chiumareli and Hubinger, 2012). Starch films are good barriers to O_2, CO_2 and oil but poor to water (Biliaderis *et al.*, 1999; Šuput *et al.*, 2013).
Starch films are often too fragile to stand handling (bending or stretching). Plasticizing agents are used to overcome film brittleness and improve its flexibility and extensibility. Starch has been blend with different biopolymers (Xu *et al.*, 2005) or hydrophobic materials such as oils or waxes (García *et al.*, 2000) which decrease interactions between biopolymer chains, such as amylose and amylopectin, thus preventing their close packing which results in lower

degree of crystallinity in the film (García *et al.*, 2000). Mathew and Dufresne (2002) revealed that polyols are a group of efficient plasticizers for starch, for example, glycerol (Yu *et al.*, 2008), xylitol (Liu *et al.*, 2011), sorbitol (Muller *et al.*, 2008), and short-chain glycols (Roz *et al.*, 2006), as well as water. The most common process to produce films on laboratory scale is casting. Solvent is evaporated from the solution in order to form the film (Anker *et al.*, 2001; Lazaridou and Biliaderis, 2002; Rindlav-Westling *et al.*, 2002).

Guar gum is a polysaccharide composed of the galactose and mannose. The backbone is a linear chain of β 1,4-linked mannose residues to which galactose residues are 1,6-linked at every second mannose, forming short side-branches. The largest market for guar gum is food industry as it acts as thickener and stabilizer, helps maintaining homogeneity and texture, functions as a binder. In Europe, guar gum has EU food additive code E412. Xantan gum, an extracellular polysaccharide produced by submerged aerobic fermentation of a pure *Xanthomonas campestris* culture, is widely used in the food industry due to its ability to form highly viscous solutions at low concentrations, besides its biodegradability. Some authors (Fang *et al.*, 2001; Mandala and Bayas, 2004) have investigated the effect of xantan on swelling, solubility and viscosity of wheat and waxy corn starch suspensions, and introduced chemical modification of xanthan gum to increase dissolution rate (Su *et al.*, 2003). In order to decrease interactions between starch biopolymer chains, besides adding glycerol, guar-xanthan modified mixture was added during film processing in three different concentrations: 0.1%, 0.3% and 0.5% with aim to optimize water vapor permeability and mechanical film properties.

MATERIALS AND METHODS

Chemicals
Starch and guar-xanthan modified mixture were kindly provided by "Palco" (Šabac, Serbia) and glycerol was purchased from „doo Laboratorija" (Novi Sad, Serbia).

Film preparation
Starch films were prepared by casting aqueous starch solution. Aqueous solution of 1.5% (w/w) modified maize starch was prepared and heated at 90 ^{0}C for 60 minutes in a water bath. A weight of glycerol equal to 40% of the original starch was added and the solution was kept hot with mechanical stirring for 10 more minutes. Finally, guar-xanthan modified mixture was added in a portion of 0.1%; 0.3% and 0.5% to initial starch weight. The film-forming solution was homogenized using homogenizer at 10000 rpm for 1 min and then degassed under vacuum to remove dissolved air and then cast into Petri dishes. Each Petri dish was coated with 50g of film forming solution on a leveled surface and left to dry at room temperature.

Film thickness
Film thickness was measured using a micrometer with sensitivity of 0.001 mm. Ten thickness measurements were carried out on each film, from which an average was obtained.

Mechanical properties
Tensile strength (TS) and elongation at break (EB) of films were measured on an Instron Universal Testing Instrument (Model No 4301, Instron Engineering Corp., Canton, MA), according to ASTM standard method D882-01. The initial grip separation was set at 50 mm, and crosshead speed was set at 50 mm/min. The TS and EB of the strips were measured in a static mode. EB was calculated as the percent of change by dividing film elongation at the moment of rupture by initial gage length of the specimen (50 mm) and multiplying by 100. TS and EB measurements for each type of film were repeated 10 times, from which an average was obtained.

Water vapour permeability
Water vapor barrier properties of films were determined gravimetrically according to the ASTM E 96-95 desiccant method.

Fourier transform spectroscopy
FTIR analysis of the film samples was carried out in the wave number range 4000 to 400 cm^{-1}, at a resolution of 4 cm^{-1}, using the IR spectrophotometer, Nicolet IS10, Thermo Scientific (Massachusetts, USA) and attenuation total reflection (ATR) extension. Each sample was scanned 32 times, while background shot was taken before the analysis of each sample. IR spectrophotometer is controlled via computer equipped with software Omnic 8.1. and TQ Analyst (Thermo Fisher Scientific, MA, USA) which were used to collect and present all the data.

Statistical analysis
Descriptive statistical analyses for calculating the means and the standard error were performed using MicroSoft Excel software (MicroSoft Office 2007). All obtained results were expressed as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

According to visual examination, obtained films were transparent, odorless and easy to handle. Films were not greasy or sticky. Film thickness was in the range from 0.068 to 0.079 mm (Table 1).

Table 1. Starch based edible films thickness (mm)

	Guar-xanthan modified mixture addition (%)		
	0.1	0.3	0.5
Thickness (mm)	0.079±0.008	0.071±0.010	0.068±0.006

Appropriate film formation contributed to thickness uniformity no matter the amount of guar-xanthan modified mixture addition Very small values of standard deviation prove film uniformity, no matter films are biologically active material.
Results related to mechanical properties are shown in Fig. 1a and Fig. 1b. Mechanical properties with guar xanthan modified mixture addition improved since tensile strength decreased from 0.049 N/15mm to 0.009 N/15mm and elongation at break increased from 2.90 to 3.45 % for samples with lowest and highest guar xanthan modified mixture addition, respectively.
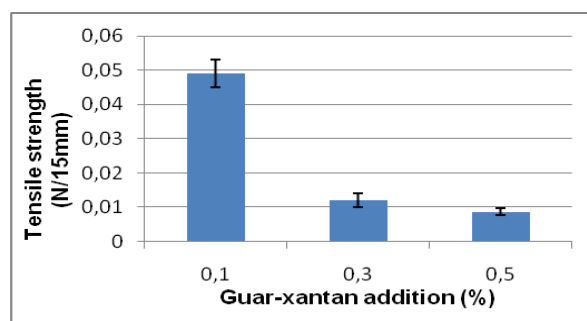
Fig. 1a. Tensile strength (N/15mm)
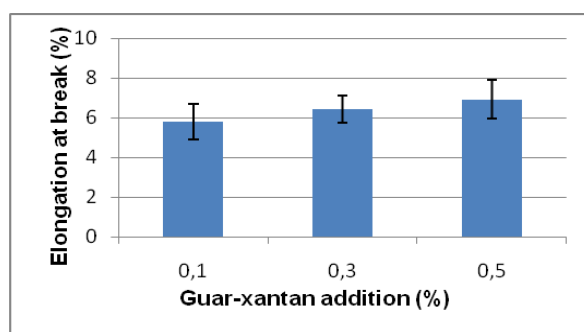
Fig. 1b. Elongation at break (%)

Regarding water vapor permeability, it was proved that guar-xanthan modified mixture addition negatively affected it (Fig 2). Water vapor permeability value increased from 205.84 g/m^2 24h, for sample with 0.1% guar-xanthan addition, over 214.33 g/m^2 24h for sample with 0.3% guar-xanthan addition to 236.35 g/m^2 24h for sample with 0.5% guar-xanthan addition.
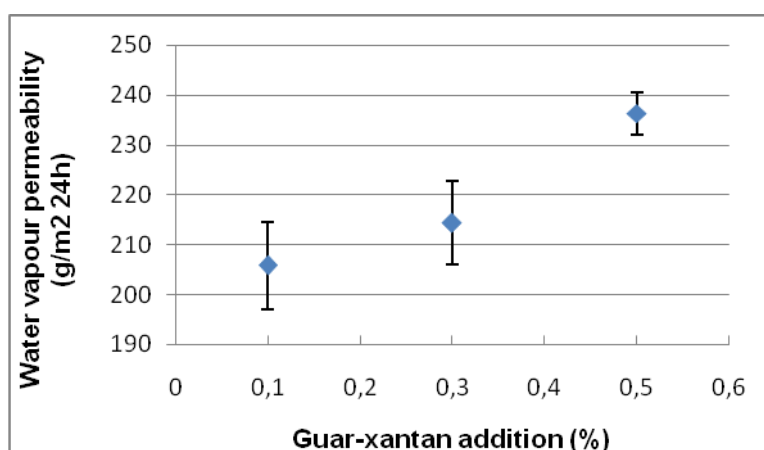
Fig. 2. Water vapor permeability (g/m^2 24h)

Obtained spectra of starch based edible films with different guar-xanthan modified mixture addition are shown in Figure 3. All characteristic peaks related to starch based edible films are observed (Šuput *et al.*, 2014). No significant differences between individual samples were noticed.
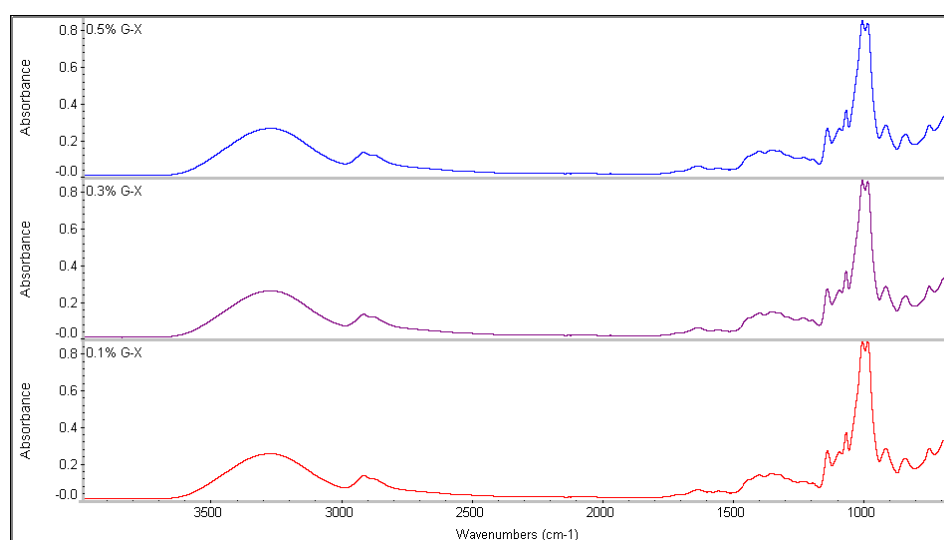
Fig.3. FTIR spectra of starch films with different guar-xantan modified mixture addition

TQ Analyst software was used for quantitative analysis. Simple Beer's law was performed on spectra of starch based edible films with growing amount of guar-xanthan gum.
Functional dependency, described by equation:

$$y=0.385x+(-0.0295) \tag{1}$$

was determined between added amount of guar xanthan modified mixture and spectra absorption values. Calculated versus actual values showed good correlation (R^2=0.86698) for the linear function (Figure 4).
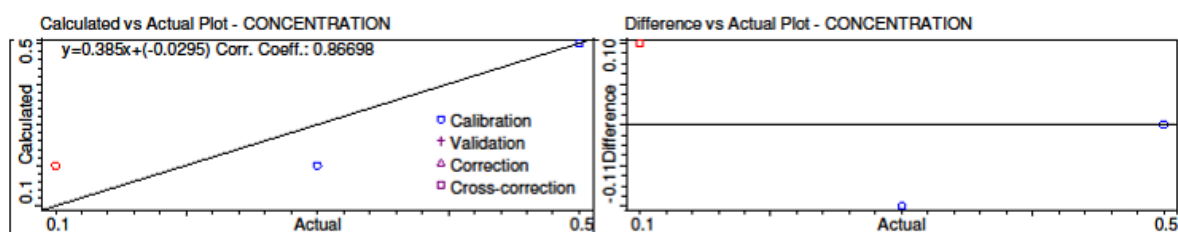
Fig. 4. Calculated verses actual values plot for Simple Beer's law performed on spectra of starch based edible films with growing amount guar-xanthan gum

CONCLUSION

A big effort has been made to extend shelf life and enhance food quality while reducing packaging waste. This trend encouraged the exploration of new bio-based packaging materials, such as edible films. The application of starch-based films in food packaging is promising because of their environmental appeal, low cost, flexibility and transparency. Present paper examined guar-xanthan modified mixture effect (used in three different concentration: 0.1%, 0.3% and 0.5%) on starch based edible films properties: mechanical, properties, water vapor permeability and structural characteristics. Obtained films were tasteless, odorless and transparent with the thickness in the range from 0.068 mm to 0.079 mm. Mechanical characteristics with guar-xanthan modified mixture addition improved (tensile strength decreased and elongation at break increased) which enabled better film folding and handling. On the other hand, guar-xanthan modified mixture addition negatively affected water vapor permeability. Water vapor permeability value increased from 205.84 g/m^2 24h to 236.35 g/m^2 24h with guar-xanthan increment. FTIR analysis proved characteristic peaks related to starch based edible films. Quantitative law dependency between added amount of guar xanthan modified mixture and obtained spectra was described by equation: y=0.385x+(-0.0295).

ACKNOWLEDGEMENTS

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HACCP - A CONDITION FOR PRODUCING OF SAFE FOOD

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ABSTRACT

The function of food in human life is multi - faceted. The food in the human organism is a source of protective and building material, a source of energy and it also provides "fuel" for all biochemical processes. Nonetheless, food has an important social role, because good and balanced diet is one of the basic terms of socially acceptable behavior, high working ability and productivity of each individual. But only safe food can fulfill the above mentioned conditions. Therefore, the safety of foodstuffs is something that is completely defined and also it is a condition that foodstuffs become a food. Very intense technological development of production of foodstuffs requires strict control, which obliges compliance with certain standards including full review of "food chain". By this, a high level of responsibility in the production and distribution of safe food is given for the producers and distributors of food. However, even in the best production conditions we cannot completely eliminate the risk of presence of pathogenic microorganisms. This article shows the results of the microbiological analysis of 826 samples of foodstuffs of animal origin and 590 food samples of plant origin. On these samples, 2754 microbiological analysis were performed according to ISO standards for certain types of microorganisms using accredited methods. The parameters of 2671 microbiological analysis were in accordance with the Pravilnik o opštim i posebnim uslovima higijene hrane u bilo kojoj fazi proizvodnje, prerade i prometa (Sl. glasnik RS br. 72/10) while 83 results of microbiological analysis were unsatisfactory. These results of microbiological analysis indicate that the implementation of the HACCP (Hazard Analysis Critical Control Points) system starting from the producing, packaging, storage and distribution of food greatly limits the presence of microbial contaminants. Because of this, food safety is raised to a much higher level.
Keywords: food safety, microbiological parameters, HACCP system

INTRODUCTION

When it comes to nutrition and its influence on human health, it primarily implies its nutritional value, quality and safety. Nutritional value is one of the most important characteristics of food, but for the health and even for the survival of humanity perhaps even greater importance has the quality and safety of food (Cilevski, 2013). It is believed that through the food and water in the human body enters 90% of environment pollution. As the international trade of food and the population of the world is rapidly increasing, many countries adopt and apply national legislation to ensure that the quality and safety of food complies with all the increasingly stringent requirements. Yet still some 800 million people don't have access to quality and safe food (Durmišević *et al.,* 2007).

The intensive growth of the population follows the intense technological development of process of food production. On the other hand, very intense technological development of process of food production requires strict control, ensuring compliance with certain standards including full control of the "food chain", conditioned upon a certain level of responsibility of the industry, manufacturers and suppliers in the marketing of safe food which provides adequate health care of the consumer.

That means that food production is a complex process that involves certain number of factors, many of those whose main goal is getting safe, high quality and hygienically approved foodstuffs. Characteristics of production of that quality foodstuffs are: good hygiene according to microbiological terms, limited presence of contaminants, especially pesticides, heavy metals, antibiotics, food additives (flavors, sweeteners, preservatives) and other

substances used in the production of food in the permitted amounts (Jovanović *et al*., 2007).

One of the modern and reliable food safety systems is HACCP (Hazard Analysis and Critical Control Point). HACCP is synonymous with food safety and world-renowned system for the production of safe food that has a systematic and preventive approach for identifying biological, chemical and physical hazards. HACCP system involves the establishment of responsibility of all participants in the chain of food production. This means that if there is a health risk to consumers, they have a duty to immediately take all measures to prevent harmful effects. So the ultimate goal of the HACCP system is the most safe product and the most economical and efficient production. The aim of this paper was to evaluate, from a microbiological point of view, how application of HACCP system through the entire food supply chain can contribute to reduction of foodborne pathogens and thus increase in food safety.

MATERIAL AND METHODS

This article presents the results of microbiological analysis of 1416 samples of foodstuffs of which 826 samples were of animal origin and 590 samples were of plant origin. Analyses were performed for external users of accredited laboratory FINSLab (Novi Sad, Serbia) whose production activity is based on the principles of HACCP system. On this number of samples 2754 microbiological analysis were performed according to ISO standards for certain types of microorganisms. Microbiological analysis included the determination of the following microbial contaminants: *Escherichia coli* (SRPS ISO 16649 - 2: 2008), the total number of microorganisms (SRPS EN ISO 4833: 2008), the number of yeasts and moulds (SRPS ISO 21527 - 1: 2011 and SRPS ISO 21527 - 2: 2011), the number of enterobacteria (SRPS ISO 21528 - 2: 2009), *Salmonella spp.* (SRPS EN ISO 6579: 2008), *Listeria monocytogenes* (SRPS EN ISO 11290 - 1: 2010), coagulase - positive staphylococci (SRPS EN ISO 6888 - 1: 2009), sulfite - reducing bacteria (SRPS ISO 15213: 2011) and *Bacillus cereus* (SRPS EN ISO 7932 - 2009).

RESULTS AND DISCUSSION

Figure 1 presents the number of microbiological analysis performed for all the samples. Most analysis were done to confirm the presence and to enumerate *Escherichia coli* (SRPS ISO 16649 - 2: 2008) - 557 analysis, followed by determination of the total number of microorganisms (SRPS EN ISO 4833: 2008) - 503 analysis, the enumeration of yeasts and moulds (SRPS ISO 21527 - 1: 2011 and SRPS ISO 21527 - 2: 2011) - 487 analysis, enumeration of enterobacteria (SRPS ISO 21528 - 2: 2009) - 458 analysis, determination of *Salmonella spp* (SRPS EN ISO 6579: 2008) - 314 analysis, detection of presence of *Listeria monocytogenes* (SRPS EN ISO 11290 - 1: 2011) - 125 analysis, determination of coagulase - positive staphylococci (SRPS EN ISO 6888 - 1: 2009) - 125 analysis, determination of the number of sulfite - reducing bacteria (SRPS ISO 15213: 2011) - 101 analysis and enumeration of *Bacillus cereus* (SRPS EN ISO 7932 - 2009) - 84 analysis.
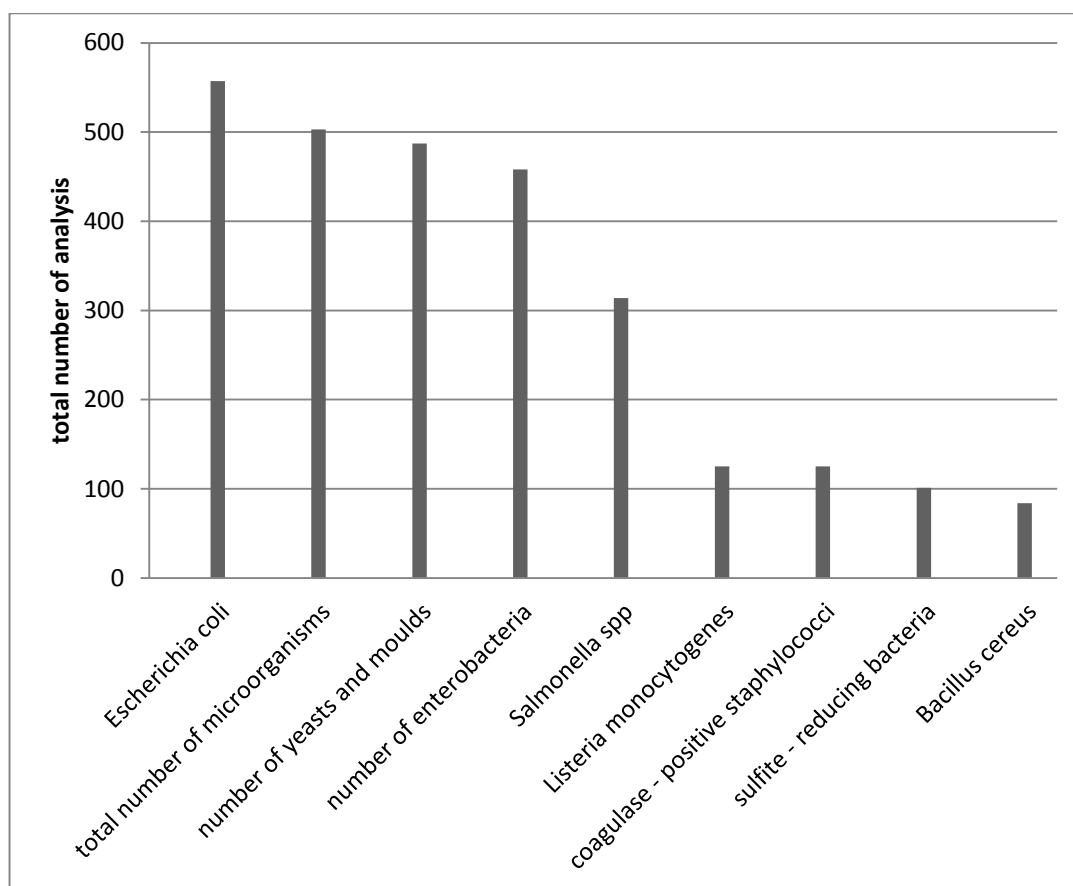
Figure 1. Number of analysis performed for different microbiological parameters

Figure 2 presents the number of microbiological analysis which parameters were in accordance with the Serbian regulation (Pravilnik o opštim i posebnim uslovima higijene hrane u bilo kojoj fazi proizvodnje, prerade i prometa;Sl. glasnik RS br. 72/10) and analysis which parameters were not in accordance with the Serbian regulation.

From the total number of performed microbiological analysis which counted 2754 analysis, the parameters of 2671 microbiological analysis were in accordance with the Serbian regulation (Pravilnik o opštim i posebnim uslovima higijene hrane u bilo kojoj fazi proizvodnje, prerade i prometa, Sl. glasnik RS br. 72/10), while 83 parameters were higher than the ones set by Serbian regulation which represents 3.01% of total number.
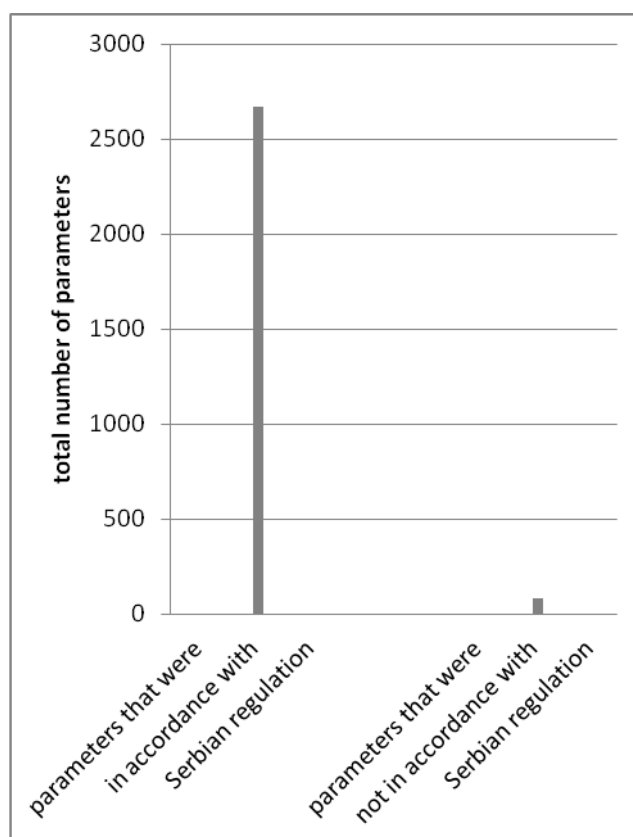
Figure 2. Presentation of microbiological parameters compliance with Serbian regulation (Pravilnik; - Sl. glasnik RS br. 72/10)

Table 1. Presentation of analyzed parameters according to Serbian regulation (Pravilnik; - Sl. glasnik RS br.72/10)

Parameters of microbiological analysis	Number of analysis that were in accordance with Serbian regulation	Number of analysis that were not in accordance with Serbian regulation
Escherichia coli	507	50
Total number of microorganisms	494	9
Number of yeasts and moulds	480	7
Number of enterobacteria	442	16
Salmonella spp	314	
Listeria monocytogenes	125	
Coagulase - positive staphylococci	124	1
Sulfite - reducing bacteria	101	
Bacillus cereus	84	

Presentation of microbiological analysis by the type of parameter, specifying the number of those that were in accordance and those that were not in accordance with the requirements given by Serbian regulation (Pravilnik; - Sl. glasnik RS br. 72/10) is given in Table 1. The results demonstrated that most of the analysis of microbiological parameters that were not in accordance with the Serbian regulation were related to *Escherichia coli* - 8.97% of the total number of analysis performed in this study.

CONCLUSIONS

Microbiological criteria are the basis for making judgments about the safety of food production methods and procedures during its storage and distribution. According to the epidemiological data, the microorganisms and their toxins are still the main cause of diseases which are transmitted by food. Therefore, implementation of the HACCP system in all phases of production, storage and distribution of food cannot be the measure of its hundred percent safety, but it should be continued to work on its improvement and development. The application of microbiological criteria, as integral part of food production, based on the HACCP principles, should also contribute to its continuous improvement.

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AROMA QUALITY OF STRAWBERRIES: OVERVIEW OF THE POTENTIALITY OF GC-OLFACTOMETRY

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ABSTRACT

Strawberry aroma is composed of more than three-hundred compounds belonging to several chemical classes. The main components are esters, providing the «sweet-fruity» odor note, along with aldehydes, alcohols, furans and sulfur compounds; other volatiles, such as the monoterpene linalool, γ-dodecalactone and some sulfur compounds are the most important contributors to strawberry aroma, along with few impact compounds, such as furaneol and its methyl ether. The odor activity of each compound is related to its odor threshold, the lowest concentration able to generate an olfactory response. The gaschromatography-olfactometry (GC-O), allowing the chromatographic separation of volatiles together with its sensory evaluation, is an appropriate tool for evaluating the aroma composition and the odor active compounds of foods. By comparing the aroma compound results of different studies on strawberries carried out at CRA-IAA, it was confirmed the major influence of the volatile compound sampling method on the odour profiles as assessed either by the calculation of OAV from quantitative data, or by GC-O analysis. Our results underlined that, whatever the sampling method, OAV patterns were well related to the GC-O profiles, even if by using GC-O a more complete odour pattern could be obtained.

Keywords: *strawberry, gaschromatography-olfactometry, odour active compounds, hyphenated analysis method, sensory evaluation*

INTRODUCTION

Strawberries are cultivated in nearly all countries of the world and are one of the most popular fruits that are consumed as fresh, conserved or manufactured products. The consumers' appreciation and further consumption of foods depend also upon good eating quality resulting from flavour, taste and aroma. Therefore, nowadays the improvement of flavour and aroma profile is taken into account in the breeding programmes (Marta *et al.,* 2004; Noguchi *et al.,* 2002) including wild strawberries as a source of many genes responsible for traits desired by consumers (Ulrich *et al.,* 2007, Ulrich and Olbricht, 2014; Diamanti *et al.*, 2014).

Strawberry aroma consists of approximately 350 volatile compounds that can be divided into several chemical classes including organic acids, alcohols, aldehydes, ketones, esters, lactones, furans, sulfur compounds, terpenes and terpene alcohols. The furanones 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol) and its methyl derivate 2,5-dimethyl-4-methoxy-3 (2H)-furanone (mesifurane) are the character impact compounds of strawberry aroma and contribute to the typical caramel-like, sweet, floral and fruity aroma (Zabetakis and Holden, 1997; Polesello *et al.*, 1993; Jetti *et al.,* 2007). Aldehydes and alcohols such as hexanal, 2-hexenal and 3-hexenol are important for the green, unripe notes associated with strawberry aroma and their concentrations are cultivar as well as ripeness dependent (Azodanlau *et al.*, 2004, Jetti *et al.,* 2007). Esters form the majority of aroma compounds and may cover up to 90% of the total number of volatiles in ripe strawberry fruit. Among the major esters are methyl butanoate, ethyl butanoate, butyl acetate, methyl hexanoate and ethyl hexanoate (Ménager *et al.,* 2004; Jetti *et al.,* 2007). Other volatiles, such as linalool, γ-dodecalactone, 2,3-butanedione and some sulphur compounds, are the most important contributors to strawberry aroma and it was shown that some of these key aroma compounds tend to be cultivar-specific (Schieberle and Hoffmann, 1997; Du *et al.,* 2011).

Not all aroma compounds are equally important for the overall aroma as the odour activity of each compound is related to its odour threshold (OT), the lowest concentration able to produce an olfactory response. The OT is inversely proportional to aroma intensity, so it can be used as an index of the sensory impact of each volatile compound to the overall aroma. In order to combine sniffing experiments with traditional threshold analysis, the ratio of the concentration of the flavour compound to its OT, such as the odour activity value (OAV, Mulders, 1973) can be used to select the most important contributors to the overall aroma (Zabetakis and Holden, 1997). The gas chromatography-olfactometry (GC-O), allowing the chromatographic separation of volatiles together with its sensory evaluation, is another valuable method to evaluate the odour significance of an individual compound to overall aroma (Van Ruth, 2001).

This work aimed at reviewing and comparing the aroma compound results of different studies on strawberries carried out at CRA-IAA over the last twenty years. The various aspects of the characterization of volatile composition aroma evaluation using both GC-O and OAV are compared for the evaluation of differences among cultivars.

MATERIAL AND METHODS

GC-O analyses: a direct intensity method based on a dynamic method in which assessors are required to rate both the odour intensity and its duration while the compound is eluting was used (Rizzolo *et al.,* 1995; Nuzzi *et al.,* 2008; Bianchi *et al.,* 2014). By using this method, a psychophysical measurement of the odour can be obtained and it was shown to be useful for discovering the most powerful odorants in a sample (Van Ruth and O'Connor, 2001). Judges were asked to characterize the detectable odours, i.e. the odorous events (OE), with a freely chosen descriptor, an indication of its intensity using a four point category scale (1=weak odour, 2=clear odour, 3= intense odour, 4=very intense odour) and its duration. When in a sample no odour was detected in correspondence of an OE of the other samples, a 0 value was assigned. In the end, the resulting outputs for each OE were: (a) duration of the odour perception, (b) maximum odour intensity (Imax), (c) area under the curve generated by the odour stimulus response (Imax×duration) and (d) odour descriptor.

Odour Aroma Value Determination: OAV were calculated according to $OAV_i=C_i/OT_i$, where C_i is the concentration of the compound *i* in the sample and OT_i is its odour detection threshold concentration found in literature (Leffingwell and Leffingwell, 2003). Compounds with OAV equal to or greater than 1 actually contribute to aroma as they are above their odour threshold concentration, whereas those with OAV smaller than 1 may not.

Fruit and experimental plans: In the experiments described by Tagliabue (1993) and Rizzolo *et al.* (1995) from three varieties of *F. vesca* and seventeen cultivars of *F.x ananassa* total aroma extracts were prepared according to Polesello *et al.* (1993), analysed by GC/MS, GC-FID and GC-O (panel of five short-term trained sniffers), and submitted to quantitative descriptive analysis (QDA) with a sensory panel of 10 judges. In the experiment described by Nuzzi *et al.* (2008) from six *F. x ananassa* cultivars coming from two localities the volatile compounds from cut fruit were sampled by dynamic headspace sampling, trapped on charcoal adsorption tubes, desorbed with dichloromethane and analysed by GC-MS, GC-FID and GC-O (three trained panelists). In the experiment described by Bianchi *et al.* (2014) from one *F. vesca*, one *F. moschata* and two *F. x ananassa* genotypes the volatile compounds from homogenized fruit were sampled by static headspace solid phase microextraction (HS-SPME) using a DVB/CAR/PDMS fiber and analysed by GC-O (three short-term trained panelists).

Data analysis: from the quantitative data from all the experiments the OAV were computed. For each GC-O analysis from each experiment the following data were extracted: (1) total number of OE for each descriptor and (2) sums of the OE intensities for each descriptor and they were compared via spider plots.

RESULTS AND DISCUSSION

For selected genotypes of each of the three GC-O experiments considered in this work, Tables 1 and 2 and Figure 2-left show the odour activity values (OAV), whereas in Figures 1-left, 2-right and 3 the GC-O patterns are compared considering for each main descriptor the total intensity, i.e. the sum of the medians of the intensities of all the OE of the GC-O analysis belonging to the same main descriptor. For all the experiments only the compounds having OAV>1 in at least one genotype are shown. Representing the volatile pattern using the OAV ratio is an approximation, as interactions between the volatile and the matrix are not taken into account, but it has a practical use in selecting the most important contributors to the overall aroma.

In total extracts 28 compounds out of the 48 identified and quantified (Rizzolo *et al.,* 1995) had OAV>1. Considering the genotypes selected (Table 1), 'Addie' (AD) fruit showed higher total OAV than the other three genotypes, of which 90.3% belonging to aldehydes and 8% to lactones; a similar pattern in OAV distribution was observed in Reine des Vallées (RDV), having 92.5% of the total OAV belonging to aldehydes and 3.4% to lactones. In contrast Bel Ruby (BR) had the lower total OAV, of which 51% belonging to aldehydes, 9.2% to lactones, 3% to alcohols and 1% to esters, while Vicoda (VI), besides 73.9% of total OAV belonging to aldehydes, had 15.3% of total OAV from lactones and 10% from esters. As for furaneol, in BR it accounted for the 32% of total OAV, whereas it was 0.35%, 0.03% and 0.06% of total OAV for RDV, AD and VI, respectively.

Table 1. OAV computed from quantitative data reported by Tagliabue (1993) and Rizzolo et al., (1995) for F. vesca 'Reine des Vallées'(RDV) and F.x ananassa cultivars 'Addie' (AD), 'Bel Ruby' (BR) and 'Vicoda' (VI) Only compounds with OAV>1 in at least one genotype are reported.

Aroma compounds	RDV	AD	BR	VI	Odour description
(E,E)-2,4-decadienal	1852	1718	553	1022	grassy
octanal	378	2103	64	56	citrus-like, green
2-methyl-propanal	319	7.6	0	0	malty
nonanal	90	22	143	34	citrus-like, soapy
pentanal	8.3	19	0.14	39	almond, malty, pungent
heptan-2-one	2.6	0	0.59	0.0039	soap
(E)-2-heptenal	1.2	5.4	1.4	0.038	green
heptanal	0.76	113	47	28	pungent
(E)-2-hexenal	0.37	40	2.6	155	green, apple-like
hexyl acetate	13	8.2	8.9	0	fruity, herbaceous
ethyl acetate	4.9	1.8	4.6	117	fruity, pleasant
pentyl butanoate	0.36	7.3	0.36	0.36	alcoholic
butyl acetate	0.21	2.6	0.011	19	fruity, pear
isoamyl acetate	0	7.6	3.8	50	banana-like
pentyl acetate	0	2.5	0	13	fruity, banana, pear
(E)-2-hexenol	3.4	2.5	0	0.39	green, leaf, walnut
(Z)-3-hexenol	0	0.62	51	0.14	lettuce-like
butanoic acid	1.9	0.059	0	4.7\	sweaty
γ-decalactone	61	15	19	68	peach-like
γ-dodecalactone	23	354	125	234	peach-like
γ-octalactone	6.4	1.2	1.6	0.86	coconut
γ-nonalactone	6.0	0.68	5.5	2.3	fatty, coconut
δ-dodecalactone	1.1	0.84	0.39	0.49	peach-like, coconut-like
δ-decalactone	1.1	0.99	0.76	0.61	creamy, coconut
linalool	45	36	1.6	8.4	citrus-like. floral
limonene	38	1.7	53	0.62	citrus-like
methyl salicylate	2.5	1.5	1.6	0.4	sweet, woody
furaneol	9.5	1.6	535	1.2	sweet, caramel-like
Total OAV	2913	4634	1664	2008	
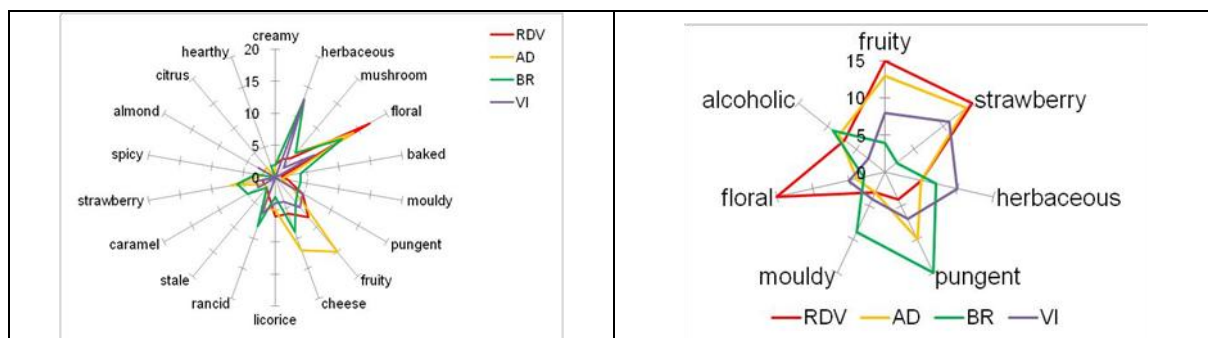
Figure 1. Total intensities for each GC-O odour descriptor (left) and quantitative descriptive analysis of total extract (right) for F. vesca 'Reine des Vallées' (RDV) and F.x ananassa cultivars 'Addie' (AD), 'Bel Ruby' (BR) and 'Vicoda' (VI) (Tagliabue, 1993; Rizzolo et al., 1995).
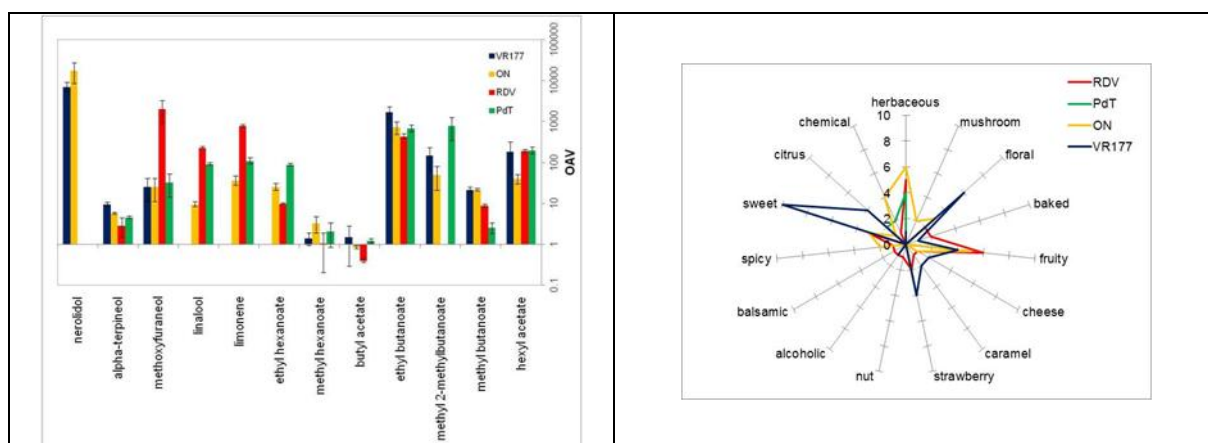
Figure 2 OAV computed from quantitative data (left) and total intensities for each GC-O odor descriptor (right) for F. vesca 'Reine des Vallées' (RDV), F.x ananassa cv 'Onda' (ON) and sel. VR177 and F. moschata 'Profumata di Tortona' (PdT). Bars refer to standard error (Bianchi et al. 2014).
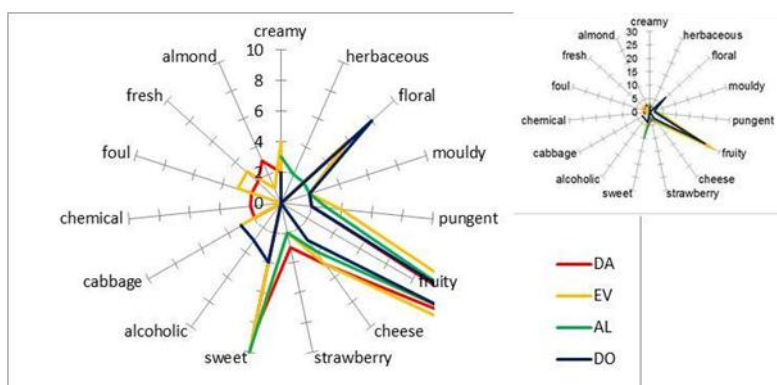
Figure 3 Total intensities for each GC-O odor descriptor F.x ananassa cultivars 'Darselect' (DA), 'Eva' (EV), 'Alba' (AL) and 'Dora' (DO) (Nuzzi et al., 2008).

Both the GC-O patterns and the QDA of total extracts (Figure 1) highlighted the different odour profiles of the four genotypes: RDV was characterized by the floral and fruity odour notes, AD by floral, fruity and cheese notes, BR by herbaceous, cheese, rancid and caramel odour and VI by herbaceous, fruity and rancid descriptors. Considering the QDA profiles, the highest strawberry characteristic odour was rated for RDV and AD and the least for BR, which had the highest score for pungent fond mouldy. Also with QDA, RDV had high scores for fruity and floral.

Table 2. OAV for F.x ananassa cultivars 'Darselect' (DA), 'Eva' (EV), 'Alba' (AL) and 'Dora' (DO) (Nuzzi et al., 2008). Only compounds with OAV>1 in at least one genotype are reported.

Aroma compounds	DA	EV	AL	DO	Odour description
Methyl 3-methylbutanoate	570	143	58	68	Fruity ethereal
Dimethyl trisulfide	512	211	208	3354	Sulfur, fish, cabbage
Ethyl 2-methylbutanoate	450	322	37	40	Sweet, fruity, strawberry
Ethyl butanoate	402	148	752	1073	apple
Ethyl 3-methylbutanoate	371	104	39	57	Cashew, fruity, anice
Methyl butanoate	327	137	755	591	Ether fruity sweet
hexyl acetate	291	301	200	257	fruity, herb
ethyl hexanoate	223	167	58	61	Apple peel, fruity
Methyl 2-methylbutanoate	96	185	51	48	apple
Butyl 3-methylacetate	40	47	6.0	30	
(Z)-3-hexenyl acetate	17	18	6.7	14	Green, banana
Ethyl 2-methylpropanoate	13	24	5.0	7.5	Sweet, fruity, strawberry
Butyl 2-methylacetate	6.3	12	0.62	2.4	
Methyl hexanoate	5.7	4.7	1.2	1.2	Fruity fresh sweet
2-methylethyl butanoate	2.7	1.2	5.4	33	Pungent, fruity
Dimethyl disulfide	2.5	1.3	0	0	Onion, cabbage, putrid
linalool	1.8	1.0	0.27	0.82	floral
2-methylpropyl acetate	1.7	7.9	0.34	5.5	Fruity, sweet, banana
Butyl acetate	1.6	1.4	3.3	12	pear
Ethyl pentanoate	1.6	0.29	0.53	2.2	Yeast, fruit
γ-decalactone	1.4	1.1	0.073	0.086	Peach, fat
Pentyl acetate	1.4	1.9	0.94	2.4	herbaceous
Butyl butanoate	0.15	0.038	1.76	3.07	Fresh, sweet, fruity
Total OAV	3335	1840	2190	5660	

If the OAV results of dynamic headspace (Table 2) and static-HS-SPME (Figure 2-left) are considered, it can be seen that no aldehydes and alcohols had OAV>1, whereas other compounds such as esters, terpenoids and sulfur compouds made up almost all the OAV. In the dynamic headspace experiment (Nuzzi *et al.,* 2008) in Darselect (DA), Eva (EV) and Alba (AL) genotypes the majority of the OAV belonged to esters (84.6- 90.5%), while in Dora (DO) sulfur compounds made up 59.3% of total OAV. These OAV patterns were also confirmed by GC-O profiles (Figure 3): in all the genotypes the GC-O profile was dominated by the ester descriptor, with the lower total intensity for DO, which had the higher intensity for cabbage descriptor. If the static-HS-SPME data are considered (Bianchi *et al.,* 2014), in Profumata di Tortona (PdT) 88% of the total OAV belonged to esters, and 10.3% to terpenoids, in contrast to RDV for which 17% of total OAV belonged to esters, 27.6 % to terpenoids and 55.2% to methoxyfuraneol. The two *F. x ananassa* genotypes were characterized by the highest value for total OAV (Onda: 17380, VR177, 9135) with 95.2% (Onda) and 77.4% (VR177) of total OAV belonging to terpenoids. These differences in the OAV patterns were confirmed by GC-O profiles (Figure 2-right): VR177 selection had the highest intensity for floral, sweet and citrus descriptors and RDV for fruity

CONCLUSIONS

Our results confirmed the major influence of the volatile compound sampling method on the odour profiles as assessed either by the calculation of OAV from quantitative data, or by GC-O analysis. In fact, in total extracts the majority of the OAV belonged to high molecular weight aldehydes and lactones, responsible the former for the herbaceous, green odour notes, and the latter for the creamy, sweet, coconut-like odour notes. In contrast these compounds are not easily sampled by the headspace techniques. Instead, by using the dynamic headspace sampling along with esters, responsible mainly for the fruity odour, it was possible to sample the sulfur compounds associated to onion and cabbage odour

descriptors, while by using the static-HS-SPME, along with esters it was possible to sample terpenoids responsible for the floral, spicy and citrus-like odours.

Whatever the sampling method, OAV patterns were well related to the GC-O profiles. However, by using GC-O a more complete odour pattern could be obtained, as the detection of strong OE was possible even for compounds present in quantities lower than the instrumental detection limit, for which, therefore, OAV could not be computed. Our data on total extracts showed also that GC-O profiles could agree with the sensory profiles obtained by quantitative descriptive analysis.

ACKNOWLEDGEMENTS

In some cases, acknowledgements (for example, for funding support) may be necessary. If so, they should be placed before the reference section.

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SENSORY PROPERTIES OF A NEW ACTIVE FOOD PACKAGING

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ABSTRACT

Packaging is no longer considered a passive component, but it has an active role, interacting with the external environment and with the food inside by the release of active molecules that will extend shelf life providing protection against microbial spoilage and oxidation.

A demonstration project was carried out with the aim of discovering new application for the Italian propolis as ingredient for active food packaging. The products arising from this research convey the propolis active compounds that are not directly embedded into the food and are dispensed over time due to the contact with the food moisture. Two addition methods were considered: surface spreading and incorporation.

Since many components of propolis have a low olfactory threshold, the sensory properties of packaging have to be checked in order to avoid the interference with food flavour. A gaschromatography-olfactometry (GC-O) analysis was carried out to characterise each type of packaging; the prevalence of odour notes such as "balsamic" and "floral" indicated the key role of terpenes in the odour profile of propolis, while other descriptors such as "chemical", "burnt" and "paper" could be referred to the packaging material. Triangle tests were carried out on cheese samples and processed meats to establish whether the developed packagings have an influence on the sensory quality of food. Results indicated that the taste and odour of packed food during a 30 days storage period at 4°C were influenced by the added propolis as well as by the addition method.

Keywords: bioactive compounds, packaging, propolis, gaschromatography-olfactometry, triangle test

INTRODUCTION

The choice of a suitable packaging system is a key factor in the diffusion of a product on the market. In fact, packaging influences the quality of food products for storage, transportation and end-use (Han, 2005). The basic functions of packaging are the containment of food, the protection from quality deterioration, the information supply to consumers (Kensley, 1985). The functions related to visual merchandising have becoming more relevant in recent years, since the consumers' expectations have increased, and packaging is defined as the "silent seller".

In order to protect food from spoilage and oxidation, active packaging systems have been formulated, based on the incorporation of natural anti-microbial and antioxidant agents that are slowly released from packaging into the food. The use of active packaging materials is not meant to be a substitute for good sanitation practices, but it should enhance the safety of food as an additional hurdle for the growth of pathogenic and/or spoilage microorganisms (Cooksey, 2005).

Propolis is a resinous substance collected by *Apis mellifera* that is used in the hive as building material and defensive agent. Due to its complex composition it has many biological properties and it is used as a component of food additives, cosmetics and over-the-counter preparations (Bankova *et al.,* 2014). Furthermore the main volatile compounds in the propolis headspace are terpenoids (Melliou *et al.,* 2007; Popova *et al.,* 2011) with a low olfactory threshold, which may influence the odour of the preparations.

The antioxidant and antimicrobial activity of propolis makes it a suitable component in the formulation of bioactive food packaging (Tosi *et al.,* 2007). It has been proposed as a

chemical preservative in meat products (Han and Park, 1995) and as germicide and insecticide for food packaging (Mizuno, 1989a, 1989b).

Since 2010, a demonstration project was funded by the Italian Ministry of the Economic Development with the aim of developing controlled release packaging systems that act as a "case" and as a "time controlled dispenser" (Mascheroni *et al.,* 2011) for active compounds, not directly embedded to the food and released after the contact with the moisture content of food (Cattaneo *et al.,* 2014).

The aims of this work were to study the sensory characteristics of different types of active packaging, made through the incorporation or the surface spreading of a propolis extract by GC-O (gaschromatography-olfactometry) and to evaluate the influence of these active packagings on the sensory properties of packed food by a triangle test.

MATERIAL AND METHODS

Materials:
- active paper by incorporation of 0.2% propolis extract (API 0.2)
- active paper by incorporation of 0.4% propolis extract (API 0.4)
- active polyethylene paper by propolis surface spreading (APP)
- paper (P) (control 1)
- active PET 12 µm by surface spreading (APLSS)
- PET 12 µm (PL) (control 2)

GC-O analysis:

The olfactometric analysis was carried out by 6 panellists aged between 35 and 55 years. Before the analysis of the samples all panellists attended three training sessions to identify the main odour categories. The following standards were used: $\tilde{}$pinene (resinous), ethyl butanoate (ether, fruity), limonene (citrus, terpene), eucalyptol (fresh, camphor), p-cymene (gasoline, citrus), (Z)-3-hexen-1-ol (herbaceous), benzaldehyde (almond, nut), ☐-caryophyllene (spicy), phenyl ethanol (floral), guaiacol (medicine).

Each sample consisted of a 5 x 5 cm square of the before listed packaging (two replicates for each sample), put in a 20 mL vial closed with an aluminium cap with silicone-rubber septum. The extraction of volatiles was performed by headspace solid-phase microextraction (HS-SPME) using a DVB/CAR/PDMS fiber (absorption step: 40°C for 30 min; desorption step in the injector port: 250°C for 5 min in splitless).

GC analyses were carried out with an Agilent 6890 N GC equipped with a FID (flame inization detector) and a DB-WAX capillary column (60 m × 0.25 mm I.D., 0.25 µm film thickness); injector and FID temperatures, 250 °C; column temperature program: 40 °C x 2 min, 4.5 °C/min until 220 °C held for 10 min. The gaschromatograph is linked to an olfactometric system that includes the Olfactory Detector Port ODP2 Gerstel (Gerstel GmbH) equipped with the ODPneumatics module to control humidification and make-up gas flows; the olfactometric data (intensity on a 5-point intensity scale where 0 = no odour and 5 = very intense odour, duration and area of each odour event [OE]) are collected with the ODP-Recorder software. The area of each OE is calculated by the software from the intensity and duration values and is shown as a chromatographic peak.

Food storage trial and sensory analysis:

Sample preparation: Two processed meat products (prosciutto crudo, dry-cured ham and prosciutto cotto, cooked ham) and three types of cheese (sweet Provolone, piquant Provolone and Fontal) were used in the packaging tests. All foods were purchased from local markets.

The samples are listed in Table 1. The meat products were cut with a slicer (ABM, Milano, Italy) and 3-4 slices per sample were manually wrapped in each paper type. The cheese samples (four Provolone 0.5 mm slices or one Fontal 4 mm slice) were packed in plastic pouches made by a vacuum packaging machine CS27S (VP Vacuum Pump Ltd., Brivio, Como, Italy) with the following operating parameters: vacuum time, 25 s; sealing time, 5 s for PL and 4 s for APLSS.

Table 1. List of packagings used in the food storage trials (subscript p= pouch; s=sheet)

Food material	Control	Tested packagings	Storage times (d)
Dry-cured ham	P	APP	2, 6
Cooked ham	P	APP	2, 6
Dry-cured ham	P	API 0.2	3, 7
Cooked ham	P	API 0.2	2, 4
Dry-cured ham	P	API 0.4	3, 7
Cooked ham	P	API 0.4	2, 4
Fontal cheese	PL_p	$APLSS_p$	7, 14, 30
Sweet Provolone cheese	PL_p+PL_s	$PL_p+APLSS_s$; $APLSS_p+PL_s$; $APLSS_p+APLSS_s$	7, 14
Piquant Provolone cheese	PL_p+PL_s	$PL_p+APLSS_s$; $APLSS_p+PL_s$; $APLSS_p+APLSS_s$	7, 14

The four Provolone slices were detached by sheets of PL or APLSS packaging materials in the four combinations reported in Table 1.

Sensory analysis: A storage period at 4 °C based on average home storage duration was established for each product and triangle tests were performed after half the storage time had passed and at the end of storage.

Tests (Pompei and Lucisano, 1991) were performed by ten semi-trained panellists, members of the CRA-IAA staff, 4 men and 6 women aged from 35 to 60 years. The experimental design was performed by means of the FIZZ software v. 2.45 (Biosystèmes, Couternon, France).

Data analysis:

The triangle test data were analyzed using the significance table indicated by Pompei and Lucisano (1991). The principal component analysis (PCA) of OE areas was carried out with the PAST software (Hammer et al., 2001) v. 2.12 (http://folk.uio.no/ohammer/past/).

RESULTS AND DISCUSSION
GC-O analysis:

The GC-O analysis allows obtaining a chromatogram with the peaks detected by the FID, together with an aromagram that shows the concurrent odorous events detected at the sniffing port. The following OE were detected by the GC-O of all tested products: Chemical (Ch), Terpene-chemical, (TerpCh), Chemical, paper (ChPa), Spicy, (Sp), Undefined (Un), Herbaceous-spicy (HeSp), Herbaceous (He), Herbaceous-burnt (HeBu), Terpene (Terp), Floral-rot (FlR), Spicy-chemical (SpCh). The descriptor "Chemical" was associated to 7 OE, 3 OE were described as "Undefined" and 2 as "Spicy".
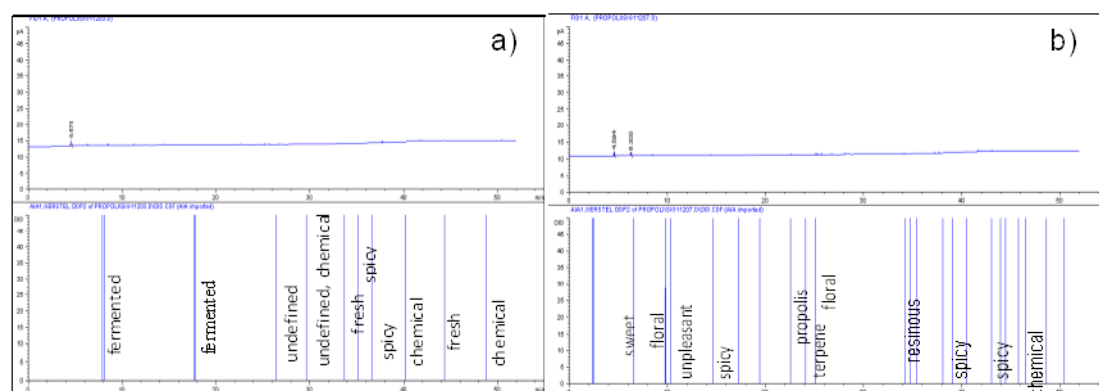
Figure 1. Aromagram of API 0.2 (a) and API 0.4 (b) papers

The FID (above) and ODP (below) peaks of API 0.2 and API 0.4 papers are shown in Figure 1. Even if the FID profile is very poor in both samples, the olfactometric profile showed the presence of several OE and the descriptors related to the propolis incorporation (propolis, resinous, spicy) were, as expected, more frequent in the API 0.4 papers.
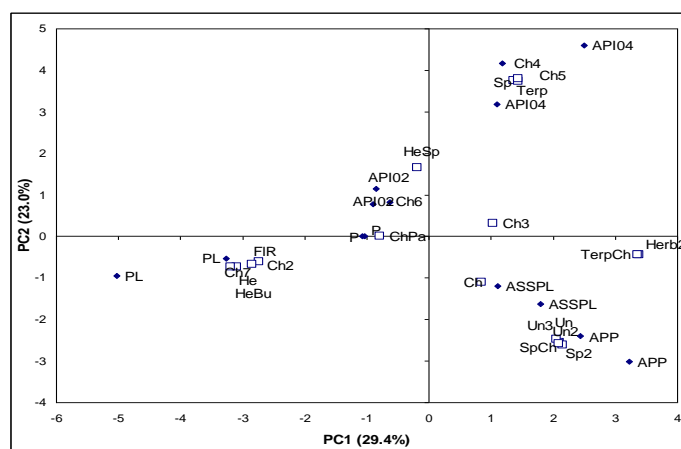
Figure 2. PCA of OE areas

The areas of the odorous events detected in all GC-O analyses of each tested product (control and active packagings) were analyzed with the PCA; 5 PCs were extracted, explaining 90% of total variance. The biplot made with the first two components explained 52% of total variance (Figure 2). PC1 divided control materials (P and PL) and API 0.2, with negative values, from all the other active packagings that showed positive values. PC2 distinguished paper materials P, API 0.2 and API 0.4, with positive values, from plastic materials and active polythene paper, with negative values. The descriptors related to the propolis, as expected, were associated with the active packaging samples: "spicy", "terpene" characterized the API 0.4 samples, while APP and ASSPL were associated with "spicy, chemical" and "spicy 2" and the untreated paper with "chemical, paper".

Food storage trial and sensory analysis:

The results of the sensory analysis of meat products are shown in Table 2. No significant differences were detected among dry-cured ham samples packed in control or active packaging. However, at the end of the storage the cooked ham samples packed in API 0.2 and APP resulted different from control, and unexpectedly not the samples packed in API 0.4. In effect, we observed in the cooked ham samples a high moisture content that could have made this product more effective than dry-cured ham in absorbing the released packaging compounds.

Table 2. Meat products: triangle test results (n.s., not significant; *, α =0.05; **, α =0.01)

	Cooked ham		**Dry-cured ham**	
Packaging	**Days of storage**	**α**	**Days of storage**	**α**
API 0.2	2	n.s.	3	n.s.
API 0.2	4	*	7	n.s.
API 0.4	2	n.s.	3	n.s.
API 0.4	4	n.s.	7	n.s.
APP	2	n.s.	2	n.s.
APP	6	**	6	n.s.

In Tables 3 and 4 the results of the sensory analyses on cheese products are shown. The panelists did not detect differences between active and control packaging of Fontal (Table 3), even after 30 days of storage. The other types of cheese (Table 4) showed a different interaction with the packaging: significant differences were detected in both cases, especially at the end of the storage period. No differences were detected in the middle of storage period between the control and the package made with control pouch and active sheets. Unexpectedly, the packaging with APLSS pouch and sheets interfered less than the APLSS pouch with PL sheets, except for the sweet Provolone in the middle of the storage period.

Table 3. Fontal cheese: triangle test results (n.s., not significant; *, α =0.05; **, α =0.01)

Packaging	Days of storage	α
APLSS	7	n.s.
APLSS	14	n.s.
APLSS	30	n.s.

Table 4. Provolone cheese: triangle test results (n.s., not significant; *, α =0.05; **, α =0.01)

Packaging	Days of storage	α	
		Sweet Provolone	Piquant Provolone
PL pouch, APLSS sheets	7	n.s.	n.s.
APLSS pouch, PL sheets	7	n.s.	**
APLSS pouch and sheets	7	**	*
PL pouch, APLSS sheets	14	*	*
APLSS pouch, PL sheets	14	**	*
APLSS pouch and sheets	14	*	n.s.

CONCLUSIONS

The GC-O results indicate that the propolis addition method to the packaging material has a major influence on the olfactometric profile. In fact the APP and APLSS packagings, made with the surface spreading method, had similar GC-O profiles, different from those of paper packagings prepared by propolis incorporation. This difference was observed also in the food storage trials: the APP packaging influenced the sensory properties of cooked ham, but not of dry-cured ham, and the APLSS pouches did not affect the sensory characteristics of Fontal chesse, but those of Sweet and Piquant Provolone cheese. These results suggest that the food composition itself palys a role in the exchange phenomena occurring between the food matrix and the active packaging.

In addition it was confirmed by the GC-O results that the human nose is more sensitive than the instrumental detectors (Falcão *et al.,* 2008), highlighting that the olfactometric analysis should be used in the development of food packaging incorporating odouros active ingredients to foresee the impact on the packed food.

As suggested by the results of this study, the triangle test can indicate which type of food is sensorially modified by the active packaging. However, this test is not descriptive. It could be worthwhile to repeat the sensory analysis with a larger panel and with descriptive tests in order to better understand the interaction between food and packaging.

ACKNOWLEDGEMENTS

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FERMENTATIVE ACTIVITY AND VIABILITY OF IMMOBILIZED PROBIOTIC STARTER CULTURE ABY-6 IN WHEY BASED SUBSTRATES

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ABSTRACT

The aim of this paper was to study fermentative activity by free and immobilized probiotic ABY-6 culture in whey substrate enriched with milk. Viability of free and immobilized probiotic cells in simulated gastrointestinal conditions was evaluated. In order to improve probiotic character of immobilized cells, the influence of beads wall material composition (alginate, alginate beads with cells coated by whey protein and chitosan–coated alginate beads) was examined. The beads were prepared using electrostatic extrusion technique. Alginate cell suspension was added dropwise by a syringe pump into a gelling solution. After 5.5h of fermentation, enumeration of viable cells was performed by determining the number of colony-forming units on MRS agar plates after incubation at 37 °C for 48 h using pour plate technique on MRS agar.
The results showed that immobilized cells beads have much better potential for survival in gastrointestinal conditions than free cells. Immobilization of probiotic cells in calcium alginate beads coated with chitosan resulted in better survival than both type of non-coated beads in simulated gastrointestinal conditions. These beads can be used to improve preservation of probiotic cells in fermented whey based beverage.
Keywords: whey, probiotic, immobilization, alginate, chitosan

INTRODUCTION

In the recent decade there has been an explosion of probiotic health-based products. After applications of probiotics in food such as milk and dairy products, new application of probiotics has been proposed: chocolate, bread, fermented vegetables. Unfortunately, many studies indicated that there is poor survival of probiotic bacteria in these products. The use of immobilized lactic acid bacteria of starter cultures in the conventional and especially nonconventional dairy products is one of the applications for next decade. Cell immobilization confers protection to sensitive probiotic lactic acid bacteria from oxygen, freezing and acidic conditions during manufacture and gastrointestinal transit. In dairy industry, immobilization has been applied to improve survival and delivery of bacterial cultures.

MATERIAL AND METHODS

Culture and media

Commercial lyophilized dairy starter culture that is known as 'Lactoferm ABY 6' used in this work, was supplied by Biochem s.r.l. (Monterotondo, Roma, Italy). Starter culture is mixture of *Streptococcus salivarius ssp. thermophilus* (80 %), *Lactobacillus acidophilus* (13 %), *Bifidobacterium bifidum* (6 %), *Lactobacillus delbrueckii ssp. bulgaricus* (1 %). Whey remained after cheese production and sterile skim milk with 0.5 % fat was obtained from domestic dairy plant Imlek a.d. (Belgrade, Serbia). After collection, whey was stored at -18 °C until use (no longer than one week). The chemical composition of whey was as follows: total solids 9.8 ± 0.03 % (w/v); protein 2.6 ± 0.012 % (w/v); fat 1.05 ± 0.08 % (w/v) and lactose 5.6 ± 0.114 % (w/v).

Extrusion technique

Alginate beads were produced using an electrostatic extrusion technique. 4 g sodium alginate (Sigma, Aldrich USA) were dissolved in 150 mL of distilled water, pasteurized at 70 °C for 30 min. 1% ABY-6 commercial culture diluted in 100 mL of whey was mixed with 150 mL of the sodium alginate solution. 250 mL of the alginate-cell suspension containing 1.66 % sodium alginate was added dropwise by syringe pump (Racel, Scientific Instruments, Stamford, CT, USA) to the solution of 2 % (w/v) $CaCl_2$ (Acros organics, USA). Alginate drops solidified upon contact with $CaCl_2$, formed beads and thus entrapped bacterial cells. The beads were allowed to harden in gelling solution for 30 min. The same technique was used for WPC-alginate beads, 2% ABY-6 culture diluted in 50mL of whey, mixed with 50mL 15 % (w/v) WPC (Whey protein concentrate) solution (DMV International, Netherlands) and then mixed with 150mL of the sodium alginate solution.

Coating drops

Coating procedures followed the methods reported by Zhou *et al*. (1998) 0.4 g of low-molecular-weight chitosan (Acros organics, USA) was dissolved in 90 mL distilled water acidified with 0.4 mL of glacial acetic acid to achieve a final concentration of 4 g L^{-1}. The pH was then adjusted to between 5.7 and 6.0 by adding 1M NaOH. The mixture was filtered through Whatman #4 filter paper. Volume was adjusted to 100 mL before autoclaving at 121 °C for 20 min. 15 g of washed beads were immersed in 100 mL of chitosan solution with gentle shaking at 100 rpm for 40 min on an orbital shaker for coating. The chitosan-coated beads were washed and used instantly.

Fermentation

Experiments were conducted in 100 mL Erlenmeyer flasks with 50 mL working volume. Medium contained 70 % pasteurized whey and 30 % sterilized milk with 0.5 % of fat. Sample was inoculated by adding 6 % (v/v) ABY-6 commercial culture, previously activated by diluting 1 % (w/v) ABY-6 commercial culture in sterile milk with 0.5 % of fat, for 30 min at 42 °C. Samples with encapsulated beads were inoculated by adding 6 % (w/v) ABY-6 commercial culture encapsulated in beads. All samples were incubated at 42 °C. Fermentations were stopped by quick cooling when pH between 5.0 and 4.5 was reached.

Acid and bile tolerance (Probiotic properties)

After fermentation, cells were inoculated at 2 % (v/v and w/v for beads) in MRS broth and incubated under anaerobic conditions at 37 °C for 0, 60, 120, 180 and 240 min. To determine acid tolerance, probiotics were grown in MRS broths with pH adjusted to 2.5 and 3.0 using 1M HCl. Bile tolerance was determined by growing the probiotics in MRS broth supplemented with 0.3 % dehydrated bovine bile (Torlak, Serbia). Probiotics were inoculated in MRS broth as control. The numbers of viable cell were determined on MRS agar plates.

Viable cell enumeration

Cell number of *S. salivarius subsp. thermophilus* was determined by pour plate counting method on M17 agar. Cell number of *L. delbrueckii ssp. Bulgaricus, L. acidophilus* and *Bifidobacterium bifidum* was determined by pour plate counting method on MRS agar (Vrbaški and Markov, 1993). Cell number expressed in log (CFU mL^{-1}) for free cells and log (CFU g^{-1}) for beads. The beads were dissolved in (2 %, w/v) sodium citrate.

Titratable acidity and pH

Titratable acidity was determined by the Soxhlet-Henkel method (Varga, 2006) and the pH value was measured using a pH meter (Inolab, WTW 82362, Wellheim, Germany).

Statistical analysis

Experiments were performed in triplicate. All values are expressed as mean ± standard deviation. Mean values were analysed using two-way ANOVA. The Tukey post hoc test was

performed for means comparison (Origin Pro 8 (1991-2007) computer package, Origin Lab Co., Northampton, USA).

RESULTS AND DISCUSSION

Fermentation of whey by commercial cultures designed for yoghurt production could be an interesting way for including whey in human consumption. Encapsulating the bacterial culture brings many benefits such as: cells can be isolated from the original substrate and used again for the fermentation. Also cells are protected from external factors and they have a greater ability to survive in the human intestinal tract. The ability to survive adverse environmental conditions and course of fermentation were examined.

Fermentation with free cells takes 1.5 h less than fermentation with encapsulated cells. It is the result of difficulties in the exchange of nutrients between medium and cells inside of drops. Titratable acidity is lower and pH decrease during fermentation is slower. Growth of culture in these conditions is also slightly lower. It is shown in Table 1.

Table 1.Cell number before and after the fermentation of whey with ABY-6 culture, pH and titratable acidity after the fermentation

	Free cells	Alginate beads	WPC-alginate beads	Chitosan coated beads
Before fermentation (log cfu g^{-1})	7.429 ± 0.22	8.133 ± 0.21	7.4487 ± 0.19	7.977 ± 0.18
After fermentation (log cfu g^{-1})	9.052 ± 0.18	9.274 ± 0.21	8.5514 ± 0.22	9.199 ± 0.21
pH	4.50 ± 0.10	4.60 ± 0.05	5.13 ± 0.08	4.77 ± 0.12
Titratable acidity (°SH)	19 ± 0.20	14.8 ±0.25	12 ± 0.20	12 ± 0.20
Fermentation time (h)	4.0	5.5	5.5	5.5

[*]*Cell number before and after the fermentation for free cells is expressed in log cfu ml^{-1}*

Probiotic property

Bile tolerance

Bile tolerance is often used as a criterion for probiotic strain selection. In order to exert a beneficial effect in the digestive tract, probiotic culture must survive passage through the stomach and be tolerant to the bile salts concentrations in the small intestine (Sanders, 2000). The relevant physiological concentration of human bile is about 0.3 % (Dunne *et al.*, 2001). Bile salt solution was used here to determine whether encapsulation and coating of the alginate beads would increase survival of cells in this environment, which is similar to that of the digestive system. The results of analysis of probiotic characteristic of beverages with free ABY-6 yogurt culture, with entrapped cells in alginate beads and coated beads are given in Figure 1. Free cells had a significant reduction in the number of viable cells in 2 hours there were 85.15 % of viable cells compared to the initial number and in the fourth hour the number of viable cells decreased to 83.0 % (Figure 1a). The Figure 1b shows that samples with alginate beads had 94.54 % surviving cells after 4 h. Samples with coated beads had 95.86 % survival rate after 4 h (Figure 1c). Samples with WPC-alginate beads had 93.0 % survival cells after 4 h. As shown in Figure 1, samples with alginate beads, WPC-alginate beads and coated beads have significantly higher probiotic viable cell count than samples with free cells, during 4 h of monitoring survival. The chitosan coating provides the best protection in bile salt solution because an ion-exchange reaction takes place when the beads absorb bile salt (Murata *et al.,* 1999) and an insoluble complex between chitosan and bile salt forms in the chitosan–alginate membrane. This can decrease the diffusion of bile salt into the beads and consequently protect encapsulated cells from interacting with the bile salt.

Krasaekoopt *et al.* (2004) reported that for *L. acidophilus*, the survival of cells in both coated and uncoated beads was significantly (p<0.05) better than that of free cells, for *B. bifidum*. Yu *et al.* (2001) also reported that *B. bifidum* entrapped in alginate beads containing chitosan had higher viability than in alginate without chitosan, which were similar to results in this paper.

Acid tolerance

Acid tolerance of probiotics is important not only for resistance to gastric pH, but is also prerequisite for their use as dietary adjuncts and enables strains to survive for longer periods of time in high acid carrier foods such as yogurt, without large reduction in viable cell numbers (Kim *et al.*, 2006). The gastric pH in healthy humans is about 2.5. This causes the destruction of most microorganisms ingested (Lankaputhra and Shah, 1995). Lactic acid starter bacteria showed low resistance to simulated gastric juice and low pH environment, *Lb. delbrueckii ssp. bulgaricus, Lb. acidophilus, S. thermophiles* showed losses in cell viability ranging from 0.7 to more than 6.0 log (Vinderola and Reinheimer, 2003) As demonstrated by Sultana *et al.* (2000) aciduric members such as *L. acidophilus,* generally could not survive in low pH environment. Low pH environments are thought to inhibit the metabolism activity and growth of *L. acidophilus*, thus reducing the probiotics' viability. Sultana *et al.* (2000) found that encapsulation of probiotics in alginate beads did not effectively protect the organisms from high acidity, but in this paper it was shown that alginate does protect probiotic culture from high acidity. The survival of free and encapsulated probiotic culture at pH 2.5 and 3.0 were compared (Figure 1). Free cells had a drastic reduction in the number of viable cells in 2 hours (50 %) and in the third hour there were no living cells at pH 2.5 (Figure 1). The Figure 1b shows that samples with alginate beads had 36.18 % surviving cells after 4 h. Samples with coated beads had 37.8 % surviving cells after 4 h (Figure 1c). Samples with WPC-alginate beads had 40.0 % surviving cells after 4 h As shown in Figure 1, samples with alginate beads, WPC-alginate beads and coated beads had significantly higher probiotic viable cell count than samples with free cells, during 4 h of monitoring survival at pH 2.5. At pH 3.0, samples with free cells had 72 % surviving cells and samples with alginate beads, WPC-alginate beads and coated beads had more than 96 % of cells survived after 4 h.
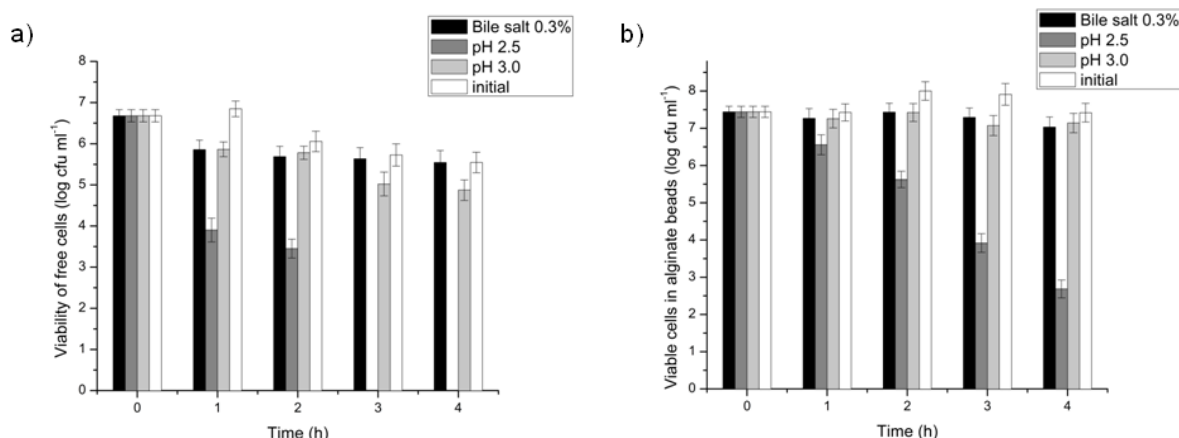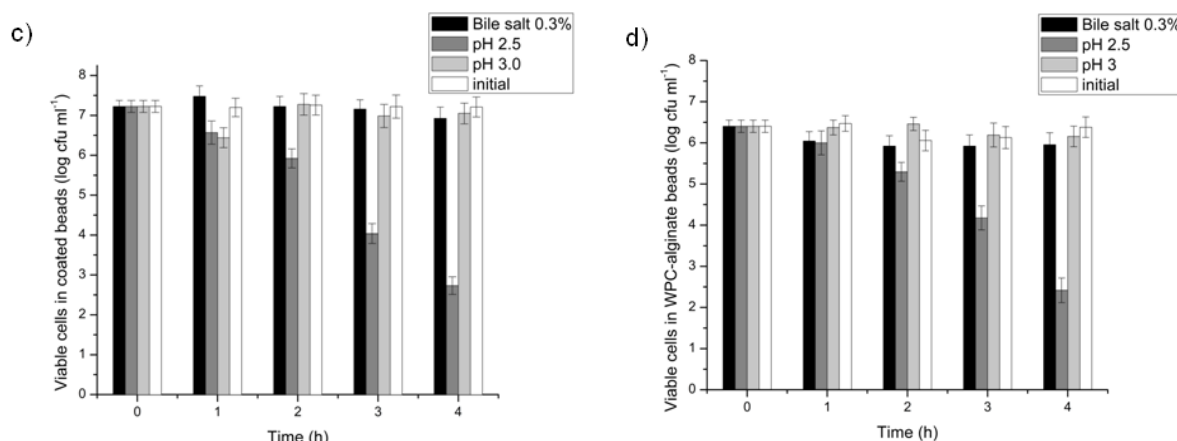
Figure 1. Number of surviving cells (log CFU/mL) for free (a), encapsulated in alginate beads (log CFU/g) (b), encapsulated in chitosan-coated alginate beads (log CFU/g) (c), encapsulated in WPC-alginate beads (d) during 4h anaerobic incubation (37 °C) in MRS broth, MRS broth with pH 2.5, MRS broth with pH 3.0 and MRS containing 0.3% bile salt. Mean values ± standard deviation

CONCLUSIONS

The present study has shown that survival of probiotic cultures is better when cells are encapsulated in alginate beads. Encapsulation significantly improves bacterial survival in simulated gastric environment, and allows viable cells to reach a beneficial level as probiotic. Consequently, chitosan-coated alginate beads could be a good way to administer these beneficial microorganisms in production of functional beverages.

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INFLUENCE OF FRUIT JUICE ADDITION ON QUALITY OF FERMENTED WHEY-BASED BEVERAGE

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ABSTRACT

The aim of this study was to evaluate the influence of fruit juice addition on the quality of fermented whey-based beverages that contain commercial ABY-6 starter culture. Milk (30%) and Cornelian cherry fruit juice (30%) were used for beverages formulation. Cornelian cherry fruit was used based on high antioxidant activity and specific texture that can improve overall quality of beverages. After 4h of whey fermentation followed by fruit juice addition, overall quality of beverages was evaluated by determining the viable cell count, pH value, titratable acidity, antioxidant activity and syneresis of produced beverages. Stability of beverages was monitored during 28 days of storage.

Based on the results fruit juice does not have statistically significant influence on viable cell count of probiotic bacteria. On the other hand, fruit significantly improves antioxidant activity and syneresis of beverages. Antioxidant activity reaches level of 88.6-94.5% in beverages formulated with fruit juice addition in contrast to the level of 36-36.7% without added fruit juice. Addition of fruit juice decreases syneresis of beverages for 9.5-11.9% after fermentation and for 9.5-25% after 28 days of storage. Titratable acidity and pH value of beverages were significantly affected by fruit juice addition but remain within recommended levels and stable during the whole storage period.

To formulate healthy beverage, supplementation of whey with 30% milk and 30% of Cornelian cherry fruit juice is advisable. Obtained fruity whey-based beverage has desirable quality attributes such as antioxidant activity of 97.0% and syneresis of 54.1%. Beverage contains 6.89 log (CFU mL^{-1}) probiotic bacteria, has a shelf life of at least 14 days and expresses quality that meets consumers' demands.
Keywords: whey, ABY-6, fruit, syneresis, probiotics

INTRODUCTION

A fermented dairy beverage, made with milk and whey, is an important nutrient source due to presence of biological valuable proteins that is mostly derived from whey as major raw material (Sanmartín *et al.*, 2011)

Fermented whey beverages represent products that are rapidly taking their place in the market of dairy products. Whey as natural substrate can replace much of significant organic and inorganic elements of body fluids lost during exercise, sweating or aging. Regardless of existing quality, whey can be further enriched by combining with fruits or fruit juices. Production of whey based fruit beverages seems to be the most logical way for enhancing the nutritional and health value of both components (Prendergast, 1985; Singh *et al.*, 2005).

The manufacture of whey based fruit beverages requires the mixing of appropriate fruit juices and minimally processed whey to develop sensory, nutritionally and medicinally acceptable beverages. Fruits are a good source of natural antioxidants, containing many different antioxidant components, which provide protection against harmful free radicals and have associated with lower incidence and mortality rates of cancer and heart diseases in addition to a number of other health benefits (Wang *et al.*, 1996; Shui and Leong, 2006).

Cornelian cherry (Cornus mas L.) is characterized by a large amount of vitamin C (up to 145 mg per 100 g of fruit) (Jacimovic and Bozovic, 2007) and it is used in traditional Chinese

herbal medicine as tonic, analgesic and diuretic. It contains pectin, tannins, organic acids, anthocyanins and antioxidants with high anti-inflammatory, anti-cancer and anti-diabetic activities acknowledged by modern medicine. Cornelian cherry fruit juice has a very impressive and attractive aroma and luxurious, quite enigmatic velvety sense in terms of taste. This spirit of taste is quite different in comparison with tastes of other fruit juices. The fruit is available in plenty during the season. Due to excellent nutritive value and medicinal properties especially in combination with whey, it shows great potential for processing into valuable products (Demir and Kalyoncu, 2003; Kean and Hwan, 1998; Mau *et al.*, 2001).

MATERIAL AND METHODS

Culture and media

Commercial lyophilized dairy starter culture 'Lactoferm ABY 6' used in this work was supplied by Biochem s.r.l. (Monterotondo, Roma, Italy). Starter culture is mixture of *Streptococcus salivarius* ssp*. thermophilus (*80%), *Lactobacillus acidophilus (*13%), *Bifidumbacterium bifidum* (6%), *Lactobacillus delbrueckii* ssp. *bulgaricus* (1%). For each experiment, 1% (w/*v*) of starter culture was gently dissolved in sterilized skimmed milk (0.5% fat) and activated 30 min at 42 °C. After activation 6% of inoculum was added into the fermentation medium.

Whey remained after cheese production and sterile skim milk with 0.5% fat were obtained from domestic dairy plant Imlek a.d. (Belgrade, Serbia). After collection, whey was stored at -18 °C until use (no longer than one week). The chemical composition of whey: total solids 9.80 ± 0.03 % (w/v), protein 2.60 ± 0.012 % (w/v), fat 1.05 ± 0.08 % (w/v) and lactose 5.60 ± 0.114 % (w/v).

Cornelian cherry fruit juice was obtained from domestic plant Foodland d.o.o. (Belgrade, Serbia) in pasteurized form. Chemical composition of Cornelian cherry fruit juice (by manufacturer's specification): proteins 0.014 % (w/v), fat 0.014 % (w/v) and carbohydrates 2.34 % (w/v).

Experimental procedure

Whey-milk mixture 70:30 (%, *v/v*) was pasteurized at 60 °C for 60 min and cooled at fermentation temperature (42 °C). Flasks containing 70 mL of formulated beverages were incubated at 42 °C in a water bath. During the incubation, samples were taken every 1h for determination of pH value. The fermentations were carried out until pH = 4.6 was attained. When pH = 4.6 was reached fermentations were stopped by quick cooling and fermented mixture were suspended with 30 mL of Cornelian cherry fruit juice. Obtained beverages were stored at 4 °C for 28 days. Analysis of titratable acidity (TA, °SH), pH value, viable cell count (log (CFU mL^{-1})), syneresis (%), antioxidant activity (%) and overall acceptability was carried out during production and 28 days of storage for whey beverage (WB) and fruit whey beverage (FB).

Chemical analysis

Titratable acidity was determined by the Soxhlet-Henkel method (Varga, 2006), and the pH value was measured using a pH meter (Inolab, WTW 82362, Wellheim, Germany).

Antioxidant activity

The antioxidant activity of fermented whey-based beverages was determined by its ability to scavenge DPPH radical, which was measured according to the method described by Balakrishnan and Agrawal (2012) with slight modifications. A stock solution of DPPH was prepared by dissolving in methanol (0.1 mM). After 4h fermentation, samples were macerated with methanol and centrifuged at 8000 rpm for 20 min at 4 °C. Methanol (1.5 mL) and DPPH (1.0 mL) were added to the supernatant (0.5 mL). Control sample was prepared by mixing methanol (1.5 mL) and DPPH (1.5 mL) while methanol was used as blank. Mixtures were allowed to stand 30 min in dark, at room temperature. The antioxidant activity was analyzed by reading the absorbance at 517 nm. Scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(A_c\ A_a)/\ A_c]\text{x}100 \qquad (1)$$

Where *Aa* and *Ac* represent absorbance of sample and control, respectively.

Microbiological analysis

One milliliter of fermented sample was diluted in 9 mL of sodium chloride (0.85%, w/v), and mixed uniformly. Subsequent serial dilutions were prepared and viable cell count was determined using pour plate technique. MRS-agar and anaerobic incubation at 37 ˚C for 48 h were used for the enumeration of viable cell count (Dave & Shah,1996).

Syneresis

Syneresis of fermented samples was determined according to the method of Keogh and O'Kennedy (1998) with slight modifications. The fermented samples (20.0 mL) were centrifuged at 1000 rpm for 10 min at 4 ± 1 °C. Collected supernatant was drained, weighed and the following equation was used for syneresis calculation:

$$\text{Syneresis (\%)} = (\text{Weight of supernatant (g)}\ /\ \text{Weight of fermented sample (g)})\text{x}100\% \qquad (2)$$

Statistical analysis

Experiments were performed in triplicate. All values are expressed as mean ± standard deviation. Mean values were analyzed using two-way ANOVA. The Tukey post hoc test was performed for means comparison (Origin Pro 8 (1991-2007) computer package, Origin Lab Co., Northampton, USA). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Changes in pH and titratable acidity (TA, ˚SH) during fermentation and storage period are specific for every product and depend on the microorganisms used for formulation as well as on the substrate composition.

Figure 1 show changes in pH, titratable acidity and viable cell count of whey beverage without fruit juice (WB) and whey beverage with added fruit juice (FB) during 28 days of storage at 4 ˚C.
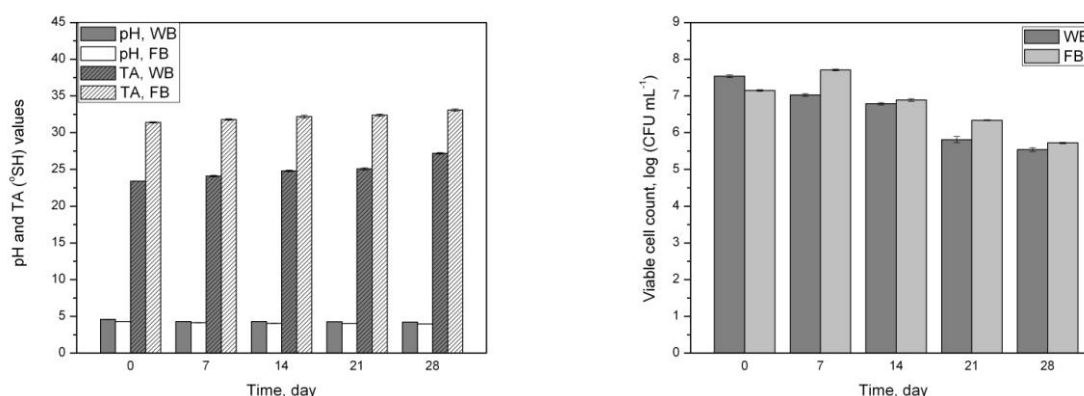
Figure 1. pH, titratable acidity and viable cell count of whey beverage without fruit juice (WB) and whey beverage with added fruit juice (FB) during 28 days of storage at 4 ˚C

As shown in Figure 1 a gradual decrease of pH was observed in all samples during 28 days of storage. Declining of pH can be attributed to the residual activity of microorganisms. The mean values of pH in sample FB, obtained by fruit juice addition, were ranged from 3.93 to 4.29 and were significantly ($p < 0.05$) lower than in sample WB. Similar pH changes was

observed by other researchers who conducted studies with yogurt prepared with *S. thermophilus*, *L. acidophilus*, *B. bifidum*, *L. casei* or other probiotic microorganisms (Gilliland *et al.,* 2002; Dave and Shah, 1997a; Gueimonde *et al.,* 2004). Observed results could be explained by fact that fruit juice (pH=3.82) additionally reduces pH value of sample FB. Additional amount of carbohydrates present in fruit juice probably promote microbial activity and increase amount of produced lactic acid. This assumption is in consistency with results obtained for titratable acidity. As shown in Figure 1 titratable acidity in sample FB was higher (32.6 ^{o}SH) than in the sample WB (27.2 ^{o}SH) at the end of storage period. Similar to pH changes, observed acidity changes could be attributed to the residual activity of the LAB.

Due to effect of low temperature used in the storage process Rojas-Castro *et al.* (2007) reported that acidification prolongs during storage but becomes less intense. The pH and titratable acidity stabilize at the end of storage period, probably due to the enzymatic activity inhibition, decreased viable cell count or possibly to depletion of carbohydrate present in the substrate. Nevertheless, titratable acidities of both samples were far below range of 53.0 ^{o}SH at which unpleasant acid taste could be detected (Pinthong *et al.,* 1980; Kehagias and Dalles, 1984).

In addition, as shown in Figure 1, viable cell count was found to be 7.54 log (CFU mL^{-1}) in sample WB and 7.15 log (CFU mL^{-1}) in sample FB at the beginning of the storage period. Difference in viable cell count between samples was not statistically significant (p>0.05). Viable cell count was different between samples during the whole storage period, probably due to the higher amount of carbohydrates present in the sample FB. Highest viable cell count was recorded in sample FB at 7th day of storage and it was 7.71 log (CFU mL^{-1}). Greatest contribution of fruit juice addition was recorded at day 21st in sample FB that still possess probiotic character in contrast to the sample WB where viable cell count drop below 6.0 log (CFU mL^{-1}). However, results recorded during whole storage period suggest that fruit juice does not have statistically significant influence on viable cell count. It could be concluded that sample FB has a satisfactory viable cell count of 6.89 log (CFU mL^{-1}) until 14th day.

Figure 2 shows changes in syneresis and antioxidant activity in whey beverage without fruit juice (WB) and whey beverage with added fruit juice (FB) during 28 days of storage at 4 ^{o}C.
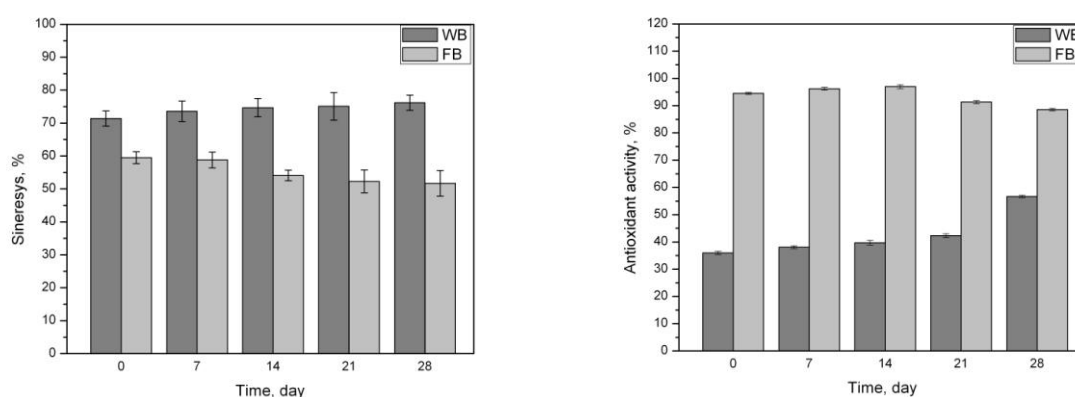
Figure 2. Syneresis and antioxidant activity in whey beverage without fruit juice (WB) and whey beverage with added fruit juice (FB) during 28 days of storage at 4 ^{o}C

As shown in Figure 2, immediately after fermentation (zero day), syneresis of sample FB was about 10.0% lower than syneresis of sample WB. In addition, syneresis of sample FB gradually decreases during 28 days of storage. At the end of storage period syneresis of sample FB was about 50.0%. On the other hand, syneresis of sample WB gradually increases during storage period and reaches about 75.0% at day 28th. Obtained results could be explained by presence of pectin in sample FB. Pectin, as anionic hydrocolloid, probably interacts with positive charges on the surface of casein micelles and rearranges

protein network formed during fermentation (Everett and Mcleod, 2005). Pectin adsorbs to the surface of micelles and promotes stabilization of gel matrix (Kouame *et al.*, 2010). Weaker polypeptide chain interaction, caused by pectin presence, leads to the uptaking of water inside the gel and therefore decreases syneresis of beverage (Zúñiga and Troncoso, 2012).

As shown in Figure 2, sample FB has significantly (p < 0.05) higher antioxidant activity than sample WB, after fermentation as well as during whole storage period. This observation could be explained by fact that antioxidant components present in the fruit juice in large quantity, were transferred to beverage thus contributes to increase antioxidant activity. In sample WB antioxidant activity increases during storage and at the 14th day (selected as shelf life according to viable cell count) of storage reaches level 39.7%. After this period, significantly (p < 0.05) faster increase of antioxidant activity was recorded in sample WB, probably due to release of intracellular antioxidant peptides by cell lysis. On the other hand, antioxidant activity increases in sample FB during first two week and reaches level of 97.0%. This value can be attributed to the combined effect of antioxidant components originating from fruit juice and bacterial metabolites. After this period antioxidant activity start to decline and at the end of storage reache value of 88.6%. Decrease of antioxidant activity is probably caused by destrucion of ascorbic acid. These findings correspond with literature data, although measured on plant based food products. Decrease in antioxidant activity of tropical fruit and juices (orange, mango) during storage was related to ascorbic acid destruction (Klimczak *et al.*, 2007, Plaza *et al.*, 2006, Yang *et al.*, 2007, Shivashankara *et al.*, 2004).

CONCLUSIONS

To formulate healthy beverage, supplementation of whey with 30% milk and 30% of Cornelian cherry fruit juice is advisable. Obtained fruity whey-based beverage has desirable quality attributes such as antioxidant activity of 97.0% and syneresis of 54.1%. Beverage contains 6.89 log (CFU mL^{-1}) probiotic bacteria, has a shelf life of at least 14 days and expresses quality that meets consumers' demands.

ACKNOWLEDGEMENTS

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QUALITY EVALUATION OF SPELT COMPOSITE BREADS PREPARED WITH VARIOUSLY PROCESSED AMARANTH SEED

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ABSTRACT

In this work, the capacity of obtaining high quality amaranth-spelt composite breads by using variously treated amaranth seed was analyzed. Amaranth grain was 1) milled to obtain raw flour; 2) popped and milled to obtain flour from popped amaranth; 3) popped and steamed to obtain steamed whole popped amaranth grain. In addition, raw amaranth flour and flour from popped amaranth were additionally steamed to obtain steamed variants of these flours. When the most important quality indicators of bread were considered (volume yield, crumb texture and structure), raw and steamed amaranth flour were the most suitable ingredients to incorporate into bread formulations. Their incorporation positively affected most quality parameters: bread yield, volume and crumb hardness. The major disadvantage of steamed amaranth flour was the impairment of crumb elasticity and porosity. The next ingredient with regards to its suitability in breadmaking is steamed whole popped amaranth grain.
Keywords: spelt, amaranth, composite bread, crumb, texture.

INTRODUCTION

The concept of composite products in breadmaking is not new. It was launched in the '60ies of 20th century with the objective to identify combinations of wheat with other non-wheat cereals or pseudocereals providing increased nutritive value and appropriate processing characteristics (Grobelnik-Mlakar *et al.,* 2009). Amaranth grain has been recognized as a promising non-wheat material for composite baked goods (Menegassi, Pilosof and Arêas, 2011; Grobelnik-Mlakar *et al.,* 2008, 2009).

Amaranth belongs to the family of pseudocereals which means that it does not botanically belong to cereals but exhibits similar properties. In ancient times, it used to be a staple crop for the pre-Colombian civilizations. Today, it is underutilized but gains increased interest due to its nutritive value and potential for growing in ecological-organic environment. Its nutritional value is higher than that of most cereals owing to high protein content, balanced amino acid composition, high mineral content, high levels of tocotrienols and squalene (Grobelnik-Mlakar *et al.*, 2009; Sanz-Penella *et al.,* 2013). It was evidenced that amaranth exerted an obvious cholesterol lowering effect in rats and chicken. Amaranth can be consumed in different ways: as toasted, popped, extruded or milled into flour. It can be consumed on its own or as an ingredient in various cereal-based products such as bread, cakes, cookies, muffins, pancakes, etc. Most reported results agree that incorporation levels of up to 25 g/100 g are the most acceptable in breadmaking (Sanz-Penella *et al.*, 2013; Lacko-Bartošová and Korczyk-Szabó, 2012).

This paper investigates the potential of variously processed amaranth grain (milled, popped, steamed and combined) in the production of composite spelt-amaranth breads. All variants of amaranth grain were included at 20% replacement level.

MATERIAL AND METHODS

Material and bread preparation
Spelt flour was purchased from a local producer commited to organic farming (ecological agricultural farm „Jevtić" from Bačko Gradište, Serbia). Amaranth seed (*Amaranthus cruentus*) was obtained from a local market. Amaranth seed was popped using an aluminium

hot plate at 200°C for 10 s. Flour from popped amaranth was obtained by milling popped seeds on a hammer mill (Lab Mill 3100 Perten, Sweden). Raw amaranth flour was prepared by passing whole amaranth grains through a Bühler mill (Switzerland). The bran fraction was discarded. Steaming was performed by mixing a material to be steamed (flour or popped seeds) with a part of hot water, boiling it for a couple of minutes. Before mixing it with other ingredients, the steamed material was cooled to room temperature.

A modified breadmaking procedure (Filipčev *et al.*, 2013) and a standard spelt bread recipe (Filipčev, 2014) was used to develop composite spelt-amaranth breads. Based on literature data and preliminary baking experiments in laboratory, spelt flour was substituted with 20% of different forms of amaranth.

Crumb hardness and elasticity

Texture analysis of crumb was completed on a TA-XTplus texture analyzer (SMS, Surrey, England, UK). Crumb hardness was determined in accordance to the standard AACCI 74-10A method. Crumb elasticity was determined by recording a recovery curve. The measurement was carried out in compression mode at constant force of 0.7N held for 300 s. After this period elapsed, the load was reduced to zero and crumb was let to recover for the same period of time. The pre-, test, and post-test speeds were 1.0/0.5/10.0 mm/s, respectively. Distance-time changes were recorded during measurement (Figure 1). From the distance-time curves, total (D), elastic (R), and plastic (P) deformation can be determined. Relative elasticity was calculated as a ratio of elastic to total deformation (Lambert-Meretei, 2012).
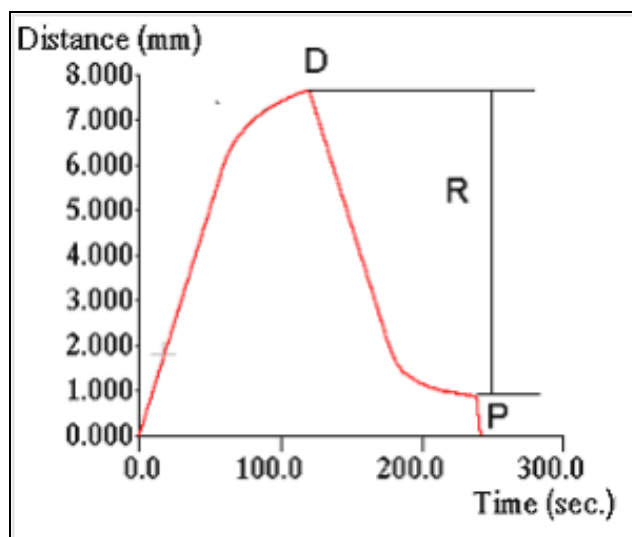
Figure 1 Schematic diagram of typical distance-time curves (elements of recovery part: D-total deformation, R-elastic deformation, P-plastic deformation) (Lambert-Meretei, 2012).

Image analysis of crumb structure

For capturing images of bread crumb, bread slices from the central part of each loaf were scanned on a flatbed scanner (CanoScan LiDE 100, Canon) at 300 dpi image resolution. The analysis of crumb images was performed using Image software. Each color image was converted to an 8-bit gray-scale. Segmentation was carried out applying the Otsu algorhytm. Crumb structure of bread samples was characterized by calculating the number of cells per cm^2, mean cell size and relative porosity (cell-to-total area ratio).

Statistical analysis

All analyses were performed at least in triplicate and mean values were reported. Analysis of variance and Tukey's test were performed to analyse data variation (Statistica 10.0 software package from StatSoft, Tulsa, OK). Means were considered statistically different at $p \leq 0.05$.

RESULTS AND DISCUSSION

The parameters that describe the baking quality of bread are presented in Table 1. The volume yield decreased for samples made with non-steamed and steamed flour from popped amaranth and popped and steamed whole amaranth grain. In other cases, volume yield did not significantly differ from that of the control. The volume yield of breads with the addition of raw and steamed amaranth flour showed even higher volume yield though the increase was not found statistically significant. Addition of raw and steamed amaranth flour and flour from popped amaranth significantly increased the bread yield (146-147.7%) as compared to the control (144%). Baking loss did not differ significantly from that of the control except for it was much lower for bread made with the addition of flour from popped amaranth.

Table 1 Baking parameters for composite spelt-amaranth breads

Amaranth seed processing type	Bread type	Volume yield (cm^3)	Bread yield (%)	Baking loss (%)
-	Control	499.47^c	144.05^a	1.11^b
Milling	Raw amaranth flour	520.89^c	147.74^d	1.00^b
Popping and milling	Flour from popped amaranth	465.28^b	149.89^c	0.45^a
Milling and steaming	Steamed amaranth flour	508.52^c	146.01^b	0.97^b
Popping and steaming	Steamed whole popped amaranth grain	491.04^{b,c}	145.01^{a,b}	1.07^b
Popping, milling and steaming	Steamed flour from popped amaranth	420.89^a	145.01^{a,b}	1.07^b

^{a,b,c} Mean values in a column followed by the same letter do not differ significantly at 5% level of significance.

Important bread quality features are crumb properties, hardness and elasticity. Addition of either raw or steamed amaranth flour significantly softened the bread crumb whereas non-steamed and steamed flour from popped amaranth and steamed whole amaranth grain significantly increased crumb hardness (Table 2). Relative crumb elasticity describes the proportion of recoverable deformation in crumb. Raw amaranth flour and flour from popped amaranth improved crumb elasticity whereas other forms of amaranth significantly decreased elasticity.

Table 2 Crumb properties of composite spelt-amaranth breads

Amaranth seed processing type	Bread type	Crumb hardness (N)	Relative crumb elasticity (%)
-	Control	8.29^{b,c}	79.9^{c,d}
Milling	Raw amaranth flour	6.26^a	78.3^{b,c,d}
Popping and milling	Flour from popped amaranth	10.68^{d,e}	83.5^d
Milling and steaming	Steamed amaranth flour	6.92^{a,b}	63.2^{a,b}
Popping and steaming	Steamed whole popped amaranth grain	8.90^c	65.9^{a,b,c}
Popping, milling and steaming	Steamed flour from popped amaranth	16.22^e	57.6^a

^{a,b,c,d,e} Mean values in a column followed by the same letter do not differ significantly at 5% level of significance.

The results of crumb grain analysis are summarised in Table 3. There were significant differences within several samples with regards to number of cells, mean cell area and relative porosity. The highest difference in cell number was between bread supplemented with flour from popped amaranth flour (101 cells/cm^2) and steamed whole popped amaranth grain (138 cells/ cm^2). Three types of amaranth additions (raw amaranth flour, steamed and

non-steamed flour from popped amaranth) showed a tendency to increase pore size although not significantly in comparison to the control; steamed amaranth flour did not differ from the control regarding pore size whereas steamed whole popped amaranth grain reduced pore size but also not significantly as compared to the control. Regarding relative porosity, bread samples could be classified into 3 groups; significantly highest relative porosity was observed for bread with the addition of raw amaranth flour whereas the lowest relative porosity was in bread with steamed amaranth flour.

Table 3 Crumb structure (digital image analysis) of composite spelt-amaranth breads

Amaranth seed processing type	Bread type	Cells/cm^2	Mean cell area (mm^2)	Relative porosity (%)
-	Control	126[a,b,c]	0.302[a,b]	38.28[b]
Milling	Raw amaranth flour	120[a,b,c]	0.398[c]	47.76[c]
Popping and milling	Flour from popped amaranth	101[a]	0.361[b,c]	36.68[a,b]
Milling and steaming	Steamed amaranth flour	110[a,b,c]	0.303[a,b]	33.35[a]
Popping and steaming	Steamed whole popped amaranth grain	104[a,b]	0.373[b,c]	38.79[b]
Popping, milling and steaming	Steamed flour from popped amaranth	138[c]	0.274[a]	38.24[b]

[a,b,c] Mean values in a column followed by the same letter do not differ significantly at 5% level of significance.

Taking into consideration the more important properties defining the quality of breads, this study showed that raw and steamed amaranth flour are the most suitable ingredients to incorporate into bread formulations. Their incorporation positively affected most quality parameters: bread yield, volume and crumb hardness. Main disadvantage of steamed amaranth flour may arise from its negative impact on crumb elasticity and relative porosity. The next ingredient with regards to its suitability in breadmaking is steamed whole popped amaranth grain as its addition did not deteriorate the volume and crumb hardness in comparison to the control.

Various effects of differently processed amaranth grain on bread quality are probably a consequence of various factors: characteristics of native amaranth starch, degree of starch damage and gelatinization during processing (milling, popping, steaming), the presence of surface-active components, etc. Positive effect of raw and popped amaranth flour on crumb elasticity is usually attributed to the high polar lipid content in amaranth which may exert gas stabilizing effect (Alvareze-Jubete *et al.*, 2010) although popping may cause a 10% decrease in the polar lipid content as shown by Gamel and coworkers (2007) although this was not specifically confirmed in the case of popping *A. cruentus*. The high lipid content in amaranth is also implicated in crumb softening effect.

The negative effect of steaming on crumb elasticity might be due to effect of higher degree of starch swelling caused by steaming. In the process of bread baking, starch in dough serves as a temperature-triggered water sink (Hoseney, 1984) as it absorbs water from gluten by gelatinization, thickens the dough, stabilizes the gas cells and prevents bread from collapse while cooling (Sandstedt, 1961). Starch practically sets the structure of a baked-system. Consequently, the state of the starch contributes greatly to the texture of baked product. In the process of steaming, starch is subjected to moisture-heat treatment. This process causes a physical modification (swelling) of starch without gelatinization and damage to granular integrity (Miyazaki *et al.*, 2006). The swollen starch granule embedded in gluten

network may have discontinued the naturally weak spelt gluten network thus impairing the crumb elasticity.

During popping of amaranth grain, partial gelatinization of starch occurs (Berghoffer & Schoenlechner, 2002). Negative impact of flour from popped amaranth, either steamed or non-steamed, on crumb hardness and elasticity is probably due to higher level of pregelatinized starch or a combination of swollen and partially pregelatinized starch which may worsen the texture of bread crumb. Hoseney (1998) found that limited starch swelling causes softer crumb in regular breads (yeast-leavened wheat breads). Whether pregelatinizated starch will positively affect crumb or not depends on many factors (balance between ingredients in the formulation, starch origin, degree of amylolitic degradation) and both positive and negative effects have been reported in literature.

CONCLUSIONS

In the composite spelt-amaranth breads, variously processed amaranth grain showed different effects on bread quality. The results showed that raw and steamed amaranth flour were the most suitable ingredients to incorporate into bread formulations as they improved bread yield, volume and crumb hardness. The only limitation of steamed flour was that it impaired crumb elasticity to a certain degree. Steamed whole popped amaranth grain is next in range of suitable ingredients.

Positive effects of flour made from raw and popped amaranth on bread quality could be explained by the high content of polar lipids in amaranth which act as surfactants. However, it seems that popping and steaming presumably caused changes in starch (swelling, partial gelatinization) that could worsen the crumb quality and decrease volume in composite breads especially when combined treatments were applied on amaranth grains.

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PROTEIN AND OIL CONTENT OF DIFFERENT AGE SEEDS OF SOME HERB SPICES

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ABSTRACT

Because they contain beneficial secondary plant metabolites , seeds of some spices are becoming more and more important in the diet of humans and animals. Seeds contain in their structure various building block substances (primary metabolites), and some of them (proteins and oils) were included in our investigation.

The objective of this study was to determine the effect of storage duration (from one to five years) on the content of protein and oil in seeds of the following medicinal plant varieties and domesticated populations: dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.), coriander (*Coriandrum sativum* L.) and caraway (*Carum carvi* L.). Laboratory studies have been performed in three replications over the course of 2014, according to accredited methods.

The storage duration had a great influence on the oil and protein contents. The protein content of 18.33% was characteristic for two-year old seeds while the seeds stored for five years had 16.66% of the oil.

Our results showed that the highest reduction in protein and in oil content was observed between the second and the third year of storage (from 18.33% to 17.54% and from 13.26% to 11.63%, respectively. On average, the highest protein content was recorded in caraway seeds (18.58%), which was for almost 2% higher than the lowest protein content in dill seeds (16.62%), while the highest and the lowest oil contents obtained for caraway and dill seeds were 14.22 and 6.79%, respectively.

Following our study, we came to the conclusion that the seeds with the highest protein and oil contents were those aged two or three years; if the seeds are to be stored longer than that period, they will certainly contain less of these two building block substances.

Keywords: oil and protein content, spice of medicinal plants, storage duration

INTRODUCTION

For centuries, spices have been playing a significant role in food preparations all over the world. Their role has become even more significant in the last fifteen years, since their production has been increasingly performed according to certified integrated and/or organic agriculture practices (Filipović *et al.*, 2010). One of the families with the highest number of spicy plant species is *Apiaceae* family, whose representatives are used both individually and as mixtures of different fresh or dried plant parts (fruit, seed, leaf, flower, root, etc.). In some spices, maximum aroma and healing properties are contained in fruits and seeds, and it is primarily because they contain the large percentage of secondary metabolites. These compounds do not participate in the primary plant metabolism (carbohydrates, vegetable oils, non-protein amino acids, proteins and vitamins) (Stanway, 2013), although the primary metabolites usually prevail over the secondary ones in the fruits and the seeds (Abdel-Azem, 2006). The oil content of the seeds is inversely proportional to their protein content.

Longer seed storage causes oxidative degradation of fats and oils in food, which leads to its organoleptic deterioration and disruption of its nutritional and sensory quality, as well as its potential toxicity due to newly formed compounds (Gordon, 2001).

The aim of this study was to determine the effects of one to five year long storage duration process on oil and protein content in the seeds of following common spicy species: *Anethum graveolens* L., *Foeniculum vulgare* Mill., *Coriandrum sativum* L., and *Carum carvi* L.

MATERIAL AND METHODS

In the experiment, seeds of the following varieties and domesticated populations were used: dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.), coriander (*Coriandrum sativum* L.) and caraway (*Carum carvi* L.). The seeds were produced in experimental fields of the Institute for Medicinal Plant Research "Dr Josif Pancic" Belgrade, Srbia. The study was conducted with seeds of different age, from five (collected in the 2009th) to one year old (collected in the 2013th).

Primary seed processing and preparation for the determination of oil and protein contents was performed in the seed laboratory of the Institute for Medicinal Plant Research "Dr Josif Pancic" Belgrade, Serbia. Oil and protein content in the seeds was determined in the laboratory "Tamiš Agrolab" of the Institute "Tamiš", Pančevo, Serbia, according to the method prescribed by the Ordinance on the methods of sampling and physical, chemical and microbiological analysis of animal feed (Službeni list SFRJ, no.15/87).

The obtained experimental data were statistically analyzed using the methods of variation statistics: mean value (I) and the coefficient of variation (CV). The statistical significance of the difference between the calculated mean values was obtained by applying the analysis of variance, using the statistical package Statistica 7.1 for Windows (Data Analysis Software System).

RESULTS AND DISCUSSION

Results of the analysis of protein content in different age seeds of tested spices were presented in Table 1.

Table 1. Protein content (%) in the seeds of tested spicy species

Seed maturity	V year	IV year	III year	II year	I year	Average
Dill (*Anethum graveolens* L.)	16,23	15,29	16,71	17,43	17,46	16,62
Fennel (*Foeniculum vulgare* Mill.)	18,15	17,64	16,55	16,12	15,76	16,90
Coriander (*Coriandrum sativum* L.)	15,69	16,27	17,63	18,91	15,97	16,73
Caraway (*Carum carvi* L.)	16,57	18,78	19,27	20,86	21,90	18,58
Average	16,66	16,99	17,54	18,33	17,77	17,21

The highest protein content was recorded in two year old seeds (18.33%) and the lowest one in five year old seeds (16.66%). Caraway seeds had the highest content of proteins (18.58%). On the other hand, fennel seeds had the lowest protein content (16.62%), almost equal to the average value of five-year coriander (16.73%) and fennel seeds (16.90%). Both of tested factors, as well as their interactions, caused statistically very significant variation in the protein content values (p <0.01).

The highest oil contents were recorded in two-year old seeds (23.17%) while the lowest in five-year old seeds (10.73%). The oil contents of the one-, three-, and four-year old seeds were very close to the average value, i.e. about 11.50%. The caraway seeds had the highest oil content (14.22%), while significantly lower oil content was recorded in dill seeds, which had averagely 6.79% oil (Tables 3 and 4). The obtained results showed significant effects of influence of the seed age and the plant species on the oil content were observed.

Table 2. Analysis of variance of protein content (%) in the seeds of tested spicy species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant species	3	89.73724	29.91241	933.69[**]	<.001
Years	4	19.53761	4.88440	152.46[**]	<.001
Interaction	12	88.77024	7.39752	230.91[**]	<.001
Residual	40	1.28147	0.03204		
Total	59	199.32656			

Cv= 1,0%, * P≤5%, ** P≤1%

	Plant species	Years	Interaction
LSD 5%	0.1321	0.1477	0.2954
LSD 1%	0.1768	0.1976	0.3952

Table 3. Oil content (%) in the seeds of tested spicy species

Seed maturity	V year	IV year	III year	II year	I year	Average
Dill (*Anethum graveolens* L.)	6,25	5,15	8,98	7,47	6,10	6,79
Fennel (*Foeniculum vulgare* Mill.)	13,78	14,87	13,15	14,28	14,53	11,93
Coriander (*Coriandrum sativum* L.)	8,27	9,69	10,55	12,92	9,84	11,10
Caraway (*Carum carvi* L.)	14,62	15,69	13,83	18,38	16,08	14,22
Average	10,73	11,35	11,63	13,26	11,64	11,01

According to previous researches, it was found that the mature fennel fruits consist of protein 9.5% and fat 10%, and dill contain 15.68% proteins (Kaur and Arora, 2010). Coriander fruits contain approximately 1-2% of essential oil and 16-28% of fatty oil, 11-17% proteins, 23-36% cellulose fibers and 13 -20% carbohydrates (Parthasarathy *et al.,* 2008; Hussain *et al.,* 2009; Pathak *et al.,* 2011) while the caraway seeds contain 1-6% of essential oil, 13-21% fatty oil rich in petroselinic fatty acid, and 25-36% protein (Sedlakov *et al.*, 2003, Ngo-Duy *et al.,* 2009; Laribi *et al.,* 2009).

Table 4. Analysis of variance of oil content (%) in the seeds of tested spicy species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant species	3	723.07006	241.02335	4701.67[**]	<.001
Years	4	46.33449	11.58362	225.96[**]	<.001
Interaction	12	62.85293	5.23774	102.17[**]	<.001
Residual	40	2.05053	0.05126		
Total	59	834.30802			

Cv= 2,0%, * P≤5%, ** P≤1%

	Plant species	Years	Interaction
LSD 5%	0.1671	0.1868	0.3736
LSD 1%	0.2236	0.2500	0.5000

Small-fruit coriander seeds (*C. sativum* var. *microcarpa*) are richer in fatty oil than the large-fruit coriander seeds (*C. sativum* var. *vulgare*) (Kiralan et al., 2009), and the fatty oil is mainly concentrated in the endosperm (22.65%), while its share in the pericarp use to be significantly lower (Sriti et al., 2010).

CONCLUSIONS

Length of storage of the seeds of tested spices caused statistically significant reduction in protein and oil contents of tested spicy seeds. On average, the best quality (i.e. the highest protein and oil contents) possessed two-year old seeds. Regarding the tested spicy species, the caraway seeds had the highest protein and oil contents (18.58% and 14.22%,

respectively), while the smallest and also statistically the most variable contents of these two building block substances was recorded in dill seeds.

ACKNOWLEDGEMENTS

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METHODOLOGY FOR NEW PRODUCT DEVELOPMENT ON THE EXAMPLE OF GEL WITH SPECIFIC PURPOSE

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ABSTRACT

The aim of this research was to develop a gel of defined sensory and thermo-reversible properties, which would allow heating it in a pot to become liquid and use for making cake with biscuit dough and cream. After short cooling, it again takes gelled consistency and other properties. Expected sensory properties of the gel are defined by descriptive method: gel is smooth with glossy surface; orange colour associated with ripe orange; sufficiently expressed aroma, pleasant and recognizable with other cake's ingredients (cream and biscuit), mild pungency reminds on fresh orange; taste moderately sweet and slightly sour; consistency moderately firm, harmonious with cream and biscuit consistency, pleasant during chewing.

Methodology of defining the new product's composition and quality parameters during development was described in this paper, on example of gel with orange juice. Sensory, physical and chemical properties of each gel sample were analyzed after production, and then recipe was modified for next product making, in order to achieve a defined quality.

Four gel samples were produced to define quantitative relationship between basic ingredients and to select orange aroma. The temperature of gel heating, before pouring in the cake was determined (~60 °C), in order to form a solid gel during cooling. Then, four samples of gel were produced and optimal concentration of pectin determined. The quality and stability of the gel in cake were analyzed applying descriptive analysis. Selected gel had a consistency compatible with the cake. The recipe for preparation of the product and quantity of the basic ingredients was defined (25% orange juice, 31.5% sugar, 42% water, 0.6% orange flavour), additives (0.7% pectin, 0.02% ascorbic acid, citric acid if necessary) and quality parameters of the gel (dry matter 35%, acidity 0.70%, pH 3.2, CIEL*a*b*), which will enable production of gel with defined sensory characteristics and quality.

Keywords: *gel for cakes, new product, sensory analysis*

INTRODUCTION

The successful development of a new product is a great challenge for any production company, especially due to the fact that its realization carries certain risks (Grujić and Grujić, 2011; 2012). Fruit has an important role in human nutrition as a source of nutrients, in maintaining normal body function, and in control of chronic and infectious diseases (Vattem *et al.*, 2005; Battino *et al.*, 2009). In the baking industry there are a need and an opportunity for the manufacture of products using improved nutritional composition using selected fruits products as ingredients (Cvetković *et al.*, 2009).

Quality of cakes is usually defined on the basis of physico-chemical and sensory properties. Sensory analysis could be used as a tool in daily quality control, during the development and improvement of product quality, for the most important defining quality characteristic of the new food products, for analytical approach in harmonization of quality parameters of the new product with defined, or for testing of acceptability of the quality of products on the market, and for successful placement and the survival of new products on the market (van Kleef *et al.*, 2006; Grujić *et al.*, 2008a,b; van Trijp and van Kleef, 2008; Burseg *et al.*, 2009; Grujić and Grujić, 2011; 2012).

The aim of this research was to give a description of methodology for the new food product development, on the model of the gel with orange juice, defined sensory and thermo-reversible properties and with a special purpose.

MATERIAL AND METHODS

The experimental part of this study was realized on the Faculty of Technology, University of Banja Luka (Bosnia and Herzegovina), in Laboratory for sensory analysis of food, which is designed according to the Standard ISO 8589:2007 and in Laboratory for analysis of food.

Material used for the experiment
Ingredients used for the realization of the experiment and gel samples preparation: pasteurized pulpy orange juice, made from concentrated orange juice (11% dry matter - DM; 1% acidity), commercial product purchased in a retail store; sugar (crystals, purchased in retail store); gelling agent amidated citrus pectin (E440) standardized with acidity regulator diphosphate (E450), and firming agent calcium chloride E509 (Pectin Amid CB 025-E, producer Herbstreith & Fox, KG, Germany); tap water (medium hard); citric acid (E330); ascorbic acid (E300); natural orange aroma A and orange aroma B (producer, Esarom Essenzenfabrik GmbH, Vienna, Austria). Cakes' biscuit dough and foamy cream with vanilla aroma, used as components for experiment, were purchased from the local cakes producer.

Method of the gel production
The sugar and fruit juice were homogenized and heated in open pot, gelling agent was added, dry meter and acidity were controlled or corrected, aroma was added, and product was hot filled in glass jars after pasteurization in pot (15 min at 80 °C). Prepared samples were closed with twist-off caps and after cooling stored in refrigerator on +4 to +8 °C.
Experiment was designed in 4 parts: (1) expected products quality defining, selection of orange aroma type and optimum concentration; (2) determining of limiting values for gel heating temperature before use, which will provide sufficiently firm gel after application and during cooling in or on the cake; (3) determining of pectin optimal concentration that will give expected consistency, firmness and elasticity of the gel; (4) testing the stability and quality of the gel as a new product, selecting the best of analyzed according to their purpose, and defining quality parameters of the gel using descriptive sensory analysis as a tool.
Sensory, physical and chemical properties of each gel sample were analyzed after production, and then recipe was modified for next level of product making, with aim to achieve a defined quality.

Analysis of the gel quality
For quality and chemical composition control of gel samples, percent of dry matter on 20 °C (Leica Abbe Mark II Refractometer Model 10480, Leica, USA), total acidity (titration with 0.1 M NaOH) expressed as % of citric acid and pH (pH-meter, ISKRA) were determined.
Colour parameters (CIEL*a*b*) were determined using a tristimulus colorimeter (Minolta CR410) with standard illumination D65, colorimetric normal observer angle of 10 ° and 50 mm measurement area. Colour parameters, expressed as CIE L*, a* and b* values, were determined as indicators of lightness, redness and yellowness. The results are expressed as the mean value of five measurements. Descriptive Statistical Analysis of all data for colour parameters of samples are realised by Microsoft Office Excel 2007 and presented as mean values ± standard deviations.

Descriptive sensory analysis was used for qualitative and quantitative testing of the gel model samples properties, according to the procedures defined by the appropriate standards (ISO 13299:2003; ISO 11035:1994; ISO 4121:2003). The corresponding attributes were used for detailed, illustrative descriptive sensory analysis of selected, important product properties, and variations in quality, which are presented in written form in the Guidelines for sensory evaluation of the gel quality.
The sensory analyses were performed, in the booths, individually by five panellists, persons selected and trained in quantitative descriptive analysis of fruit gel and similar products. The scoring forms (questionnaire) with attributes definition and possible defects description were

used for gels sensory quality evaluation. Panellists were instructed to evaluate intensity for selected sensory attributes using 5-point scoring system (in scale from 5 for very good quality, to 1 for very bad, unacceptable quality). In initial preparation of the gel sensory analysis, coefficients of significance (Cs) for the most important sensory attributes were determined: appearance (Cs = 4), colour (Cs = 3), consistency (Cs = 7), aroma (Cs = 3), and taste (Cs = 3), where sum of them were 20. Appropriate coefficient of significance (Cs) was multiplied with score given after sensory evaluation of each selected attribute and after that, addition of all results for evaluated sensory attributes gives corrected score, expressed as percentage of maximum possible product quality (MPQ) or 100% for the best quality, used for products quality comparing.

Gel appearance and colour were evaluated visually; consistency was evaluated by sight, with fingers, teaspoon and oral in the mouth; aroma and taste were evaluated after putting portion of sample in the mouth, during chewing. Sensory analyses of the samples were performed at least 24 hours after the production and storage of the gel at temperature of 4-8 °C. Samples of the gel (approximately 30 g) were presented on coded white plates, on the tray with teaspoon, white paper napkins, and glass cups with water for the mouth rinsing.

The quality, visual impression, consistency, aroma and taste of gel samples (N.13, N.15, N.16, N.17) were evaluated by descriptive sensory analysis, as well as the stability of quality and harmony of their sensory properties, combined with cream for cakes: 1, 3 and 5 days after cakes producing and storage at 4-8 °C as expected cake durability, and after 7 days.

RESULTS AND DISCUSSION

New product development is a complex process, carried out in the several stages and requires the simultaneous achievement of the objectives within defined period. No matter how complex, creating a new product is useful, as it could open the way for sustainable development and improving the strategic position of the company (Grujić and Grujić, 2012).

The first part of the experiment consisted of defining the expected quality of the product and production of model samples of fruit gel, selecting the best orange aroma and determining its optimal concentration, which will give expected recognition of the gel in cake.

Expected sensory properties of the gel were defined by descriptive method: gel should be smooth with glossy surface; orange colour associated with ripe orange; sufficiently expressed aroma, pleasant and recognizable with other cake's ingredients (cream and biscuit), mild pungency reminding on fresh orange; taste moderately sweet and slightly sour; consistency moderately firm, harmonious with cream and biscuit consistency, pleasant during chewing. The gel should have thermo-reversible properties. During heating in a pot, it becomes liquid, and may be used for making cake with biscuit dough and cream. After short cooling, it again takes gelled consistency and keeps other properties.

Orange juice is especially appreciated product because of a refreshing taste, pleasant, pronounced aroma and a nice, stable colour (Dluzewska *et al.,* 2004). The type and quantity of aromatic substances have an impact on the acceptability of quality of fruits or products manufactured from it, so it is very important to choose properly the type and concentration of aroma that will be used in a particular food product, as a specific medium.

During the product thermal treatment, volatile aromatic compounds contained in the natural orange juice are partially lost. To make the finished product with the expected, pleasant and distinctive aroma, orange flavour can be added. It was the variable in the first part of the experimental production of the gel model samples N.1, N.11, N.12, and N.13 (Table 1).

Descriptive sensory analysis of gel with orange juice showed that the natural aroma of orange A in the sample N.1 is not sufficiently expressed (evaluated with 2 points), so that the overall gel quality was evaluated with 89.5% MPQ, and the sample N. 11 with 98.5% MPQ, had a very pleasant aroma of orange juice (5 points) and gentle pale yellow colour, like the colour of fresh orange juice and 4.5 points. Sensory analysis of the gel sample in the cakes cream showed that the sample N.1 had unacceptable quality, and aroma in the sample N.11 was not sufficiently pronounced, the colour was too bright for the purpose of the gel, so this sample was rejected from further consideration.

Sensory analysis of sample N.12 produced with aroma B (emulsion with concentrated aroma of orange, intense orange colour), evaluated as relatively lower overall quality of the gel (91% MPQ), because of a little more intense the aroma (4 points) and colour (4 points) than expected.

Sample N.13 had the best quality (100% MPQ). The aroma was pleasant, expressed, pungent and associated with juice and rind of fresh oranges (5 points), retained in the mouth with a pleasant prolonged refreshing impression after ingestion of the sample. The colour of the sample was adequate, pure yellow-orange (5 points). Comparing the results of sensory evaluation of samples N.12 and N.13 aroma, together with cream for cakes, it was found that sample N.13 had expected quality, and that aroma B in concentration 0.6% (Table 1) could be used for further modelling of the gel quality parameters

Table 1. The quantity (m/m) of the ingredients in percents and quality parameters of gels

Sample code	Orange juice (%)	Sugar (%)	Pectin[a] (%)	Aroma (%)	Ascorbic acid (%)	Water (%)	Quality parameters of gel		
							Dry matter (%)	Acidity[d] (%)	pH
N.1	25.0	31.5	0.80	**0.025[b]**	0.02	43.0	35.9	0.84	3.04
N.11	25.0	31.5	0.80	**0.125[b]**	0.02	43.0	35.9	0.84	3.04
N.12	25.0	31.5	0.80	**0.80[c]**	0.02	42.0	33.8	0.80	3.00
N.13	25.0	31.5	**0.80**	**0.60[c]**	0.02	42.0	36.0	0.89	2.97
N.15	25.0	31.5	**0.70**	0.60[c]	0.02	42.0	35.2	0.85	2.92
N.16	25.0	31.5	**0.60**	0.60[c]	0.02	42.0	35.0	0.84	2.90
N.17	25.0	31.5	0.70	0.60[c]	0.02	42.0	**35.0**	**0.70**	**3.20**

[a] Pectin (Pectin Amid CB 025-E, producer Herbstreith & Fox, KG, Germany);
[b] Orange aroma A; [c] Orange aroma B; [d] Total acidity of the gel sample, expressed as % of citric acid

In the second part of the experiment, the limiting values for the gel heating temperature before its pouring and application in or on cake (between 65 and 55 °C) were selected which will provide sufficiently re-formation of solid gel during cooling (on the temperatures 4-8 °C). The gel was heated to the temperatures 70-80 °C, cooled and shaped in the mould at an initial temperatures as follow: 60 °C; at the 45 °C (gel did not maintain shape of the mould, it was thick, viscous and easily disintegrated by pressing); at the 35 °C and 32 °C (gels were very soft and watery). If the sensory analysis are carried out according to the standard procedures, quantitatively measured value of each observed sensory property and scientific evaluation of the product quality level during development for target market were obtained (Grujić and Grujić, 2011; Grujić *et al.,* 2008a,b; Sanz *et al.,* 2008; 2009; Iannario *et al.,* 2012). To determine the optimal concentration of pectin, which will provide the necessary gel firmness and elasticity, 4 models of gel with orange juice were produced **in the third part** of the experiment, with pectin as variable in concentrations: 0.8% (sample N.13); 0.7% (sample N.15); 0.6% (sample N.16), and other ingredients used for samples production were in the same relationship (Table 1). Their quality were analyzed after production and conditioning.

The highest scores (5 points) for all selected properties (100% MPQ) are assigned to the sample gel N.13 during sensory analysis. Sample N.15 had a high level of quality, the gel was moderately firm, gentle, with consistency very little softer than expected; it easy tears on the juicy pieces during morsel manipulation (4.5 points), and other analyzed sensory characteristics had the expected quality level (5 points) and overall quality of the gel was high (96,5% MPQ). The consistency of sample N.16 was too soft, a little soggy, and during

morsel manipulation in the mouth, it reminded on an overcooked fruit (2 points), which resulted in a relatively small overall assessment of gel quality (79% MPQ).

The fourth part of the experiment was aimed on testing the stability and quality of 4 gel samples as a new product (N.13, N.15, N.16 and N.17) in the cake, according to the purpose, using the descriptive sensory tests. The gel model samples were used in cakes made with light yellow biscuit and moderately firm, gentle yellow foamy cream with vanilla aroma. The gel rapidly undergoes gelling after pouring in the space mechanically formed inside the cream, which lies between two biscuits, or on the upper side of the cake. During the sensory analysis of aroma and taste (sweetness and acidity) quality in gel sample N.15, it was concluded that it is the best of compared, but if slightly reduce the product acidity from 0.8% to 0.7%, better harmony of sweetness and acidity of gel and cream could be achieved.

The amount and ratio of basic ingredients were defined for manufacturing of sample N.17 and a new product (25% orange juice, 31.5% sugar, 42.0% water, 0.6% orange aroma), food additives (0.7% pectin, 0.02% ascorbic acid, and citric acid if it is necessary), quality parameters of the gel as final product (35% dry matter, acidity of 0.70%, pH 3.2) as shown in Table 1, and colour parameters expressed as mean values ± standard deviations in the CIEL*a*b* System (L*=38.48±0.04 for lightness; a*=3.27±0.03 for share of red; b*=9.78±0.04 for share of yellow), which will allow the production of gel for special purpose with defined sensory properties and quality. CIEL*a*b* System could be useful for daily quality control on-line, as it describes all the colours visible to the human eye and can serve as a device-independent model to be used as a reference. The three coordinates represent the lightness of the colour ($L*$ = 0 yields black and $L*$ = 100 indicates diffuse white), its position between red and green ($a*$ negative values indicate green while positive values indicate red) and its position between yellow and blue ($b*$ negative values indicate blue and positive values indicate yellow).

It was found that the gel sample N.17 had the best quality, stability and harmony of consistency with the cream used in the cake after 7 days of cake storage, under normal conditions, and expected shelf life of the cake was 5 days. During chewing of mouthful of cream and gel, significantly different in nature and composition, they were blended and mixed simultaneously and formed a pleasant unit. Orange aroma was clearly recognizable during and after swallowing mouthful, the aroma and pungent impression retained in the mouth for a short time giving a refreshing effect during the cake consumption.

CONCLUSIONS

The success and survival of new food product is possible only if quality is harmonized with the identified needs on the target market, in the given time and the business environment. Product development includes procedures that idea selected for the products manufacturing in a given time and the business environment, transform into a model product, and then after numerous analysis, harmonization and quality testing, lead to the development, definition and realization of technological processes of manufacturing and marketing of the new product on target market. During new product development, testing, correction and product quality parameters harmonization were successfully completed by modelling the quantity of ingredients and food additives, until reaching the aim, a defined level of the sensory quality of the gel with specific purpose. The new product, a gel with thermo-reversible gelling properties, intended for use in industrial conditions, may be interesting also as a product for domestic making cakes and deserts, because of its ease use and specific quality.

ACKNOWLEDGEMENTS

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SENSORY ANALYSIS AS A TOOL IN THE NEW FOOD PRODUCT DEVELOPMENT

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ABSTRACT

Jellied fruit products are interesting food category because of their sensory properties, favourable and biologically acceptable nutritional composition and content of certain ingredients that give them properties of functional food. The aim of this research was to develop a new food product, on a model of fruit topping, with quality harmonized with market needs and consumers' expectations. Fruit topping should have roughly chopped fruits, pleasant fruity aroma, refreshing sweet-sour taste, dark red colour, to be transparent with moderate density and viscosity, suitable for decorating ice cream and similar confectionery products.

A series of 6 model samples of fruit topping with wild berries (raspberry, blackberry, blueberry), sugar, water, food additives and aroma as ingredients, were produced in laboratory conditions. For quality control of topping, dry matter, total acids and pH were determined. Quantitative descriptive analysis of topping selected sensory properties were realised by consensus method, with five trained panellists. Sweet-sour taste acceptability of two products was evaluated by 58 selected assessors, and overall acceptability of final product was evaluated by 55 assessors, using the affective sensory test. The fruit topping was presented to each consumer with vanilla ice cream as a carrier.

Descriptive and affective sensory tests were used as a tool for development of the new products, with quality harmonized with consumers' expectations. After that, ingredients content, quality parameters of fruit topping as new product (dry matter 40%; total acidity 0.80%; pH 3.00) and processing parameters were defined. Prepared as finished product, it could be used for decorating the dessert just before serving and consumption, as is usual. Results of the research confirmed that an appropriate viscosity, sensory characteristics and acceptable quality of new product were achieved by the proper selection and modelling of quantitative relationship of ingredients and selected food additives during product development.

Keywords: sensory analysis, new product, consumers

INTRODUCTION

New product development and quality management are the strategically important activities and components of any successful business system (Grujić and Grujić, 2011; Hansen and Hamilton, 2011). To make a new product which will found its place and kept it on the market, it is essential to be created in accordance with the needs of the target market and end-user of the product (Recurreccion, 1998; Sijtsema *et al.*, 2004; Gielens and Steenkamp, 2007; Grunert *et al.*, 2008). The need for fruit toppings, as new food products are identified due to the fact that there are no similar commercial fruit products, with moderate sweetness, a defined viscosity and transparent appearance. Justification for developing this kind of fruit products gives the fact that the World Health Organization and experts in the field of nutrition, consider that there are not consumed sufficient amounts of fruit, despite recommendations for proper nutrition and evidence that fruit consumption may decrease the risk of appearing and development of many chronic diseases of humans (Seeram, 2008; Battino *et al.*, 2009; Ali, 2012; Barrett and Lloyd, 2012).

Sensory properties of the product are important quality parameters, which influence on status of the finished product on the market, and whether consumers, whom it is intended, will like and buy product. Results of scientific research have shown that quality and important sensory properties of the product, can be identified and controlled with the descriptive

analysis or by consumers testing can be examined if the overall product quality or the selected property of the product were affected by carried out modification (Bahamonde *et al.*, 2007; Grunert *et al.*, 2008; Grujić and Spaho, 2010; Grujić and Grujić, 2011).

Jellied fruit products are interesting food category because of their sensory properties, favourable and biologically acceptable nutritional composition and content of certain ingredients that give them properties of functional food. The aim of this research was to develop a new food product, on a model of fruit topping, with quality harmonized with market needs and consumers' expectations, and to test the null hypothesis (H_0): quality control and evaluation of the acceptability of the sensory properties of the product, using the affective sensory tests, can be used as a tool for development and harmonization of the new products quality.

MATERIAL AND METHODS

The experimental part of this research was realized on the Faculty of Technology, University of Banja Luka (Bosnia and Herzegovina) in Laboratory for food sensory analysis, which is designed according to the Standard ISO 8589:2007 (E) and in Laboratory for food analysis.

Material used for the experiment

Following ingredients were used for fruit topping production: frozen fruit raspberry (SM 13%), blackberry (SM 12%), blueberry (SM 12%), producer „Frattelo trade" Banja Luka; sugar (crystals, purchased in retail store); gelling agent (E440): **pectin A** (Pectin Classic AF703, producer Herbstreith & Fox, KG, Germany); **pectin B** (OBIPEKTIN Blue Ribbon 150US, producer, Naturex Company, France); firming agent calcium chloride (E509), $CaCl_2*2H_2O$,p.a, M=147.02 g/mol, (producer Lach-Ner, s.r.o. Czech Republic); citric acid (E330); tap water (medium hard).

Production of fruit topping

Production of fruit topping model samples as new product consists in preparing necessary ingredients, mixing, heat treatment, quality investigation (using method of physical, chemical and sensory analysis), filling in glass jars, hermetic closing, cooling and storage.

The first part of the experiment was done in previous studies and included descriptive sensory analyses, determining the following parameters: the initial amount ratio of fruit blackberry (*Rubus sp.*), raspberry (*Rubus ideaus*) blueberry (*Vaccinium myrtillus*), as ingredients for products making; the degree of sweetness and dry matter; and acidity of fruit topping with berries, samples code S.11, S.12, S.21, S.22 (Tables 1 and 2).

The second part of the experiment included activities on the product development using descriptive sensory, physical and chemical analyses which define product quality parameters and the ratio of ingredients, making fruit topping samples that will have the expected consistency and aroma, according to the purpose of fruit topping.

The third part of the experiment *was designed to test the acceptability of the new product, using the affective sensory tests. Fruit topping was served to the assessors with ice cream (temperature -10 °C), as one of the possible combinations for topping use and consumption.*

Analysis of product's quality

Percent of dry matter on 20°C (Leica Abbe Mark II Refractometer Model 10480, Leica, USA), total acidity (titration with 0.1 M NaOH) expressed as % of citric acid and pH (pH-meter, ISKRA) were determined for quality and chemical composition control of fruit topping.

Colour parameters (CIEL*a*b*) were determined using a tristimulus colorimeter (Minolta CR410) with standard illumination D65, colorimetric normal observer angle of 10° and 50 mm measurement area. Colour parameters, expressed as CIE L^*, a^* and b^* values, were determined, where measured parameters represent the lightness of the colour ($L^* = 0$ yields black and $L^* = 100$ indicates diffuse white), its position between red and green (a^* negative

values indicate green while positive values indicate red) and its position between yellow and blue (*b** negative values indicate blue and positive values indicate yellow). The results are expressed as the mean value of five measurements. Descriptive Statistical Analysis of all data for colour parameters of samples are realised by Microsoft Office Excel 2007 and presented as mean values ± standard deviations.

Descriptive sensory analysis was carried out by 5 tested, skilled and trained assessors using the method of consensus, according to the procedures defined by the relevant standards (ISO 13299:2003; ISO 11035:1994; ISO 4121:2003) and was used as a tool for the evaluation and comparison of the achieved quality of food products samples. In addition to descriptive sensory evaluate the quality of each model sample fruit topping as product, for testing their suitability for use with ice cream, was analyzed the quality, visual impression, viscosity, aroma and taste, as well as the balance the sensory properties in combination with ice cream.

Evaluation of acceptability of the achieved quality of fruit topping samples was realized in multiple sessions, using the affective sensory tests. The level of product sweetness was defined, also the optimal value for dry matter and acidity of the product ranking (ISO 8587:2006) and selecting one of two offered samples (S.12, S.22) by engaging 58 assessors, and the acceptability of the final product (sample S.35) was evaluated by engaging 55 assessors, selected as permanent consumers of similar products, who prefer and like to eat ice cream with fruit topping.

Chilled sample of topping (~4 °C) were served with vanilla ice cream (in the usual serving conditions, in relation: 20 g of ice cream with 5 g of fruit topping) to the respondents for sensory analysis of topping acceptability. Samples were served in the booths, in white plastic cups (volume ~200 ml) with white plastic teaspoons for single use, white paper napkins, glass cup with water room temperature (20–23 °C) for the senses regenerating, pencil and evaluation sheet.

Respondents were introduced with the purpose of the analysis just before the evaluation of acceptability of topping as final product. The task of analysis (Mark the answer that expresses your opinion about the quality of the product) was clearly defined in the evaluating sheet, with the possibility of choosing one of three answers (I really like; I like; I do not like it).

RESULTS AND DISCUSSION

Jellied fruit products are interesting food category because of their sensory properties, favourable and biologically acceptable nutritional composition and content of certain ingredients that give them properties of functional food (Levaj, 2012; Ali, 2012; Barrett and Lloyd, 2012). As the aim of this research was a new food product development on a model of fruit topping, with quality harmonized with market needs and consumers' expectations, specific procedure should be followed. Expected quality was identified and described. Fruit topping should have roughly chopped fruits, pleasant fruity aroma, refreshing sweet-sour taste, dark red colour, to be transparent with moderate density and viscosity, suitable for decorating ice cream, fruit salads and similar confectionery products. To achieve so quality level, it is necessary to do following: identify and define important sensory characteristics of the new product; choose ingredients that will be used for new products manufacturing; realizing a series of experiments, by modelling relation of the ingredients and food additives to create the product models until it reaches the quality of the product close to defined; applying affective sensory tests to examine the acceptability of the new products quality; define the quality parameters of the new product.

Fruit toppings with berries were produced of blackberries, raspberries and blueberries in a defined relationship, as types of fruit that consumers like to eat. **In the first part of this research**, 4 model samples of fruit topping were produced (S.11, S.12, S.21, S.22), with different sweetness and acidity (Table 1), physical and chemical parameters were analyzed, as well as the sensory properties by descriptive analysis. After that, samples with most acceptable quality (S.12, S.22) were selected, which will be base for new product development, as suggested (Grunert *et al.*, 2008; Grujić and Grujić, 2011).

In the second part of the research, selecting of type, relationship and the amount of the ingredients (fruit, sugar, pectin, $CaCl_2$, correcting of aroma and acidity) series of model samples of fruit topping were made (Table 1, Table 2). The addition of calcium ions should provide additional strength of pectin network of low esterified pectin and stability of the gel after gelling (Peleg, 2006; Willats *et al.*, 2006; Van Buggenhout *et al.*, 2009).

It was found that with the appropriate choice of the type and amount of pectin, and providing optimal conditions for gelling, can be achieved smooth, transparent structure and consistency of the product, with glossy surface and juiciness. The color intensity and concentration of aroma in fruit topping must be such to give an impression that the product contains a sufficient amount of fruit from which it is produced and to be pleasant for consuming. The concentration of fruit aroma in the topping, in a moment of dessert consuming, should be similar to the concentration in the fresh ripe fruits. In that producing conditions, the balance of fruity aromas and aromas of vanilla, milk, ice cream and other ingredients that give the rich taste and aromas in dessert could be achieved, and make it more distinctive and enjoyable for consuming. After testing and evaluation of selected sensory characteristics of the product, accurate information about its real quality can be obtained and compared to the expected or defined product quality (Gielens and Steenkamp, 2007; Grujić *et al.*, 2008a,b; Grunert *et al.*, 2008; van Trijp and van Kleef, 2008).

Product quality has been assessed and the product with the expected sensory properties was selected, using the descriptive analysis. S.31 sample was too viscous, acidity and aroma are not expressed, and the dominant sweetness. Sample S.32 was moderately viscous, with harmonious and expressive aromas of fruit. Commercial offer and suitability for pectin purchase has been checked and S.3.2 sample was selected for further modelling and product development. Analyzing topping with ice cream found that it should increase the share of fruits, flavours and acids in the total amount of the product (Table 1), to produce a sample S.35. After that, ingredients content, quality parameters of fruit topping as new product (dry matter 40%; total acidity 0.80%; pH 3.00) and processing parameters were defined. Sample S.35, chilled to +4 °C, had expected the viscosity, colour, taste and aroma.

Table 1. The quantity relation (m/m) of the basic ingredients of fruit topping with berries, in percents

| Sample code | Fruit | | | Sugar (%) | Pectin | | CaCl₂x2H₂O (%) | Citric acid (%) | Aroma (%) | Water (%) |
	Raspberry (%)	Blackberry (%)	Blueberry (%)		Code	Quantity (%)				
S.11	5.50	12.00	3.10	32.00	n	n	n	0.25	n	47.50
S.12	5.50	12.00	3.10	32.00	n	n	n	0.30	n	47.50
S.21	5.50	12.00	3.10	42.00	n	n	n	0.25	n	37.50
S.22	5.50	12.00	3.10	42.00	n	n	n	0.30	n	37.50
S.31	5.50	12.00	3.10	37.00	B	0.70	0.064	0.30	n	42.00
S.32	5.50	12.00	3.10	37.00	**A**	**0.60**	n	**0.30**	**0.10**	42.00
S.34	5.50	12.00	3.10	37.00	B	1.00	n	0.30	0.10	42.00
S.35	**8.00**	**17.50**	**4.50**	36.40	**B**	**0.60**	n	**0.35**	**0.20**	33.00

A - Pectin A (Pectin Classic AF703, producer Herbstreith & Fox, KG, Germany)
B - Pectin B (OBIPEKTIN Blue Ribbon 150US, producer, Naturex Company, France)
n - not used

Fruit topping was stable, moderately viscous after several successive mixing (with teaspoon) in the packaging, there was no disintegration or destabilization, which is vital for normal conditions of fruit topping use. The temperature of the ice cream that was used as a carrier was lower than –5 °C and may affect the viscosity of the topping during the serving and

consumption. During ice cream decorating, topping gently glided down the spoon and lazily descended on the slopes of the shaped dessert of ice cream, leaving a thin layer of compact fruit topping on a road that passes. It had intense, clean bordeau red colour, glassy shine, visible fruits of blueberry, seeds of blackberry and raspberry evenly distributed in the fruit topping. Sweetness and acidity were moderately expressed, giving to the fruit topping the impression of refreshing and juiciness. Aroma was assessed as characteristic for the fruit of which topping was made, while the aroma of blackberry and raspberry mixed together providing the expected balance during consumption of fruit topping served in a defined relationship with vanilla ice cream.

Table 2. Quality parameters of fruit topping with berries

Sample code	Dry matter (%)	Acidity [a] (%)	pH	L* (average ± SD)	a* (average ± SD)	b* (average ± SD)
S.11	35.00	0.60	-	28.77±0.01	5.35±0.04	-2.45±0.01
S.12	35.00	0.70	-	28.72±0.02	5.12±0.03	-2.37±0.02
S.21	45.00	0.60	-	28.51±0.02	4.76±0.09	-2.56±0.03
S.22	45.00	0.70	-	28.65±0.02	5.07±0.07	-2.45±0.03
S.31	40.50	0.69	3.05	28.80±0.01	5.60±0.10	-2.29±0.03
S.32	40.90	0.70	3.00	28.69±0.01	5.03±0.04	-2.44±0.02
S.34	40.10	0.66	3.13	28.77±0.01	5.76±0.04	-2.28±0.02
S.35	41.40	0.79	3.01	28.65±0.01	5.33±0.04	-2.37±0.01

[a]Total acidity of the fruit topping sample, expressed as % of citric acid
L*, a*, b* - colour parameters in the CIEL*a*b* System (mean values ± standard deviations)

After preparation test samples of fruit topping, it is necessary to perform testing in cooperation with representatives of the target group of consumers (Grujić and Spaho, 2010; Recurreccion, 1998; van Trijp and van Kleef, 2008; Grujić and Grujić, 2011; 2012). **The third part of the experiment** consisted in testing the acceptability of the quality of new products in accordance with their intended purpose. Results of affective sensory tests showed that 34 respondents really like, 20 respondents like, and 1 does not like topping proving that the achieved quality of new product was harmonized with the expectations of consumers. In an interview with respondents it was found that they like and would like to buy and consume the product. The level of expressed preference depended on the kind of fruit that they prefer to consume, as some of them more like other fruits.

CONCLUSIONS

The results obtained during realization of this study confirmed that **the null hypotheses should not be rejected and** that the quality control and evaluation of the acceptability of the sensory properties of the product by affective sensory tests can be used as a tool for the development and harmonization of new products' quality. An appropriate viscosity, sensory characteristics and acceptable quality of new product were achieved by the proper selection and modelling of quantitative relationship of ingredients and selected food additives with different functional properties, during new food product development.

ACKNOWLEDGEMENTS

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CONTENT OF FREE AMINO GROUPS IN WHEAT FLOUR AS INDICATOR OF WHEAT ENZYMATIC STATUS

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ABSTRACT

The quality of starch and protein fractions in wheat flour has the most dominant role in the formation of quality of wheat-based products. The level of protein quality as well as the end-use flour quality is determined by different chemical, physical and rheological methods. The aim of this study was to determine the biochemical status of maturated wheat flour originating from different localities in terms of the content of free amino groups as an indicator of the protein hydrolysis degree.

Samples of five wheat varieties were collected from six localities in northern Serbia in 2010/2011 production year.The content of free amino groups was determined after the wet gluten samples incubation for three hours at two different temperatures (30° and 37°C).The selected temperature of 30 °C is commonly used for processing of the dough in practise, while the temperature of 37 °C was chosen as the optimum temperature for present proteolytic enzyme activity.

The obtained results indicated significant differences in the content of free amino groups between the temperature treatments (at 30 and 37 °C) as well as the differences between varieties. The content of free amino groups increased with the increase in incubation temperature of gluten from 30 °C (0.110826 µg/mg) to 37 °C (0.132867 µg/mg) ($p < 0.05$). The lowest average of free amino groups' content was determined in Kikinda, while the highest content was determined in Sombor locality.

The further research should be carried out covering investigated wheat varieties from different production years.

Keywords: wheat flour quality, free amino groups, proteolytic activity

INTRODUCTION

Several factors may determine wheat quality, including: physical grain properties, the protein content and quality, and starch content and composition. Among the quality characteristics, the content and quality of gluten proteins have a primary influence on the technological quality of wheat flour (Wieser, 2007). Any alterations of these proteins may have a beneficial or detrimental effect on the quality of wheat flour, wheat dough and the final product-bread. The alteration of gluten proteins could be caused by proteolytic activity. Although these enzymes are inactive during grain and flour storage, when water is added they become active influencing the functional attributes of flour (Rani *et al.,* 2001). Endogenous proteolytic enzymes and storage proteins are both synthesized during development of the wheat kernel. After harvest and wheat milling, obtained flour undergoes a maturation process. Pyler (1973) described the complex biochemical changes during the flour maturation which started 4–5 days after milling and lasted for approximately 3 weeks. Those biochemical changes include activity of several enzymes groups present in wheat grain and flour: amylases, proteases, oxygenise polyphenol oxidases and peroxidases (Evers and Redman, 1973). Each time when a peptide bond is hydrolysed by peptidase enzymes a free amino group and a free carboxyl group are released. The progress of hydrolysis is determined on the basis of the increase in the concentration of these groups. Also, the increase of their content indicates on the degradation of the polymer structure of protein which may not has negative effects on wheat flour quality. During flour maturation, the proteolytic activity decreases as a result of flour aeration. The relative amounts of the glutenins and gliadins fractions at the beginning and in the end of wheat and flour maturation were statistically significantly different, so it is possible that the final redistribution and polymerization of the protein macromolecules occurred in this phase (Janić Hajnal *et al.,* 2014). Besides normally present proteolytic

enzymes, the increasing content of damaged wheat grain by various insects could also contribute to the higher proteolytic activity (Aja *et al.,* 2004; Pérez *et al.,* 2005). During processing of dough with higher proteolytic activity, softening of gluten occurs causing the decrease of final product quality (Every *et al.,* 1998; Aja *et al.,* 2004). The processing properties of wheat flour can significantly vary as a result of climate and cultivation factors (Prieto *et al.,* 1992; Weegels *et al.,* 1988). Johansson *et al.* (2013) reviewed the influence of genotype and environment, as well as their interaction on breadmaking quality which additionally complicates the understanding of the effects of individual factors. Taking into account all mentioned wheat flour quality parameters, the aim of this study was to determine the biochemical status of matured wheat flour, especially the content of free amino groups as an indicator of the protein hydrolysis degree, in samples originating from different localities.

MATERIAL AND METHODS

Material
Five wheat varieties (*Triticum aestivum*) Pobeda (Pob), Zvezdana (Zve), Apache (Ap), Gordana (Gord) and Gora (Go) grown in 2011 in six regions in Serbia: Bačka Topola (BT), Sremska Mitrovica (SM), Vršac (VR), Kikinda (KI), Subotica (SU) and Sombor (SO) were selected for the study. Pobeda, Zvezdana, Gordana and Gora were bred by the Institute of Field and Vegetable Crops, Novi Sad, Serbia, whereas Apache was bred by Limagrain, Chappes, France. The samples were stored in craft paper bags under laboratory conditions (22 °C, 70% RH) during 50 days. The samples were cleaned and tempered and milled to laboratory flour using a Bühler MLU 202 (Bühler, Uzwil, Switzerland) according to AACC methods (1999). Flour (matured flour) obtained after 50 days of wheat storage was analysed after 14 days of storage at the above mentioned conditions.

Content of besatz of wheat
The content of besatz of wheat was determined according to the ICC standard method 102/1 (ICC, 1972). We analyzed sprouted wheat kernels (SWK), kernels damaged by pests (KDP), wheat bug damaged kernels (WBDK), *Fusarium* infested kernels (FIK) and kernels with black point (BPK).

Free amino groups content
The content of free amino groups was determined according to the procedure described by Perez et al. (2005) from wet gluten washed out from flour samples according to standard ICC method 106/2 (ICC, 1984).The content of free amino groups was determined from wet gluten immediately after washing out from flour samples (at 25 °C, treatment I) and after the wet gluten samples incubation for three hours at two different temperatures (30 °C and 37 °C, treatment II and treatment III, respectively). Every treatment was applied on flour samples of each examined wheat variety (Pob, Zve, Ap, Gord, Go) from all six areas (BT, SM, VR, KI, SU and SO). The determination of free amino groups was carried out in four replicates, where the results were calculated against a serine standard curve. The spectrophotometric readings were performed at 340 nm (GBC CINTRA 303UV/VIS).

Gluten index
Gluten index was measured in two different ways: according to the ICC standard method No 155 (ICC, 1994) (GI) and by modified method (GI(37 °C)) which includes incubation of dough ball at 37 °C for 90 min (Torbica *et al.,* 2007).

Statistical analysis
The effects of factors (incubation temperature, time, variety and locality) on free amino groups content was determined by ANOVA. Where the F-test for the ANOVA reached statistical significance ($p < 0.05$), the differences among specific means were assessed by Least Significant Difference (LSD) tests. The Principal Component Analysis (PCA) was used

for the estimation of the relation between the content of free amino groups and selected quality parameters of wheat/flour samples. Statistical methods were performed using the StatSoft. Inc. (2013) STATISTICA (data analysis software system) version 12.

RESULTS AND DISCUSSION

The content of free amino groups significantly increased with the increase in incubation temperature of gluten from 25 °C (0.000–0.1407 µg/mg), 30 °C (0.0001– 0.2894 µg/mg) to 37 °C (0.0186–0.2834 µg/mg) ($p < 0.05$) (Figure 1.).
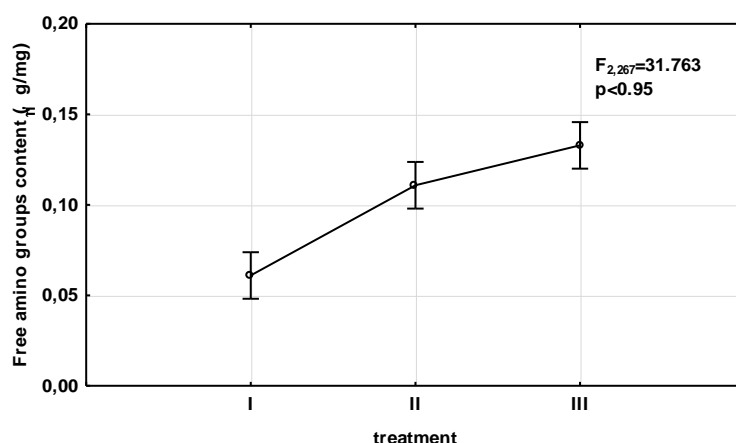
Figure 1. Difference of free amino groups between the temperature treatments (at 25 (treatment I), 30°C (treatment II) and 37 °C (treatment III))

According to Figure 2. and Figure 3. it could be noticed that content of free amino groups expressed the greater variability between varieties than the localities. This conclusion was confirmed by statistical analysis where the results showed that the content of free amino groups was significantly different between all varieties while in terms of locality there were minor differences.
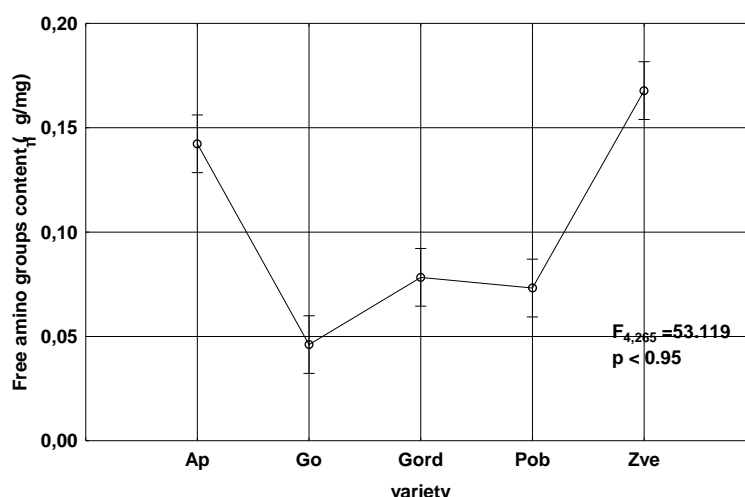
Figure 2. Difference of free amino groups depending on the wheat varieties

The lowest average value of free amino groups content was determined in Kikinda (0.0854 µg/mg), while the highest content was determined in Sombor locality (0.1267 µg/mg) (Figure 3.). Regarding the average values of free amino groups content there were not statistically significant differences between Bačka Topola, Sremska Mitrovica and Subotica localities.

Table 1 shows the structure and content of inseparable admixtures, values of GI and GI 37 °C of analyzed wheat samples from Bačka Topola, Kikinda, Sremska Mitrovica, Sombor, Subotica and Vršac localities. Only Fusarium infested kernels content was above acceptable limit (according to Serbian Regulation Regulation of methods of physical and chemical analysis for quality control of grain, milling and bakery products, pasta and quickly frozen dough, 1988,) and GI values were in the wide ranges.
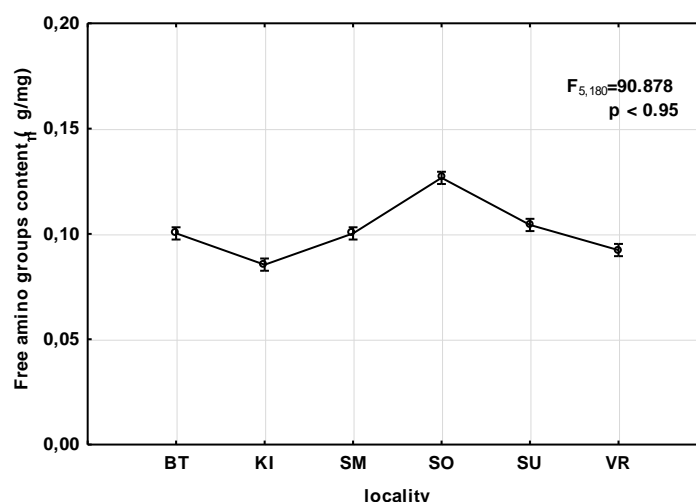
Figure 3. Difference of free amino groups depending on the locality

Table 1. The content of inseparable admixtures, GI and GI 37°C values of analyzed wheat samples from different localities

Locality	SWK (%)	KDP (%)	WBDK (%)	FIK (%)	BPK (%)	GI	GI(37°C)
BT	0	0	0.4-1	0.2-1.04	0.29-0.86	85.0 – 98.19	60.06-88.72
KI	0-0.16	0	0.21-0.62	0.0-0.72	0.76-3.16	74.0 – 92.0	58.2-82.26
SM	0-0.20	0	0.3-0.70	0.18-1.18	2.54-6.88	81.0 – 98.39	70.2-91.3
SO	0	0	1.04-1.52	0.14-0.42	0.9-2.74	82.0 – 95.0	22.84-81.44
SU	0 – 0.76	0-1.06	0.42-1.02	0.56-1.24	0.18-2.24	87.0 – 92.0	3.15-55.95
VR	0 – 0.16	0-0.06	0.26-1.32	0.32-0.92	1.64-4.7	81.0 - 98.65	59.29-85.47

Fig. 4 shows PCA loading and score plots for maturated wheat flour where the first two components explained 50.99% of the total variance in the biochemical indicators of protein properties determined. The first PC1 (31.26%) was related to the free amino groups content of wet gluten with the content of wheatbug damaged kernels (WBDK) and GI 37°C.
This indicated that the content of free amino groups was partly derived from gluten degradation caused by the activity of exogenous proteolytic enzymes originating from WBDK (lower values of GI 37 °C). This means that most of the registered free amino groups were the consequence of protein redistribution on a macromolecular level, which was primarily a characteristic of the variety. This was supported by the fact that the analyzed samples of flour from the 2010/2011 production year had good technological quality which was measured by conventional rheological methods-farinograph, extensograph, amylograph, alveograph and Mixolab (data not shown). The locality influence could be seen in the score plot where wheat samples from Subotica were grouped. Those samples had poor technological quality (data not shown) and the lowest values of GI 37 °C (Table 1). This also could be the consequence of protein redistribution on a macromolecular level because the chosen trade quality parameters were within acceptable limits.
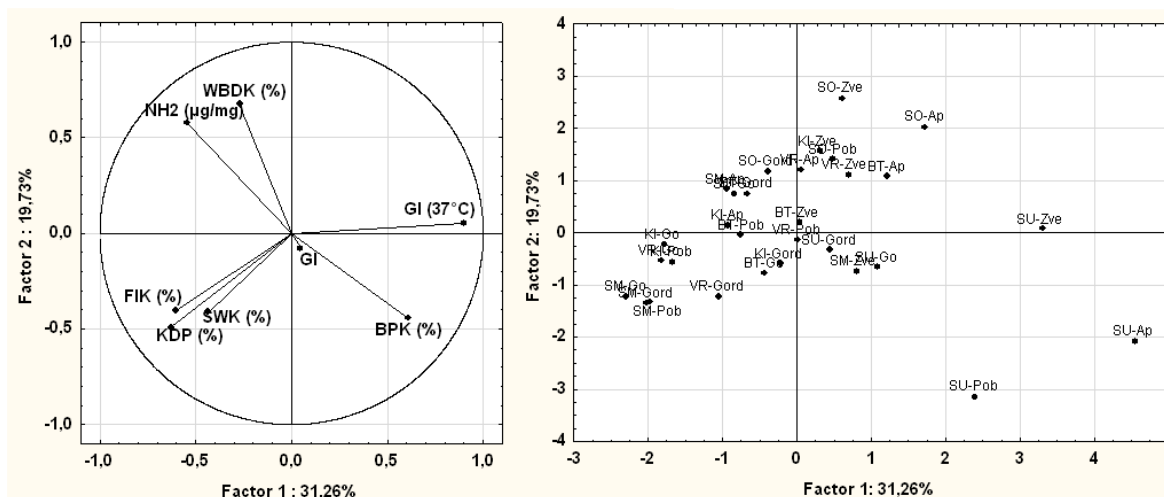
Figure 4. Principal component loading plot (a) and score plot (b) for maturated flours for quality indicators: NH2– free amino groups average content at different treatments (µg/mg); GI – standard gluten index; GI(37°C) –modified gluten index; WBDK – wheat-bug damaged kernels (%); SWK-sprouted wheat kernels (%); KDP- kernels damaged by pests (%); FIK- Fusarium infested kernels (%) and BPK- kernels with black point (%)

CONCLUSIONS

The highest content of free amino groups was determined at 37 °C (treatment III), suggesting that the damage of the proteins' primary structures occurred due to proteolytic enzymes activity. The increase in the free amino groups content determined at the ambient temperature could be explained by changes on a macromolecular level of the wheat flour protein complex structure.

ACKNOWLEDGEMENTS

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Regulation of methods of physical and chemical analysis for quality control of grain, milling and bakery products, pasta and quickly frozen dough – Pravilnik o metodama fizičkih i hemijskih analiza za kontrolu kvaliteta žita, mlinskih i pekarskih proizvoda, testenina i brzo smrznutih testa – Službeni list SFRJ, 74 (1988), 1854–1887 (in Serbian).

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POSSIBILITIES OF VISUAL AND INSTRUMENTAL IDENTIFICATION OF WHEAT INFECTION WITH FIELD FUNGI

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ABSTRACT

Cereals are the primary source of human diet, wheat being the third most produced grain worldwide. Recent studies have shown that besides *Fusarium* spp., fungi of the genus *Alternaria* spp. became the dominant contaminants on wheat kernels. Besides pathogenicity and reduction of quality of kernels, several *Alternaria* spp. are known producers of toxic secondary metabolites *Alternaria* mycotoxins, which might be harmful for human and animal health. The aim of this work was to explore the possibility of determining the intensity of field fungi infestation by application of visual evaluation (using scale 1-6, where 1 represents the lightest sample) and instrumental analysis (using Minolta Chromameter CR-400) of wheat ears and kernels colour. Experiment was carried out on the wheat protected by fungicide and wheat inoculated by *Alternaria* spp., while non treated wheat was used as a control. Wheat ears used for the establishing of visual scale were measured instrumentally in order to explore the correlations between these two methods. Grouping of wheat ear samples by values of L* colour parameter (lightness) was in accordance with the established scale. L* values were also in the highest negative correlation (-0.97, p < 0.001) with the visual scale. Significant difference was observed between all three treatments using visual scale. Protected wheat samples were significantly different from other samples in terms of all measured colour parameters (L*, a*, b*, C*, hue angle, and dominant wavelength). Inoculated and control wheat samples were significantly different in terms of lightness, hue angle, and dominant wavelength. The colour of wheat kernels was only instrumentally measured. The kernels of inoculated wheat samples differed significantly from other samples in terms of L*, a*, b*, and C* parameters. Identification of field fungi in the all examined wheat samples showed that the dominant toxicogenic fungus was *Alternaria* spp., followed by Fusarium spp.
Keywords: *colour, sensory evaluation, wheat ears, wheat kernels, field fungi*

INTRODUCTION

Cereals are the primary source of human diet, wheat being the third most produced grain worldwide. Recent studies reported that fungi of the genus *Alternaria* dominates on wheat kernels (EFSA, 2011). The two major features of *Alternaria* species are the production of melanin, especially in the spores, and the production of host-specific toxins in the case of pathogenic species (Thomma, 2003). Apart from a role in conidial development (Kawamura *et al.,* 1999), melanins appear to have an indirect as well as a direct function in virulence. On the one hand they act as 'body armour', protecting fungi against environmental stress or unfavourable conditions like extreme temperatures, UV-radiation and compounds secreted by microbial antagonists, thus adding to longevity and survival (Kawamura *et al.,* 1999; Rehnstrom and Free, 1996). There are various types of discolouration that can affect common (*Triticum aestivum* L.) wheat kernels. Black point and dark smudge, mostly associated with *Alternaria alternata* (Fr.) Keissl., and *Cochliobolus sativus* (Ito and Kurib.) Drechs. ex Dast. [anamorph *Bipolaris sorokiniana* (Sacc.) Shoemaker] (Fernandez *et al.,* 1994) are common discolourations of cereal seed, which occur in most regions where these crop species are grown. The condition in wheat or barley known as black point is a dark discoloration at the embryo end of the kernel, resulting in downgrading of the grain. In severe cases, the discoloration occurs in the outer pericarp and inner seed coat tissue, and may extend along the groove on the ventral side of the grain (Mónaco *et al.,* 2004; Williamson,

1997). These types of kernel discolouration vary significantly in incidence and severity depending on environmental conditions during kernel maturation. Numerous other studies indicate that black point may be a result of abiotic stresses, as symptoms are more likely to occur after extreme environmental conditions such as heavy rain, high humidity and extremes of temperature (Clarke *et al.,* 2004; Conner, 1989; Conner and Davidson, 1988; Fernandez *et al.,* 1994; Kumar *et al.,* 2002; Sadasivaiah *et al.,* 2004a, b). However, a recent study showed that although abiotic factors, such as high humidity levels, can promote the occasional development of black point or dark smudge on durum wheat kernels under controlled-environment conditions, fungal infection by *C. sativus* or *A. alternata* was the main factor associated with their development (Fernandez *et al.,* 2011).

In this context, the aim of this work was to explore the possibility of determining the intensity of field fungi infestation by visual scale application and instrumental measurement of wheat ears and kernels colour.

MATERIAL AND METHODS

Material and climate conditions
Experiment was carried out in the 2012/2013 season in the region of Vojvodina, north Serbia on the wheat (*Triticum aestivum* cv. Sirtaki) protected by fungicide and wheat inoculated by *Alternaria* spp., while non treated wheat was used as a control. May 2013 was characterized by weather fluctuations, warmer and more humid weather conditions than multiannual average, with surplus precipitation (ΣPrec. 125 mm). According to Standardized Precipitation Index (SPI), Z index, and Palmer Drought Severity Index (PDSI) values (1.9, 4.8, and 4.0, respectively), this month had extreme humid weather conditions.

Fungus culture and inoculation
The isolates of *A. tenuissima* were multiplied in 8 L Chapek medium without agar at room temperature for 14 days. For the purpose of inoculation, a conidial suspension 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. tenuissima* conidia was $0,13025 \times 10^6$ infective particles/mL. At the full flowering stage inoculation was performed with 8 L of aqueous suspension of fungal isolates. Inoculated spikes were immediately covered with plant protection cover (Stocker, Italy) for 48 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.

Visual scale establishing and colour measurement
Visual scale (1-6) of wheat ear colour was established, where 1 represented the lightest sample and 6 represented the darkest sample. These samples were also measured instrumentally in order to explore the correlations between these two methods. The colour of wheat kernels was only instrumentally measured.

The colour of all samples was directly measured with Konica Minolta Chroma Meter CR-400, using different attachments; for the measurement of wheat ears colour, Light Protection Tube CR-A33f was used, while the colour of wheat kernels and hulls was measured using Granular Attachment CR-A50. The CIE L* (lightness), CIE a* (red-green) and CIE b* (yellow-blue), C* (chroma), h° (hue angle) and dominant wavelength (DWL) were read using a D_{65} light source and the observer angle at 2°. The tristimulus values of CIE L*, a*, b* readings were calibrated against a standard white plate (Y=84.8; x=0.3199; y=0.3377). Each wheat ear sample was divided in four subgroups, and one hundred ears from each subgroup (400 ears from one sample) were measured on 6-8 locations, depending on ear size. Each wheat kernel sample was also divided in four subgroups, and ten replications were measured from each subgroup (40 replications per sample in total).

Percent of kernel infection

According to the method proposed by Pitt and Hocking (1985) 100 wheat kernels were randomly selected from each treatment. The samples were disinfected 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 25°C and after seven days intensity of infection was assessed. Confirmation of fungi genera was carried out on potato dextrose agar (PDA) and malt extract agar (MEA) media after 7 days of incubation at 25 °C.

Statistical analysis

Pearson correlation coefficients on different significance levels (5%, 1%, and 0.1%) between visual scale and measured parameters were calculated. Analysis of variance (ANOVA) and Duncan's multiple range tests were applied to compare means at 5% significance level. Principal Component Analysis (PCA) was applied to explore the relationships among the colour parameters and to group the wheat ears used for visual scale establishing. Data analysis was performed using the statistical data analysis software system STATISTICA (StatSoft, Inc. (2013), version 12.0 (www.statsoft.com).

RESULTS AND DISCUSSION

The aim of this work was to explore the possibility of determining the intensity of field fungi infestation by application of visual evaluation and instrumental analysis of wheat ears and kernels colour. Wheat ears used for the establishing of visual scale were measured instrumentally in order to explore the correlations between these two methods. Pearson correlation coefficients between visual scale and instrumental measurement showed that L* values were in the highest negative correlation (-0.97, $p < 0.001$) with the visual scale. Other colour parameters (b*, C*, hue angle, and DWL) were also in high correlation ($p < 0.001$) with the visual scale (-0.72, -0.72, -0.75, and +0.74, respectively). Colour parameter a* was also significantly positively correlated with the visual scale at 5% significance level. Six wheat ears used for visual scale establishing differed significantly among each other only by L* (lightness) values.

PCA results showed that high percentage of total variance is explained by the first two components (96.29%) (Figure 1). Colour parameters b* and C* were close to the circle line and almost overlapping, which indicated high correlation (*r* close to +1) between them. L* value was also highly correlated with these two parameters, leading to the connection between yellow tone, saturation, and lightness of examined wheat ears. Hue angle and dominant wavelength were on the opposite sides of the centre, implying that they are significantly negatively correlated (*r* close to -1). Wheat ears that comprised the visual scale were completely separated by colour parameters (Figure 2). Wheat ears assessed as 1 and 2 were distinguished mostly by hue angle, while wheat ear assessed as 3 was separated mostly by high L* values. Wheat ear assessed as 6 was almost in opposite to the wheat ear marked as 3, indicating low L* values. DWL and a* caused differentiation of wheat ears marked as 5 and 4, respectively, which could be explained by more prominent red tone. Measured colour parameters were in most cases in accordance with appearance of wheat ears used for visual scale establishing.

Considering differences between wheat samples, all three treatments differed significantly among each other by scores obtained using visual scale (Table 1), with protected wheat sample assessed with lowest scores, and inoculated wheat sample with highest scores. Protected wheat samples were significantly different from other samples in terms of all measured colour parameters (L*, a*, b*, C*, hue angle, and dominant wavelength). Inoculated and control wheat samples were significantly different in terms of lightness, hue angle, and dominant wavelength.

Results of kernel colour measurement were slightly different: kernels of inoculated wheat differed significantly from other samples in terms of L*, a*, b*, and C* parameters (Table 2), whereas all samples belonged to the same homogenous group by hue angle and dominant wavelength values.

Considering all mentioned above, it can be concluded that infection entered the kernel in greater extent in inoculated wheat samples, while in non treated samples it was less prominent. Protected wheat samples were generally characterized by higher lightness and more prominent yellow tone. Results obtained by instrumental colour measurement are in accordance with the visual scale assessment of treated wheat samples.
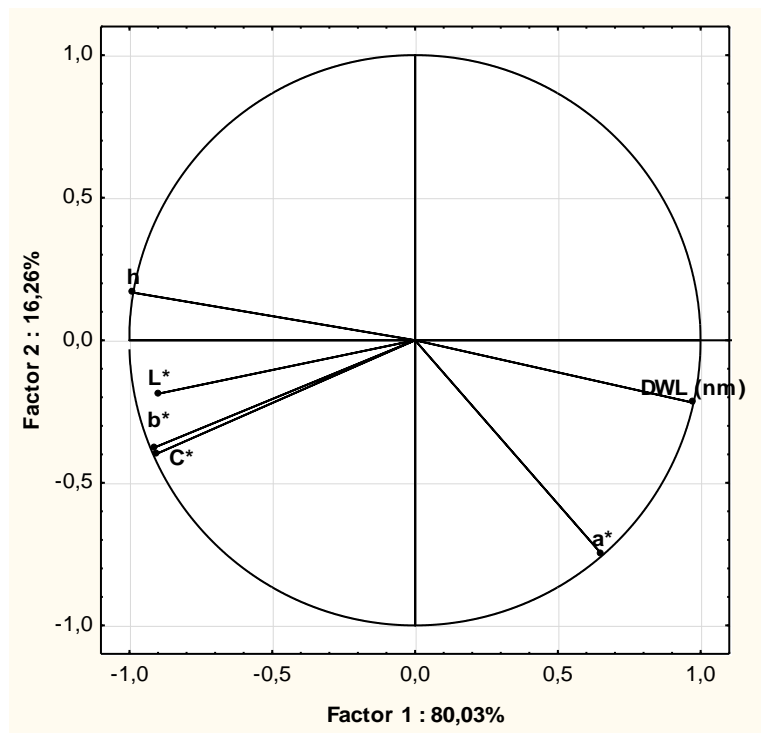
Figure 1. PCA: Projection of the variables on the factor plane
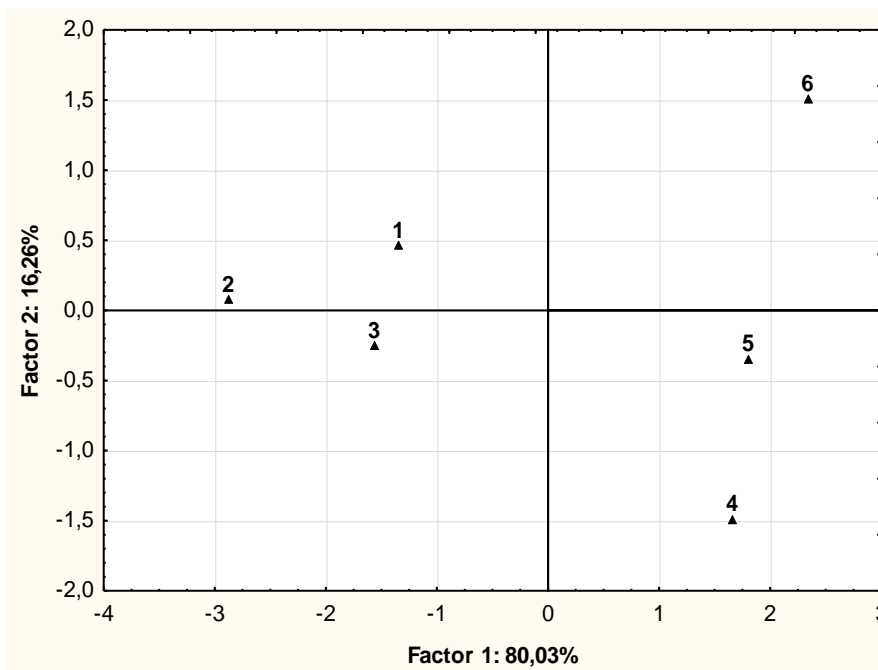
Figure 2. PCA: Projection of the cases on the factor plane

Identification of field fungi in the all examined wheat samples showed (Table 3.) that the dominant toxicogenic fungus was *Alternaria* spp., followed by *Fusarium* spp. Percent of infection by *Alternaria* spp. was the highest for inoculated samples as expected. This caused the differentiation of this wheat sample by its dark colour as determined both by application of visual scale and instrumental measurement, which was expected due to the the fact that *Alternaria* spp. produce melanin pigments.

Table 1. Colour parameters of differently treated wheat ears

Sample	L*	a*	b*	C*	h (°)	DWL (nm)	Visual scale
0	60.91±3.05[b]	2.89±0.70[a]	24.07±3.89[b]	24.25±3.89[b]	83.03±1.81[c]	578.33±0.52[a]	2.90±0.50[b]
1	63.16±3.46[a]	2.49±0.69[c]	25.41±3.32[a]	25.54±3.31[a]	84.35±1.62[a]	577.94±0.49[c]	2.35±0.74[c]
2	60.09±3.87[c]	2.81±0.71[b]	24.11±4.01[b]	24.28±4.00[b]	83.19±2.05[b]	578.29±0.59[b]	3.01±0.75[a]

0 - non treated wheat sample; 1 - protected wheat sample; 2 - inoculated wheat sample.
Results are presented as mean±standard deviation (n≈2400). Values with the different superscript within the same column are statistically different (P<0.05).

Table 2. Colour parameters of differently treated wheat kernels

Sample	L*	a*	b*	C*	h (°)	DWL (nm)
0	53.05±1.63[a]	8.73±0.91[a]	23.81±1.60[a]	25.36±1.78[a]	69.91±1.05[a]	582.64±0.41[a]
1	53.34±1.88[a]	8.87±0.88[a]	24.09±1.84[a]	25.67±2.00[a]	69.81±0.97[a]	582.68±0.37[a]
2	52.20±1.53[b]	8.30±0.96[b]	22.25±1.84[b]	23.75±2.03[b]	69.58±1.02[a]	582.67±0.40[a]

0 - non treated wheat sample; 1 - protected wheat sample; 2 - inoculated wheat sample.
Results are presented as mean±standard deviation (n=40). Values with the different superscript within the same column are statistically different (P<0.05).

Table 3. Presence of certain genera of fungi in wheat samples

Sample	Fungi genus (%)					
	Fusarium	*Alternaria*	*Cladosporium*	*Rhizopus*	*Aspergillus*	*Penicillium*
0	2	31.5	1	65.5	-	-
1	2	29	-	69	-	-
2	4	44.5	-	50	-	1.5

0 - non treated wheat sample; 1 - protected wheat sample; 2 - inoculated wheat sample.

CONCLUSIONS

Pearson correlation coefficients between visual scale and instrumental measurement showed that L* values were in the highest negative correlation with the visual scale. Samples used for visual scale establishing differed significantly among each other only by L* (lightness) values. Results obtained for the non treated, protected and inoculated wheat samples showed that instrumentally measured colour parameters are in accordance with the visual scale assessment. Identification of field fungi in the all examined wheat samples showed that the dominant toxicogenic fungus was *Alternaria* spp., followed by *Fusarium* spp. It can be concluded that higher degree of infection by *Alternaria* spp., higher score on visual scale and lower L* (lightness) values were directly related due to the production of melanin pigments by this genus of fungi.

ACKNOWLEDGEMENTS

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AGARICUS SILVATICUS - PROMISING FUNCTIONAL FOOD

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ABSTRACT

Objectives of this study were a) to determine the antibacterial ability of crude hot water extract (SV) and hot alkali extract (SNa) obtained from the mushroom *A. silvaticus* against selected foodborne Gram-positive and Gram-negative pathogenic bacteria by microdilution assay; b) to evaluate their antioxidant ability by measuring DPPH free radical scavenging activity assay, inhibition of lipid peroxidation, reducing power and chelating ability; c) to determine their cytotoxic effect on malignant human breast cancer MDA-MB-453, cervical adenocarcinoma HeLa and myelogenous leukemia K562 cells. Antiproliferative activity of investigated compounds was assessed, measuring cell survival in standard, 72 h, by MTT test.

Gram-positive bacterial strains were more susceptible to the tested extracts than Gram-negative. SNa possessed higher activity than SV (MIC - 0.3125 - 5 mg/mL and 5 - 10 mg/mL). At 10 and 2.5 mg/mL, scavenging abilities of SV and SNa toward DPPH radicals increased to 76.8 ± 1.2 and 74.8 ± 2.3%. The antioxidant activities of SV and SNa reached the levels of 62.7 ± 3.1 and 81.8 ± 1.9% at 0.1 mg/mL. At 5 mg/mL SV and SNa chelated 87.7 ± 2.7 and 81.8 ± 1.4% of ferrous ions. The reducing power of SV and SNa was 1.4 ± 0.8 and 2.2 ± 1.6 at 5 mg/mL. Extracts derived from *A. silvaticus* displayed dose dependent antiproliferative action towards all investigated tumor cell lines, with IC_{50} values ranging from 0.7 to 1.7 mg/mL. The most cytotoxic effect showed SNa extract (IC_{50}=0.7 mg/mL for HeLa cells).

The results of this study confirm a high biological potential of mushroom *A. silvaticus*. At a time of increasing resistance of microorganisms to conventional antibiotics, naturally-derived antimicrobial substances are very desirable. Due to its very pleasant taste and nutritional value, antibacterial potential as well as a high content of antioxidant components it could be considered as functional food and might be able to contribute to the reduction of cancer risks.

Keywords: *Agaricus silvaticus, antimicrobial, antioxidant, antiproliferative, functional food*

INTRODUCTION

Agaricus silvaticus Schaeffer is often found in groups in mixed woodland and under trees in parks. Recent investigations suggest highly efficient biological properties of this common, edible mushroom.

Since ancient times wild edible mushrooms are appreciated for their texture, flavor and medicinal properties. Recent studies confirmed their very important chemical and nutritional characteristics - higher protein, mineral and vitamin contents (in particularly, vitamin D) and less fat. Due to this favorable chemical composition mushrooms are sources of nutrients and important therapeutic foods, useful in preventing of much health related disorders. Barros *et al.,* (2008 a) reported that *A. silvaticus* is important source of nutrients and nutraceuticals.

Mushrooms have become attractive as functional foods and their functional characteristics are mainly due to the presence of several active substances, including polysaccharides, dietary fiber, in particular chitin and beta glucans, as well as triterpenoids, specific proteins and phenolic compounds (Cheung, 2008).

There are a number of microorganisms that can cause different diseases, spoil food, cosmetic and pharmaceutical products whose use might cause intoxication. A common way to fight against microorganisms is the application of appropriate antibiotics, but there are also significant problems caused by long-term use of antibiotics since the microorganisms become resistant to them. Additionally, there are many side effects, especially during

prolonged and improper use of antibiotics. Therefore, the search for new antimicrobial agents without adverse effects, such as various herbs and mushrooms, is of major importance.

The last three decades wild mushrooms are increasingly attracting attention as organisms with strong antioxidant properties (Kozarski *et al.,* 2014). Among several *Agaricus* species, tested in chemical, biochemical and electrochemical assays by Barros *et al.* (2008 b), *A. silvaticus* was the most efficient exhibited the highest antioxidant power. Using more mushrooms in the daily diet could reduce oxidative damage in cells, caused by free radicals, that cause oxidation of nucleic acids, proteins and lipids and thereby possibly to aging and cause and acceleration of very serious illness.

Mushrooms are also recognized as functional foods for their polysaccharides derived from fruiting bodies as well as from liquid - cultured mycelium. These polysaccharides have been identified as many types of glucans (e.g., β-1,6 and β-1,3). High molecular weight glucans exhibit more effective antitumor and immunostimulating properties than those of low molecular weight (Wasser, 2002).

MATERIAL AND METHODS

Crude hot water extract (SV) and hot alkali extract (SNa) obtained from *A. silvaticus* were prepared as described before (Klaus *et al.,* 2011).

Antimicrobial activity testing

Gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Listeria monocytogenes*) and Gram-negative bacterial species (*Escherichia coli*, *Salmonella enteritidis*, *Shigella sonnei*, *Yersinia enterocolitica*) were challenged in this study to ascertain the antibacterial properties of SV and SNa. Selected species of foodborne pathogenic bacteria originate from ATCC (American Type Culture Collection, Rockville, Maryland). Working concentrations of approximately 10^5-10^6 cfu/mL were used for antibacterial activity assay.

SV and SNa were dissolved in dimethylsulfoxide DMSO (2%) to prepare stock solutions at a concentration of 80 mg/mL, sterilized by filtration through a 0.22 µm membrane filter (Sartorius, Germany), and further diluted in Mueller-Hinton broth (MHB) to working solutions. DMSO was chosen as a non-toxic solvent.

Broth microdilution method

Broth microdilution method was employed to determine minimum inhibitory (MIC) concentrations (Klančnik *et al.,* 2010). Concentrations of mushroom extracts ranged from 20.0 to 0.0097 mg/mL. Test bacterial culture (50 µL) in a MHB was added to the wells of a sterile 96-well microtiter plate (Sarstedt, Germany) already containing 50 µL of two-fold serially diluted extract in MHB. The final volume in each well was 100 µL. The microplates were incubated aerobically, for 24 h at 37°C. Positive controls were wells with a bacterial suspension in 50 µL of MHB and wells with a bacterial suspension in a MHB with DMSO in amounts corresponding to the highest quantity present in the broth microdilution assay. A microplate shaker (Lab Companion, VM-96B, Korea) was used for mixing the content of each well at 900 rpm for 1 min prior to incubation in the cultivation conditions described above.

To indicate cellular respiration, TTC (0.05%) was added to the culture medium. The MIC was defined as the lowest sample concentration that exhibited complete inhibition of bacterial growth.

Evaluation of the antioxidant properties

The inhibition of lipid peroxidation was determined by the conjugated diene method (Lingnert, Vallentin, Eriksson, 1979).

DPPH free radical scavenging activity assay was done according to Ekanayake et al., 2005, and modified as described before (Klaus *et al.,* 2011).

Ferric-reducing antioxidant power assay was determined according to Oyaizu (1986).

Chelating ability on ferrous ions was determined according to the method of Dinis, Madeira, Almeida (1994), which was modified by us (Klaus *et al.,* 2011).

Cytotoxicity analysis

Cell culture
Stock solutions of SV and SNa were prepared in a nutrient medium. Human cervix adenocarcinoma HeLa and breast carcinoma MDA-MB-453 were cultured as monolayers in the nutrient medium, while myelogenous leukemia K562 cells were maintained as suspension culture. The cells were grown at $37^{o}C$ in 5% CO_2 and humidified air atmosphere.

Cell sensitivity analysis
HeLa (2.000 cells per well) and MDA-MB-453 (3.000 c/w) cells were seeded into 96-well microtiter plates and 20 h later, five different concentrations of investigated extracts were added to the wells. Final concentrations were in the range from 0.1875 to 3 mg/mL. Investigated compounds were added to a suspension of K562 cells (5.000 c/w), 2 h after cell seeding, in the same final concentrations applied to HeLa and MDA-MB-453 cells.

Determination of target cell survival
Cell survival was determined by MTT test according to the method of Mosmann (1983) and modified by Ohno and Abe (1991), 72 h after the investigated extracts were added.

RESULTS AND DISCUSSION

Evaluation of antimicrobial activity
Broth microdilution method as a rapid quantitative determination of MIC, based on the color change caused by the enzymatic activity of viable microorganisms, was applied. Well defined endpoints appeared as the results of the metabolic activity of bacteria, i.e. TTC reduction (Table 1).

Table1. Antibacterial activity of crude hot water extract (SV) and hot alkali extract (SNa) from A. silvaticus expressed as MIC (mg/mL) determined by the broth microdilution method

Bacterial strain	Source	MIC (mg/mL)	
		SV	SNa
Staphylococcus aureus	ATCC 25923	$5.0 \pm 0.0^{a*1}$	2.5 ± 0.0^{b}
Enterococcus faecalis	ATCC 29212	5.0 ± 0.0^{a}	0.3129 ± 0.0000^{b}
Bacillus cereus	ATCC 10876	5.0 ± 0.0^{a}	1.25 ± 0.00^{b}
Listeria monocytogenes	ATCC 19115	5.0 ± 0.0^{a}	2.5 ± 0.0^{b}
Escherichia coli	ATCC 25922	5.0 ± 0.0^{a}	5.0 ± 0.0^{a}
Salmonella enteritidis	ATCC 13076	10.0 ± 0.0^{a}	5.0 ± 0.0^{b}
Shigella sonnei	ATCC 29930	5.0 ± 0.0^{a}	2.5 ± 0.0^{b}
Yersinia enterocolitica	ATCC 27729	10.0 ± 0.0^{a}	5.0 ± 0.0^{b}
Escherichia coli (0157:H7)	ATCC 12900	5.0 ± 0.0^{a}	5.0 ± 0.0^{a}

Data are expressed as mean ± standard deviation (n=3)
[1]*Within the same row, means followed by different letters are significantly different at α=0.05 (ANOVA, Tukey`s HSD Test)*

Both extracts, SV and SNa, inhibited the growth of all tested Gram-positive and Gram-negative bacteria. In most cases SNa possessed higher activity than SV (MIC - 0.3129 ± 0.000 - 5.0 ± 0.0 mg/mL and 5.0 ± 0.0 - 10.0 ± 0.0 mg/mL, respectively); exceptions were both tested strains of *E. coli* which were equally sensitive to the SV and SNa. The highest antibacterial potential of SNa was achieved against *E. faecalis* (MIC - 0.3129 ± 0.0000 mg/mL), indicating promising potential of SNa to inhibit this causer of serious infections in humans, which can be very useful, especially in case of antibiotic-resistant strains.

In general, Gram-positive bacteria have demonstrated a greater sensitivity to the tested extracts than Gram-negative bacteria, probably due to the difference in the structure of the cell wall.

Evaluation of antioxidant activity

An increase in antioxidant activity with increasing concentration of the extracts was confirmed in all applied assays. SNa was better antioxidant shown by the lower EC_{50} values of inhibition of lipid peroxidation, ferric-reducing antioxidant power, DPPH scavenging ability, and ferrous ion-chelating ability (Table 2).

Table 2. EC_{50} values of crude hot water extract (SV) and hot alkali extract (SNa) from A. silvaticus in antioxidant properties

	EC50a (mg extract / mL)	
	SV	**SNa**
Inhibition of lipid peroxidation	0.25 ± 0.0	< 0.1
Reducing power	1.5 ± 0.1 A[b]	0.72 ± 0.2 B
Scavenging ability on DPPH radicals	2.22 ± 0.2 A	0.26 ± 0.1 B
Chelating ability on ferrous ions	0.33 ± 0.0 A	0.09 ± 0.0 B

[a]EC_{50} value: The effective concentration at which the inhibition of lipid peroxidation was 50%; the absorbance was 0.5 for reducing power; 1,1-diphenyl-2- picrylhydrazyl (DPPH) radicals were scavenged by 50%; and ferrous ions were chelated by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.
[b]Each value is expressed as mean ± standard deviation (n = 3). Within the same row, means followed by different letters are significantly different at α=0.05 (ANOVA, Tukey`s HSD Test)

At particularly low concentration of 0.1 mg/mL SV and SNa inhibited peroxidation of 62.7 ± 3.1 and even 81.8 ± 1.9% lipids. Excellent antioxidant capacity of the SNa and SV is expressed through very low EC_{50} value (<0.1 and 0.25 ± 0.0 mg/mL, respectively). Based on our previous report (Klaus et al., 2011) it seems that monosaccharide composition of the polysaccharide chains, emerged during the hot alkaline treatment, directly affects on the prevention of peroxidation of linoleic acid.

The reducing power of SV and SNa was 1.4 ± 0.8 and 2.2 ± 1.6 at 5 mg/mL, and EC_{50} value of SNa (0.72 ± 0.2 mg/mL) was signicantly lower than that of SV (1.5 ± 0.1 mg/mL). These results suggest that hot alkaline extraction could especially contribute to the ability of the extract to reduce Fe^{3+} to Fe^{2+} by donating an electron.

At 10 mg/mL scavenging ability of SV toward DPPH radicals increased to 76.8 ± 1.2%, while 74.8 ± 2.3% was achieved in the presence of four times lower concentration (2.5 mg/mL) of SNa. Significantly higher potential of SNa toward DPPH radicals was confirmed by approximately ten times lower EC_{50} value (0.26 ± 0.1 mg/mL), compared with SV (2.22 ± 0.2 mg/mL), and might be due to higher levels of hydrogen-donating components occurred during a very intense alkaline treatment.

At 5 mg/mL SV and SNa chelated 87.7 ± 2.7 and 81.8 ± 1.4% of ferrous ions, and in this case, also, EC_{50} value of SNa (0.09 ± 0.0 mg/mL) was signicantly lower than that of SV (0.33 ± 0.0 mg/mL). It appears that extraction in hot sodium hydroxide solution contributed to higher ability to form complexes with Fe^{2+}.

In vitro cytotoxic activity

The cytotoxicity of the two *A. silvaticus* extracts, crude hot water extract (SV) and hot alkali extract (SNa), was tested against selected cancer cell lines: human cervix adenocarcinoma HeLa, human myelogenous leukemia K562 and human breast carcinoma MDA-MB-453 cells. Both investigated extracts exerted selective dose-dependent cytotoxic actions on malignant cells. The decrease in survival of target cancer cells induced by the extracts is shown in Figure 1 and Table 3.

With IC_{50} values (concentration of extract that is required for 50% inhibition *in vitro*) ranging from 0.7 to 1.7 mg/mL, following continuous incubation, both examined extracts possess

moderate cytotoxicity. The highest cytotoxicity was found in a SNa extract treated HeLa cells (IC_{50}=0.7 ± 0.1 mg/mL).

Data from this *in vitro* study demonstrate moderate antitumor potential of two extracts prepared from the *A. silvaticus*. Cancer-suppressive effects of the tested extracts should be further evaluated in *in vivo* experiments, and also, extracts should be fractionated to identify the active antitumor compounds.
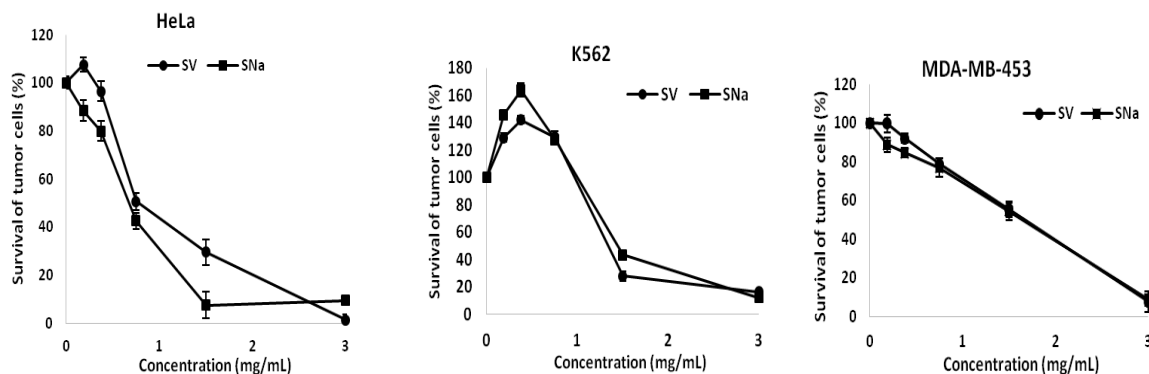
Figure 1. Survival of tumor cells as determined by MTT test, after 72 h of continuous action of applied concentrations of SV and SNa. It is given as a function of different concentrations of investigated extracts.

Table 3. Concentrations of crude hot water extract (SV) and hot alkali extract (SNa) from A. silvaticus which induced 50% decrease (IC_{50}) in malignant cell survival

IC_{50} [mg/mL] ± SD			
Extract	**HeLa**	**K562**	**MDA-MB-453**
SV	0.8 ± 0.1	1.3 ± 0.5	1.7 ± 0.5
Na	0.7 ± 0.1	1.4 ± 0.2	1.6 ± 0.4

CONCLUSIONS

Crude hot water extract and hot alkali extract obtained from *A. silvaticus* possess higher antibacterial potential against tested Gram-positive than Gram-negative bacterial strains. The highest activity exhibited hot alkali extract towards *E. faecalis*. As infections caused by this bacterium are very difficult to treat due to its frequent resistance to multiple antibiotics, the use of *A. silvaticus* extracts as supplements to certain types of food might lead to the destruction of bacteria in food and thus to contribute to the reduction of poisoning with this type of bacteria.

The results from different *in vitro* assay systems, including the inhibition of lipid peroxidation, the scavenging effects on DPPH radical, the reducing power and the ferrous ions chelating effect, demonstrated that these polysaccharide extracts have effective antioxidant activities. These findings could be important in terms of development of natural, easily accessible sources of antioxidant agents which are able to protect the human body from free radicals and to slow down the progress of many chronic diseases.

According to this investigation, crude hot water extract and especially hot alkali extract obtained from the mushroom *A. silvaticus* showed very desirable biological properties. In order to improve health condition it would be very useful to include this mushroom in our daily diet, since it possesses potential as a functional food.

ACKNOWLEDGEMENTS

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SENSORY PROPERTIES AND SHELF LIFE OF SPREADABLE CREAM WITH SOYBEAN OIL

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ABSTRACT

Spreadable cream is confectionery product based on powdered sugar, vegetable fat, cocoa powder, milk powder and other ingredients. Beside hidrogenated fats, cream product contains sunflower oil rich in essential fatty linoleic acid, but almost does not contain the essential α-linolenic acid. Soybean oil contains 85–88% unsaturated fatty acids of which up to 10% of α-linolenic acid. Oils rich in unsaturated fatty acids have beneficial nutritional attributes that make them desirable for incorporation into various foods, but, on the other hand, these oils might have lower oxidative stability.

This research examined sensory properties and shelf life of cocoa spreadable cream with partial and complete replacement of sunflower oil, which is exclusively used in Serbia in the production of cocoa spreadable cream, with soybean oil. Color on the surface of cocoa spread samples was monitored by colorimetric method and sensory analyses 24h after cream production and every month in the period of six months of storage in the dark and room temperature. Oxidative stability was evaluated applying gas chromatographic analyses of the formed aldehydes as secondary products of lipid oxidation.

The results showed that partial and complete replacement of sunflower oil with soybean oil doesn't have any influence on surface color and shelf life of cocoa spreadable cream. Soybean oil improved the taste of cocoa spread cream and had no influence on other sensory parameters.

Keywords: spreadable cream, soybean oil, sensory properties, color, shelf life

INTRODUCTION

Raw material usually used for cocoa cream spread manufacturing consists of powdered sugar, cocoa powder, milk powder, hydrogenated vegetable fat, and may contain some other ingredients. The most important technological and sensory characteristics of this type of product are: good spread ability in a wide temperature range, rich creamy taste, smooth homogeneous structure with no fat phase separation, and good oxidative stability (Petković *et al.,* 2013). Beside hidrogenated fats, cream product can contain sunflower oil in order to improve the spread ability of cocoa spread cream (Lončarević, 2013). Fat phase comprises over 30% of cocoa spread cream and therefore its physical and sensory properties are strongly influenced by the behavior of fat phase (Pajin *et al.,* 2007).

Nowadays, consumers more and more believe that foods contribute directly to their health. The development of new functional food products turns out to be increasingly challenging, as it has to satisfy the consumer's expectancy for products that are simultaneously tasty and healthy. For that reason, the food industry takes into consideration many variables to develop functional products, such as sensory acceptance, price, physical and chemical characteristics, functional properties, stability (Betoret *et al.,* 2011). According to the American Dietetic Association, functional food is defined as: any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients that contains (El Hadad *et al.,* 2011). Oilseed medicinal is a new term that are used to describe a compound that is found in edible oil, often also cited as the active ingredient in a pharmaceutical reference text commonly used by health professionals (Reichert, 2002).

Oils rich in unsaturated fatty acids have beneficial nutritional attributes that make them desirable for incorporation into various foods. Soybean oil contains 12–15% saturated fatty acids (mostly palmitic) and 85–88% unsaturated fatty acids (mostly oleic, linoleic, and linolenic). On the other hand, these oils have a lower oxidative stability (Chen *et al.,* 2012).

Off-flavors in fat based food occur mainly as a result of the formation of volatile compounds, representing a small proportion of the formed lipid oxidation products. Some of them, such as aldehydes, are highly specific for the oxidative degradation of a particular polyunsaturated fatty acids and chromatographic analyses of the formed aldehydes as secondary products of lipid oxidation. Static head space gas chromatography (SHS GC) analysis is considered to be an easy, reliable and fast method for the determination of the main volatile compounds (Mandić *et al.,* 2013).

Soybean oil is considered to have good nutritive value mainly because of its high concentration of essential polyunsaturates. This oil contains about 55% linoleate and 8% linolenate, both recognized as essential fatty acids (Hammond *et al.,* 2005).

This research examined sensory properties and shelf life of cocoa cream spread with partial and complete replacement of refined sunflower oil, which is exclusively used in Serbia in the production of cocoa spread cream, with soybean oil.

MATERIAL AND METHODS

Materials used as ingredients
- Cocoa-cream mass that passed through 3 roll mill in industrial conditions (mixture of powdered sugar, cocoa powder, milk powder, vegetable fat)
- Vegetable fat NTFCP – produced in Oil Factory "Dijamant", Serbia
- Refined sunflower and soybean oil, produced by Oil factory Victoriaoil, Serbia
- Native fluid sunflower lecithin – produced by Oil Factory "Victoriaoil", Serbia
- Hazelnut and vanilla flavor

The composition of cocoa spread cream, used as control, includes: 50% of powdered sugar, 24% of vegetable fat, 6% of refined sunflower oil, 7% of cocoa powder, 12% of milk powder, 0.5% of lecithin, vanilla and hazelnut flavors in trace.

Plan of experiments
Partial and complete quantity of sunflower oil was replaced with soybean oil in order to get the following samples:

Control sample (C/control) - cocoa spread cream with 100% of sunflower oil
C/50 – cocoa spread cream with 50% of soybean oil (and 50% of sunflower oil)
C/70 - cocoa spread cream with 70% of soybean oil (and 30% of sunflower oil)
C/100 - cocoa spread cream with 100% of soybean oil

Preparation of cocoa spread cream samples
Raw materials were added into a laboratory ball mill with a capacity of 5 kg. The temperature in the ball mill was 40°C, with a speed of 50 rpm. Retention time of milling was 40 minutes.

Fatty acid composition
The fatty acid content in oils and cocoa cream spread samples were determined by gas chromatography (ISO 5508:1990), using gas chromatograph Becker 409, equipped with a packed steel column (3 m x 3 mm) coated with 10% SP^{TM} 2330 stationary phase immobilized on a Chromosorb W/AW of 60-80 mesh particle size. Nitrogen was used as an inert carrier (15 ml/min), whereas for the detection of eluted compound flame ionization detector was used. Methyl-esters were separated under isothermal regime applying the oven temperature of 170 ºC, while detector temperature was 250 ºC.

Color
Color on the surface of cocoa spread samples was monitoring by colorimetric method and sensory analyses 24 h after cream production and every two months in the period of six months of storage in the dark and on room temperature.

Color measuring was performed using a Minolta Chroma Meter CR-410 (Minolta Co., Ltd., Osaka, Japan) colorimeter (8 mm Ø contact area). The instrument was calibrated using a standard light white reference tile and the measurements were performed under standard

illuminant D65. The obtained results were expressed in terms of L* (lightness), a* (redness to greenness - positive to negative values, respectively), and b* (yellowness to blueness – positive to negative values, respectively) values.

Sensory analyses of color included scale method, where a group of 10 trained panelists, who were familiar with sensory analysis techniques, tested the following attributes: color on the surface (1 - extremely bright, 4 - optimal, 7 - extremely dark), and surface gloss (1 - mat, 4 - optimal; 7 - separation of oil on the surface). The samples were kept at room temperature and served in a plastic cups in laboratory for sensory analysis made of ten boxes in which each panelist tested all of the samples at room temperature.

Oxidative stability

Oxidative stability of cocoa spreadable samples were monitored using static head space gas chromatography and sensory analyses 24h after cream production and every two months in the period of six months of storage in the dark and on room temperature.

Static headspace gas chromatographic analyses were performed on Agilent 7890A GC System (Agilent Technologies, USA) equipped with a capillary split/split less inlet, total electronic pneumatic control of gas flow, headspace autosampler and FID. Chromatographic data were collected and analyzed using Agilent ChemStation Software.

Static headspace sampling was performed with the headspace sampler, CombiPAL System (CTC Analytics, Zwingen, Switzerland). A 2.5-mL headspace syringe for CombiPAL was used for the injection of 2.0 mL from the 10 mL headspace vials. The auto sampler conditions were set as follows: incubation temperature, 90 °C; incubation time, 10 min; syringe temperature, 100 °C; agitator speed, 500 rpm; fill speed, 100 µL/s; pullup delay, 1 s; injection speed, 500 µL/s; pre- and post-inject delay, 500 ms; flush time, 10 s. After each injection, carryover in the syringe was eliminated by automatic flush of the syringe with carrier gas.

Sensory analyses of oxidative stability included scale method, where a group of 10 semi-trained panelists, who were familiar with sensory analysis techniques, tested the following attributes: color on the surface, odor (1 - extremely bad; 7 - extremely good), taste (1 - extremely bad; 7 - extremely good). The samples were kept at room temperature and served in a plastic cups in laboratory for sensory analysis made of ten boxes in which each panelist tested all of the samples at room temperature.

Statistical analysis

Results were expressed as mean of triplicate analyses. The results were statistically tested using ANOVA method and the means were compared by one-factor analysis at variance with subsequent comparisons by Duncan's test at a significance level at 0.05 using software Statistica 12.0 (Statsoft, USA).

RESULTS AND DISCUSSION

Fatty acid composition of sunflower and rapeseed oil
Composition of fatty acids in sunflower and soybean oil is given in Table 1.

Table 1. Fatty acid composition of sunflower and soybean oil

Fatty acid (%)	Oil	
	Sunflower	Soybean
C14:0	0.15±0.01	0.07±0.01
C16:0	6.91±0.17	10.57±0.26
C16:1	n.d.	0.04±0.01
C18:0	4.00±0.15	7.19±0.04
C18:1	31.68±0.36	28.37±0.21
C18:2	56.79±0.52	46.51±0.52
C18:3	n.d.	7.02±0.19

Sunflower oil is rich in ω-6 fatty acids (56.79% of linoleic), contains 31.68% of ω-9 fatty acids (oleic), but no detected α-linolenic ω-3 fatty acids.

On the other hand, soybean oil contains a lower proportion of ω-6 fatty acids (46.51%), and ω-9 fatty acids (28.37%) compared to sunflower oil, but on the other hand, contains 7.02% of ω-3 fatty acids. Examined oils contain saturated palmitic fatty acid, which is more present in soybean oil (10.57%).

Color on the surface of cocoa spreadable cream

Table 2 shows the presence of particular color tones (red tone - a* and yellow tone - b*) and lightness (L*) on the surface of cocoa spread cream with replacing 50, 70 and 100% of sunflower oil with soybean oil, during 6 months of storage.

Table 2. Color on the cocoa spread cream surface

Sample	CIE L*a*b*			Sensory analyses of color	
	L*	a*	b*	Surface color	Surface gloss
	After 24 hours				
C/control	34.41±0.31[d,e,f]	9.95±0.12[a,b]	11.31±0.33[a]	4.4±0.12[a,b]	4.3±0.15[d]
C/50	34.18±0.51[c,d,e,f]	10.54±0.48[a,b,c,d,e]	11.14±0.74[a]	4.4±0.24[a,b]	4.4±0.22[e]
C/70	34.30±0.27[d,e,f]	10.03±0.09[a,b,c]	10.81±0.09[a]	4.4±0.15[a,b]	4.4±0.24[e]
C/100	34.48±0.03[d,e,f]	9.94±0.24[a,b]	10.20±0.32[a]	4.4±0.34[a,b]	4.5±0.16[f]
	After two months				
C/control	34.63±0.32[e,f]	9.90±0.47[a]	10.55±0.53[a]	4.3±0.12[a]	3.8±0.33[b,c]
C/50	33.11±2.26[a,b,c,d,e]	10.13±0.83[a,b,c]	10.32±1.14[a]	4.4±0.12[a,b]	3.8±0.26[b,c]
C/70	31.90±0.64[a]	10.13±0.12[a,b,c]	10.80±0.19[a]	4.4±0.17[a,b]	3.8±0.32[b,c]
C/100	32.41±1.56[a,b,c]	10.12±0.81[a,b,c]	10.26±1.11[a]	4.4±0.24[a,b]	3.8±0.14[b]
	After four months				
C/control	35.82±0.70[f]	10.82±0.41[b,c,d,e]	10.98±0.58[a]	4.3±0.27[a]	3.7±0.12[a,b,c]
C/50	33.25±1.77[a,b,c,d,e]	11.11±0.85[e]	11.10±1.31[a]	4.4±0.13[a,b]	3.7±0.14[a,b,c]
C/70	33.11±0.31[a,b,c,d,e]	10.68±0.09[a,b,c,d,e]	10.78±0.09[a]	4.4±0.09[a,b]	3.7±0.26[a,b,c]
C/100	33.76±0.67[b,c,d,e,f]	10.80±0.26[c,d,e]	10.59±0.49[a]	4.5±0.16[a,b]	3.8±0.13[b]
	After six months				
C/control	35.89±0.30[f]	10.61±0.28[a,b,c,d,e]	11.18±0.29[a]	4.3±0.52[a]	3.7±0.16[a,b,c]
C/50	32.31±1.03[a,b,c]	11.07±0.35[d,e]	11.32±0.57[a]	4.4±0.22[a,b]	3.7±0.12[a,b,c]
C/70	32.66±0.30[a,b,c,d]	10.29±0.07[a,b,c,d]	10.68±0.09[a]	4.4±0.54[a,b]	3.7±0.15[a,b,c]
C/100	34.06±0.88[c,d,e,f]	10.22±0.19[a,b,c]	10.18±0.38[a]	4.5±0.36[b]	3.7±0.23[a,b,c]

Values represent the means; n=3. Values followed by different lower-case letters in the same column are significantly different from each other (p<0.05).

Control sample with the addition of 100% of sunflower oil and all samples with addition of soybean oil had very similar values of lightness (L*) in the first week after production, whose values are not statistically significantly different at 95% confidence interval of the mean value of three measurements. During storage, the color on the surface of the control sample becomes slightly brighter, while the samples with the addition of soybean oil have darker surface. Increasing the share of soybean oil decreases the red (a*) and yellow (b*) content in the first week of production and these values do not change statistically significantly (p<0.05) during the 6 months of storage.

In the first week after production all samples had intrinsic color of cocoa, without the presence of white and gray color on the surface. After six months, the color on the cocoa spread cream surface was not significantly changed (p<0.05). All samples have a shiny surface, with no oil separation at the surface. Upon six months of storage there was a minor loss of intensity of gloss, with no migration of the oil to the surface.

Oxidative stability of cocoa spread samples

Oxidative stability of cocoa spreadable cream samples, determined by sensory analyses and static head space gas chromatography, is presented in Table 3. The flavour of all cocoa cream samples was aromatic, without presence of smell of certain oils, fat and other raw

materials. During the storage the flavour became slightly worse, whereby it was still pleasant, but it was less pronounced. Addition of soybean seed oil improved the taste of the cocoa spreadable cream samples in comparison to the control sample produced with sunflower oil. After two months of storage there was no chemical changes in the fat phase of samples and no changes in the taste. However, after four months of storage the taste was slightly worsened, where the fat was emphasized. After six months of storage the taste of the control sample and samples with the addition of soybean oil got worse, with emphasizing a sense of sweetness and fatty taste.

Table 3. Oxidative stability of cocoa spread cream

Sample	Sensory analyses		Aldehydes (mg/kg)
	Flavor	Taste	
	After 24 hours		
C/control	6.8±0.13[a]	6.2±0.33[c]	1.13±0.02[a]
C/50	6.8±0.25[a]	6.4±0.14[b]	1.05±0.03[a]
C/70	6.8±0.14[a]	6.4±0.12[b]	1.07±0.01[a]
C/100	6.8±0.23[a]	6.4±0.22[b]	0.48±0.01[b]
	After two months		
C/control	6.7±0.23[a,b]	6.6±0.13[a]	1.06±0.01[a]
C/50	6.7±0.15[a,b]	6.2±0.23[c]	1.11±0.03[a]
C/70	6.7±0.22[a,b]	6.2±0.15[c]	1.09±0.04[a]
C/100	6.8±0.15[b]	6.4±0.16[b]	0.46±0.02[b]
	After four months		
C/control	6.6±0.25[b]	5.8±0.12[d]	1.10±0.02[a]
C/50	6.6±0.14[b]	5.7±0.14[d,e]	1.07±0.03[a]
C/70	6.7±0.22[a,b]	5.7±0.25[d,e]	1.09±0.01[a]
C/100	6.6±0.11[b]	5.7±0.15[d,e]	1.06±0.01[a]
	After six months		
C/control	6.6±0.12[b]	5.7±0.26[d,e]	1.12±0.01[a]
C/50	6.6±0.22[b]	5.6±0.13[e]	1.09±0.03[a]
C/70	6.6±0.14[b]	5.6±0.12[e]	1.11±0.04[a]
C/100	6.6±0.22[b]	5.6±0.23[e]	1.08±0.02[a]

Values represent the means; n=3. Values followed by different lower-case letters in the same column are significantly different from each other (p<0.05).

Static head space gas chromatography method showed no significant difference in aldehydes content, which ranged between 0.46 to 1.13 mg/kg during 6 months of storage.
The exception are cocoa spread cream samples containing only soybean oil in the formulation, which showed significantly lower content of aldehydes in the first two months of storage comparing to control sample and the samples containing less percentage of soybean oil.

CONCLUSIONS

The main objective of the study was to compare sensory properties and shelf life of spreadable cream, produced with sunflower oil and with replacement of partial and complete quantity of sunflower oil with soybean oil.
Results showed that soybean oil contains 7.02% of ω-3 fatty acids while essential α-linolenic acid wasn't detected in sunflower oil, which has been exclusively used in the production of cocoa spread cream in Serbia. Soybean oil improved the taste of cocoa spreadable cream and had no influence on oxidative stability of the product during six months of storage.

ACKNOWLEDGEMENTS

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THE INFLUENCE OF DEFFATED WHEAT GERM ADDITION ON PHYSICAL CHARACTERISTICS OF THE COOKIES DOUGH

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ABSTRACT

Wheat germ is one of the main by-product of milling, nutritionally it is the most valuable part of the grain of wheat. It is a rich source of vitamin E, B vitamins, protein, dietary fiber and minerals (calcium, iron, potassium, magnesium, zinc). Compared to wheat flour it provides more than three times of proteins, up to 15 times more sugar and more than 6 times of minerals. Proteins of wheat germ are particularly rich in essential amino acids such as lysine, methionine and threonine which lack in the majority of the grains. Stabilization of wheat germ can be carried out by removal of the fat, thereby increasing the protein content to 38%, the soluble fiber content from 2.07 to 3.01% and insoluble fiber content from 14.4 to 24.4%. Cookies are inexpensive and very popular food, so it is important to make them more nutritionally rich. Cookies with high protein and fibre content can be produced by replacing part of the flour with germ in the amount up to 40%. In this work, the modern instrumental methods will be applied to examine the physical characteristics of the cookie dough, depending on the amount of the replaced flour with wheat germ (5%, 10% and 15%), different particle sizes of wheat germ (<150 μm, 150 - 1000 μm, 800-2000 μm) and varying the moisture content of the dough (20%, 22% i 24%).
Keywords: wheat germ, cookies, texture

INTRODUCTION

Wheat germ is a by-product separated during milling, but it is the nutrient-richest part of the wheat grain, with high content of protein, minerals, vitamins, dietary fibers and some relatively functional phytochemicals (Rao et al., 1980, Amado and Arrigoni, 1992, Pietrzak and Collins, 1996, Al-Hooti et al., 2002). Wheat germ proteins have a high nutritive value comparable to that of animal proteins (Yiqiang et al., 1999).
There were several studies about influence of defatted wheat germ on nutritional and physical characteristics of biscuits (Bansal and Sudha, 2011) and cakes (Majzoobi et al., 2012), but there are not many studies about influence of wheat germ on physical characteristics of the cookie dough. Cookies are widely consumed ready-to-eat, long shelf life food (Lorens et al., 1979). Therefore, it is important to nutritionally enrich them. Cookies with high protein and fibre content can be produced by replacing part of the flour with germ in the amount up to 40% (Bansal and Sudha, 2011). As any change of ingredients may modify textural properties of the dough, this work investigates the effect of defatted wheat germ flour addition on the physical characteristics of the dough for cookies. Dough characteristics will be examined depending on the amount of the replaced flour with wheat germ (5%, 10% and 15%), different particle sizes of wheat germ (<150 μm, 150 - 1000 μm, 800-2000 μm) and varying the moisture content of the dough (20%, 22% i 24%).

MATERIALS AND METHODS

Materials
Wheat flour for cookies and biscuits (T-500 "Ratar", Pančevo) was used for preparation of the cookie dough. Defatted wheat germs were supplied by Hochdorf Nutrifood AG (Hochdorf, Switzerland): VIOGERM®1055 (microfine, granulation <150μm) - fraction 1, VIOGERM®1080 (coarse, granulation 150-1000μm) - fraction 2 and VIOGERM®1115 (fine granules,

granulation 800-2000µm) - fraction 3. Vegetable fat "Vitalina" was obtained from "Dijamant", Zrenjanin. Salt, sodium bicarbonate and ammonium bicarbonate were obtained from "Centrohem", Stara Pazova. Powdered sugar ("Centroproizvod", Beograd) was purchased in a local food store.

Preparation of the dough for cookies
Cookie dough was prepared according to the following formula: flour (i.e. flour blend) 200.00 g, vegetable fat 42.00 g, sugar 70.00 g, $NaHCO_3$ 0.6 g, NH_4HCO_3 0.4 g and NaCl 1.1 g. The measured amount of flour was mixed in a mixer (ZD2245, Stephan - Werke GmbH and Co., Hamelin, Germany) for 0.5 min, and after the addition of the total amount of fat and powdered sugar, the mixing was continued for 5.5 min at low speed (60 min^{-1}). All other components dissolved in distilled water were added into the mixer, the mixer closed and the dough mixed for 15 min (Pajin, 2009).

In the samples, 5%, 10% and 15% of wheat flour was substituted with wheat germ of different particles sizes. The amount of water was calculated in relation to the water content of the flour blends in order to obtain dough samples with 20%, 22% and 24% moisture content. The Box - Behnken experimental design (Montgomery, 2001) was used to evaluate influence of these three variables (3 input factors) arranged at three levels (Table 1).

Table 1. Variables and levels in the Box-Bahnken design

Input factors	Levels		
	Low (-1)	Medium (0)	High (1)
A: fraction	1	2	3
B: supstitution (%)	5	10	15
C: water content (%)	20	22	24
Dependent responses			
R1: Hardness (g)			
R2: Resistance to extension (g)			
R3: Extensibility (mm)			

The quadratic model obtained by the design is given as:

$$R = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \qquad (1)$$

where R is a measured response; β_0 is an intercept; β_1 to β_{33} are regression coefficients; A, B and C are the coded levels of input factors. The terms AB, AC and BC represent interactions of input factors, while A^2, B^2 and C^2 represent quadratic terms. Higher order terms or more than one factor terms represent nonlinear relationship between responses and factors. The analysis was carried out using Statistica 12 and Design-Expert 7 (trial version) (Anderson, 2007).

Dough texture analysis
Textural properties of dough were determined by Texture Analyser TA. XT Plus (Stable Micro Systems, Surrey, U.K.). Dough hardness was determined using the penetration test by application of measuring accessories P/6 (cylinder diameter of 2 mm) which penetrate to a depth of 2 mm in the dough set to a solid metal platform. The maximum force registered at the depth of 2 mm represents hardness. Measurements were performed in five replicates at 25°C using load cells of 5 kg and following operating parameters: pre-test speed: 1 mm/s; test speed: 2 mm/s; post-test speed: 10 mm/s; distance: 2 mm. To define extensibility and resistance to extension of dough, micro-method Kiffer Exstensibility Rig (Kiffer *et al.,* 1981) was used. Measurements were performed in five replicates at 25°C using load cells of 5 kg and following operating parameters: pre-test speed: 2.0 mm/s; test speed: 3.3 mm/s; post-test speed: 10 mm/s; distance: 75-mm; trigger force: auto-5 g.

RESULT AND DISCUSSION

Total of 15 experiments recommended by Box - Behnken design and obtained results are shown in Table 2.

Table 2. The Box - Behnken experimental design and obtained responses

Factor A	Factor B	Factor C	R1	R2	R3
fraction	supstitution	water content	Resistance	Extensibility	Hardness
	%	%	(g)	(mm)	(g)
1	5	22	25.666	12.372	99.664
1	10	20	16.586	7.830	144.701
1	10	24	20.330	17.292	64.616
1	15	22	38.992	15.668	115.152
2	5	24	19.559	16.923	59.819
2	10	22	18.790	11.939	90.999
2	10	22	18.757	11.049	101.438
2	10	22	18.457	11.023	117.927
2	15	20	11.289	5.912	183.598
2	15	24	19.165	14.431	74.581
3	5	20	13.840	5.868	124.184
3	5	22	20.217	10.147	118.119
3	10	20	14.374	6.033	151.161
3	10	24	13.242	10.329	83.017
3	15	22	17.932	10.758	119.214

Mean values, standard deviation and range of results for the analyzed responses as well as ratio between maximum and minimum value are shown in Table 3.

Table 3. Descriptive statistic and ANOVA results for responses

Response	Min	Max	Mean	Std. Dev.	Ratio	Lack of fit	CV	R^2	Adj R^2
R1: Resistance (g)	11.29	38.99	19.15	6.3	3.45	0.0020	0.18	0.8593	0.7187
R2: Extensibility (mm)	5.87	17.29	11.17	3.64	2.95	0.1191	0.12	0.9230	0.8803
R3: Hardness (g)	59.82	183.6	109.88	32.5	3.07	0.7975	0.10	0.9293	0.9011

Using a fitted full quadratic model, given in Eq. (1), a response surface regression analysis for each of responses was performed. Significance of input factors and interactions in observed model are determined by statistical method of analysis of variance (ANOVA). Using 5% level of significance, a factor is considered to affect the response if the p-value is less than 0.05. Sum of squares obtained by ANOVA are used to calculate corresponded contributions. Quadratic models for R1, R2 and R3 in coded terms are expressed by regression equations:

$$R1 = 18.67 - 4.48 \cdot A + 1.01 \cdot B + 2.03 \cdot C - 3.9 \cdot A \cdot B + 3.6 \cdot A^2 + 3.43 \cdot B^2 - 6.14 \cdot C^2 \qquad (2)$$

$$R2 = 11.85 - 1.99 \cdot A + 0.18 \cdot B + 4.17 \cdot C - 1.29 \cdot A \cdot C - 1.29 \cdot C^2 \qquad (3)$$

$$R3 = 109.88 + 5.92 \cdot A + 11.34 \cdot B - 40.2 \cdot C - 11.16 \cdot B \cdot C \qquad (4)$$

ANOVA results (Lack of fit, CV, R^2, adj R^2) given in Table 3, verify the adequacy of models. Positive (negative) sign of a coefficient in the Eqs. (2, 3 and 4) represent that the response increases (decreases) with the factor.

Contributions of all three factors on textural characteristics of the dough are shown at Figure 1, where linear and quadriatic contributions are jointed.
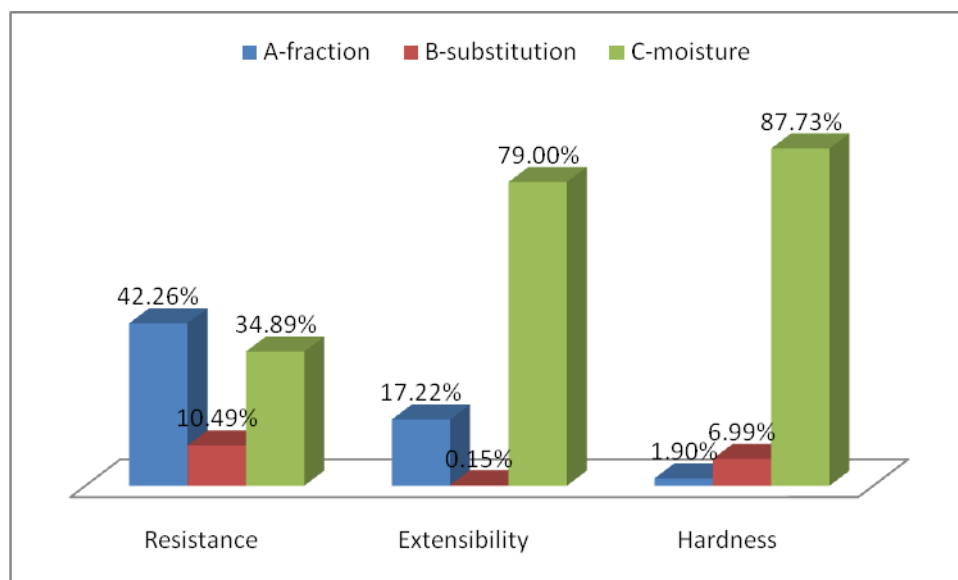
Figure 1. Contributions of main effect on textural characteristics of the dough for cookies

At Figure 1. and Eq. 1., it can be seen that the largest (negative) influence on the resistance to extension of the dough has a germ fraction (particle size). Germ proteins and fibers absorb water, so they have influence on amount of available water for hydration of flour ingredients and for formation of strong dough structure (moisture content has significant impact on resistance to extension, too). Smaller particles hydrate faster and so contribute to the rapid formation of strong structure of the dough (Wang and Flores, 2000). Zucco *et al.* (2011) concluded that difference in particle size have an effect on chemical bonds between protein and starch. Also, air bubbles that are included in the structure of the dough during mixing are larger as larger are particles of the flour (Majzoobi *et al.,* 2012). That may affect the formation of chemical bonds between various constituents of the dough.

Moisture content has a very significant impact on dough extensibility. Water provides hydration of flour particles and formation of protein chains in the dough. If there is enough water, protein chains rapidly react with each other to form cross links between the particles of flour and form a quality gluten structure.

The increasing water content in dough samples, as expected, contributes to the decrease of dough hardness. Starch granules need a sufficient amount of water for hydrating, which is not case at the dough samples containing 20% of water, so the hardness of these samples increases.

CONCLUSIONS

It can be concluded that on textural characteristics of the dough for cookies with wheat germ greatest impact has water content. Particle size of wheat germ has an impact on resistance to extension and on extensibility of the dough. Wheat germ content up to 15% has no influence on textural characteristics of the dough for cookies. In further work, it should be examined the influence of wheat germ on dough rheological characteristics and final product characteristics. Also, it should be examined whether the greater amount of wheat germ will have an impact on the dough features.

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PRESENCE OF POTENTIALLY TOXIGENIC MOLDS IN GRAIN FLOURS

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ABSTRACT

Concerning the agricultural products, grains present the most important staple food in the human diet. The most commonly used grains that are used for the processing include: wheat, rye, corn, rice, buckwheat, etc. Presence of microorganisms is inevitable on the cereal grain surface and consequently in their products. Contamination is most frequently caused by molds which in turn could produce a numerous secondary metabolites. Mycotoxins are secondary metabolites of molds, which are toxic for humans and animals. Aim of this work is to conduct a determination of molds, which are present in buckwheat, corn and rice flour, as well as a frequency of genera, species and potentially toxigenic species. All the samples were contaminated by molds. Mycopopulation of buckwheat, corn and rice flour consists of species from genera: *Absidia, Acremonium, Alternaria, Aspergillus, Cladosporium, Emericella, Eurotium, Mucor, Penicillium, Paecillomyces, Rhizopus*. Dominant species were: *Aspergillus flavus, Penicillium aurantiogriseum, P.expansum, Eurotium chevalieri, A.niger*. The most frequent species was *Aspergillus flavus*, which is a potential aflatoxin producer. Mycotoxicologycal examination of *A.flavus* didn't show the presence of aflatoxins.
Key words: *grain flours, fungal contamination, toxigenic molds*

INTRODUCTION

Cereals and derived products make an important part of the human diet. The milling process is a wheat basic technological procedure. The whole grain is an ingredient in the technological procedure, and the final products are: flour, grits, bran, and also the admixtures that are separated during the preparation. The flour based products present a basis of the diet.
Lately, the consumer dietary habits have changed, and create an impact on the market products assortment. Beside the usual types of wheat used in the diet, there is an increasing demand for the products of buckwheat, corn and rice.
The whole grain wheat flour is a high quality food in accordance with nutritional characteristics, due to high content of vitamins, minerals and especially dietary fibers. On the other hand, the cereal grain is susceptible to a whole range of contamination during the ripening, harvesting, storing and processing. A mold characteristic is a great adaptability to the outside conditions such as temperature and humidity (Žeželj, 1995). The flour is considerably susceptible to the microorganisms acting in comparison to the cereal grain, owing to lack of protective coat, whose function is to protect the whole and undamaged grain. The flour molding occurs when the relative humidity exceeds 79% (Plavšić *et al.,* 2007; Stojanović and Psodorov, 2007).
The most frequent isolated mold species from flour belongs to genera *Aspergillus*, *Penicillium*, *Eurotium*, *Alternaria, Cladosporium*, *Rhizopus, Mucor*, *Emericella* and *Fusarium*. Species of genera *Aspergillus, Penicillium* and *Eurotium,* are so called "storage" molds,which develops around the water activity (a_w value) of 0.85 or perhaps lower. Species from genera *Fusarium* and *Alternaria* are "field" molds, and for their development is necessary a higher water activity value of the substrate and lower temperatures. These species could be found in/on the cereal grain and cereal products (Maletić, 2005).

In according to the research of Pitt and Hocking (2009), the number of "field molds" which could be isolated form flour is lower from molds that are isolated on the cereals before the milling process. On the other hand the presence of *Aspergillus* and *Penicillium* is higher. Field molds could frequently be found on the grain surface considering the fact that needs higher water content and the lower temperatures.

Molds could act directly on the organism, causing some diseases called mycoses. However, molds could produce a numerous secondary metabolites, mycotoxins, that are toxic for humans and animals, and causing diseases called mycotoxycoses. Aflatoxins are very toxic coumarins derivates biosynthesized by the *A. flavus* and *A. parasiticus.* Fungus could biosynthesize those mycotoxins on the large amount of the substrate, such as oil seeds, cereals and their products, grain fruits, subtropical fruits, spices. Beside, fungus could most frequently be found in the products that are not dried well after the harvest, or during the storage at higher temperatures. From these groups of mycotoxins AB1 is the strongest carcinogen, followed by AG1, AM1 and AB2 (Kocić-Tanackov and Dimić, 2013).

Aim of this work is to conduct a determination of molds, which are present in buckwheat, corn and rice flour, as well as a frequency of genera, species and potentially toxigenic species.

MATERIAL AND METHODS

Mycological researches

Five samples of buckwheat, corn and rice flour were examined. Mycological researches were contained the determination of total number of molds in 1 g and determination of molds.

The total number of molds was determinate by the Koch method of dilutions. For dilutions preparation, 0.1% of sterile solution of peptone water was used.

Isolation and determination of the total number of molds was conducted on the two surfaces:

1. Dichloran 18% glycerol agar (DG18) (Biokar Diagnostics, France) for isolation of xerotolerant molds grows below 0.9 a_w value;
2. Malt yeast 50% glucose agar (MY50G) (malt extract 10 g, yeast extract 2.5 g, agar 10 g, glucose 500 g, distilled water 500 ml) for isolation of extremely xerophile molds grows below 0.7 a_w value (Samson *et al.,* 2004; Pitt and Hocking, 2009).

The seeded substrates were incubated at 25 °C. The results were measured after 5 and 7 days. Researches were conducted in the triplets.

Monocultivation of molds was conducted in the way that conidia and hypha fragments from molds colonies were translated on Czapek yeast (autolisate) extract agar (CYA)($NaNO_3$ 3 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4$ $7H_2O$ 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g, yeast extract 5 g, sucrose 30 g, solution of microelements 1 ml ($ZnSO_4$ $.7H_2O$ 1g, $CuSO_4$ $.7H_2O$ 0,5g, distilled water 100ml), agar 20 g, distilled water 1000 ml) or Malt extract agar (MEA) (malt extract 20 g, peptone 1 g, glucose 20 g, agar 20 g, distilled water 1000 ml). Colonies which presumed to belong in accordance to macromorphological characteristics to genera *Penicillium*, *Aspergillus*, *Eurotium* and *Emericella* were seeded on CYA, and others on MEA. Seeded surfaces were incubated 7 days at 25 °C. The criteria described by Samson *et al.* (2004), Samson and Frisvad (2004) and Pitt and Hocking (2009) were used for species identification.

Mycotoxicological researches

Examination of aflatoxin B1, B2, G1, G2 synthesis ability by the *Aspergillus flavus* molds isolate was conducted with ELISA method. Cultures of potentially toxigenic species *A. flavus* isolated from flour samples were seeded on angled Sabouraud dextrose agar (SDA)(Himedia, India) and incubated 7 days on 25 °C. After that, over the developed culture was spilled 5 ml of Yeast extract sucrose bouillon (YES) (yeast extract 20 g, sucrose 150 g, distilled water 1000 ml) bouillon and colonies were taken off with the sterile bacteriological needle. The incubation has lasted 14 days at 25 °C, on sieve shaker, in order to achieve a

constant aeration of surface. After expiration of incubation period, YES bouillon with mold cultures were sterilized in autoclave 10 minutes at 121 °C and filtrated after trough the filter paper (Whatman No 1). The obtained filtrate was used for aflatoxin content determination. Determination of total aflatoxin (B1, B2, G1 and G2) content in filtrates of *A.flavus* isolate was conducted with an application of validated ELISA method. Quantitative Aflatoxin High Sensitivity (HS) test (# 8031, Neogen Veratox®, Lansing, USA) was applied. The procedures of sample preparation also as ELISA method were conducted in accordance to manufacturer manual.

RESULTS AND DISCUSSION

Mycological researches
By examination of buckwheat, corn and rice flour contamination, the presence of molds was confirmed in all the samples.

Table 1. The total number of molds in flour samples*

Sample	Total number of molds in 1g	
	DG18 (cfu/g)	MY50G(cfu/g)
Buckwheat flour		
1	2.6×10^4	2.0×10^4
2	4.8×10^2	2.4×10^2
3	3.3×10^2	2.1×10^2
4	7.3×10^2	2.7×10^2
5	3.6×10^2	1.7×10^2
Corn flour		
1	2.2×10^2	1.1×10^2
2	6.0×10^1	2.0×10^1
3	4.1×10^2	2.0×10^2
4	6.2×10^2	1.1×10^2
5	1.5×10^3	1.2×10^3
Rice flour		
1	1.0×10^1	0
2	1.0×10^1	0
3	2.0×10^2	1.8×10^2
4	4.2×10^2	2.8×10^2
5	2.0×10^1	4.0×10^1

Legend:*- the result presents the average value of three repetitions

By comparing the obtained results for all the samples, it could be concluded that the largest mycopopulation was found in the buckwheat flour samples, especially in sample 1. The result wasn't surprise, considering the fact that sample was from buckwheat whole grain flour, which includes the protective grain layer. The buckwheat flour, due to structure and appearance, is a susceptible environment for mycotoxin population growth, which could be also found in flour (Plavšić *et al.,* 2007; Plavšić *et al.,* 2012). In accordance to researches of Halt *et al.* (2004), in 58 samples of flour the total number of molds was from 0 to 12.3×10^3 cfu/g. In results obtained by Plavšić *et al.* (2007) maximal amount of molds in samples was 1.5×10^3 cfu/g.

The total mycopopulation of all examined samples was sorted in 12 genera and 31 species.

The most dominant genera was *Aspergillus,* isolated from 14 samples, also as *Penicillium* whose presence was confirmed in 11 species, followed by genera *Eurotium* and *Paecilomyces* isolated from 7 samples, *Rhizopus* (5), *Absidia* (3).

Molds, which appears as contaminants of flour are mostly from cereal grain. There are several contamination sources for cereal grain during production, harvest, storing, and transport. Beside bacteria and yeasts, a phytopathogen microorganism also includes filamentous molds (*Alternaria, Aureobasidium, Cladosporium, Epicoccum, Fusarium,*

Helminthosporium, Claviceps). A potential contamination could also occur after harvest. The grain could get contaminated during the further processing. Usual contaminants includes xerophile *Aspergillus glaucus* group and *Penicillium* species, which needs minimal a_w value of 0.68 (14% moisture) for their growth and development (Oliveira *et al.,* 2014).

Table 2. The frequency of molds genera appearence in the flour sample

Genera	Number of examined samples/Number of contaminated samples	Frequency of genera(%)
Absidia	15/3	20.00
Acremonium	15/2	13.33
Alternaria	15/1	6.67
Aspergillus	15/14	93.33
Cladospirium	15/1	6.67
Emericella	15/1	6.67
Eurotium	15/7	46.67
Fraseriella	15/1	6.67
Mucor	15/1	6.67
Paecilomyces	15/7	46.67
Penicillium	15/11	73.33
Rhizopus	15/5	33.33

An aspergillus genus, as the most dominant, was present trough 10 species. Among *Aspergillus species, A. flavus* was the most frequently isolated. Among *Penicillium* species, the most frequently isolated species were P*. aurantiogriseum* and *P. expansum*. The obtained results were in accordance to literature results. In Demirel and Sariozlu (2013) work, the most dominant mold species isolated from flour were from *Aspergillus* (42.82%) and *Penicillium* (42.65%) genera, followed by (5.63%), *Fusariuma* (5.5%), *Paecilomyces* and *Cladosporium (*3.6%), while the other genera such as *Acremonium, Alternaria, Mucor, Rhizopus* were isolated in smaller amount. Sureka *et al.* (2011) were examined a fungal contamination of rice during 12 months. The frequency of *A.flavus* presence was detected in 100% during complete period of the research.

Beside *Aspergillus* and *Penicillium* species, a *Eurotium* genera was also present in the significant amount in mycopopulation of experimental samples. *E.chevalieri* and *E.herbariorum* species were isolated. Species of this genera are an extremely xerophile molds (minimal a_w 0.7). According to a_w value of cereals and their milling products, it wasn't surprise that *Eurotium* species were present in certain amount, right after *Aspergillus* and *Penicillium* species (Pitt and Hocking, 2009).

Mycotoxicological researches

From isolated *Aspergillus* species, *A.flavus* was present in the highest amount. *A.flavus* is the potential aflatoxin producer. Twelve isolates of *A.flavus* were examined on the aflatoxin synthesis.

Examined isolates of *A.flavus* wasn't show an aflatoxin synthesis capability. A multiple factors, beside genetic predispositions, affect the mycotoxin synthesis. Molds presence by itself doesn't necessary presume the mycotoxin synthesis. Škrinjar *et al.* (2013) was explained that aflatoxin synthesis happens on an optimal temperature of 30 °C or more, and relative humidity among 88 and 95%. Aflatoxin synthesis in *A.flavus* significantly varies from strain to strain. The toxin production is the result of interaction genotypes of strains and environmental conditions (Moreau and Moss, 1979; Muntañola-Cvetković, 1990; Šarić, 2007).

Table 3. Isolated species of molds and potential toxigenic moulds in examined flour samples and ther mycotoxins(Samson et al.,2004; Pitt and Hocking,2009; Kocić-Tanackov,2012)

Genera	Species	Frequency of species (%)	Mycotoxin
Absidia	*A.corymbifera*	20	-
Acremonium	*A.strictum*	13.33	-
Alternaria	*A.alternata*	6.67	Alternariol, alternariol monomethyl ether, alterotoxin I and II, altenuene, tenuazonic acid
Aspergillus	*A.niger*	26.67	Naphtho-4-pyrones, malphomins, ochratoxin A
	A.penicillioides	6.67	-
	A.versicolor	13.33	Sterigmatocistin, nidulotoxin
	A.candidus	33.33	Terphenyllin, xanthoascin
	A.flavus	60	Kojic acid, 3-nitropropionic acid, cyclopiasonic acid, aflatoxin B1, aspergillic acid
	A.fumigatus	6.67	Gliotoxin, verrucologen, fumitremorgin A&B, fumitoxins, tryptoquivalins
	A.sydowii	6.67	-
	A.glaucus	13.33	Echinulin, physcion, sterigmatocistin
	A.terreus	13.33	Terrein, patulin, citrinin, citreoviridin, territrem
	A.wentii	13.33	Emodin, ventilacton
Cladospirium	*C.cladosporioides*	6.67	-
Emericella	*E.nidulans*	6.67	Sterigmatocistin, nidulotoxin, penicillin
Eurotium	*E.chevalieri*	26.67	Echinulin, neoehinulin, physcion
	E.herbariorum	26.67	Echinulin, physcion, sterigmatocistin
Fraseriella	*F.bisporus*	6.67	-
Mucor	*M.circinelloides*	6.67	-
Paecilomyces	*P.fulvus*	46.67	Patulin, byssochlamic acid, byssotoxin
Penicillium	*P.aurantiogriseum*	40	Penicillic acid, verrucosidin,nephrotoxic glycopeptides,anacine, auranthine,aurantiomine, ochratoxin A
	P.exspansum	40	Roquefortine C, patulin, citrinin, communesins, chaetoglobosin C
	P.chrysogenum	6.67	Roquefortine C, meleagrin,chrysogine, penicilline,ochratoxin A
	P.commune	6.67	Cyclopiasonic acid, rugulovasin A & B, cyclopaldic acid
	P.griseofulvum	13.33	Roquefortine C, cyclopiasonic acid, patulin, griseofulvin
	P.glabrum	6.67	Citromycetin
	P.rugulosum	6.67	Rugulosin
	P.confertum	6.67	-
	P.oxalicum	20	Secalonic acid D&F, oxaline
Rhizopus	*R.oryzae*	26.67	-
	R.stolonifer	6.67	-

Table 4. Aflatoxin (B1, B2,G1,G2) synthesis from A.flavus isolates examined by ELISA method

Mold isolates	Isolate mark	Sample	AF (B1,B2,G1,G2) concentration µg/kg
A.flavus	15H3	buckwheat flour	< 1[*]
A.flavus	22H4	buckwheat flour	< 1
A.flavus	29H4	buckwheat flour	< 1
A.flavus	48H4	buckwheat flour	< 1
A.flavus	53K1	corn flour	< 1
A.flavus	58K1	corn flour	< 1
A.flavus	9K5	corn flour	< 1
A.flavus	20K5	corn flour	< 1
A.flavus	4P1	rice flour	< 1
A.flavus	5P2	rice flour	< 1
A.flavus	17P3	rice flour	< 1
A.flavus	18P3	rice flour	< 1

Legend: <1- aflatoxin presence wasn't detected

CONCLUSIONS

The total mycopopulation of examined samples (15) was sorted in 12 genera and 31 species. The most dominant species were *Aspergillus* (93.33%) and *Penicillium* (73.33%), followed by *Eurotium* and *Paecilomyces* (46.67%) and *Rhizopus* (33.33%). The most frequent isolated species were *A.flavus,* followed by *P.aurantiogriseum, P.expansum, E.chevalieri* and *E.herbariorum*. Examined *A.flavus* isolates didn't show the aflatoxin synthesis capability.

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DESCRIPTIVE SENSORY ATTRIBUTES AND CONSUMER ACCEPTANCE OF CHERRY PIE FILLING

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ABSTRACT

The aim of this study was to investigate what sensory attributes, measured by a sensory panel, are important to consumer acceptance of cherry pie filling in pastry products. Six samples of cherry pie fillings commercially available on the Serbian market were evaluated by a descriptive sensory panel comprised of the University staff and students. Descriptive sensory analysis was carried out using a defined vocabulary of 14 attributes. For the purpose of acceptance testing different samples of cherry filled pastry rolls were prepared in a local bakery using the 6 cherry pie fillings. Consumers rated the pastry samples on three 9-point hedonic scales and four 9-point just-about-right scales. Sensory descriptive data were subjected to generalized Procrustes analysis (GPA) resulting in consensus data matrix which was then subjected to principal component analysis (PCA). Extracted principal components were used as explanatory variables (predictors) in further linear multiple regression analysis against the acceptance (hedonic) values for the cherry pie filling. This technique is referred to as external preference mapping (PREFMAP). The regression coefficients (i.e. consumer scores) obtained from the regression model were segmented using K-means cluster analysis. Pronounced sourness, cherry flavour, thin gel viscosity, lower degree of fruit disintegration, and low level of adhesiveness of the cherry pie fillings were the drivers of liking for the most of the tested consumers. None of the pastry samples did receive significant advantage against the others, considering overall acceptability. In general, all of the pastry samples were recognized as insufficiently sweet by consumers.

Keywords: descriptive sensory analysis, consumer acceptance, external preference mapping, cherry pie filling

INTRODUCTION

Sensory tests are often conducted to study how product modifications such as ingredient, processing, and packaging changes will create perceived changes to human observers (Lawless and Heymann, 2010). A company's ability to produce a product which satisfies the consumers' sensory requirement has a distinct lead to success and profitability (McEwan, 1996) and a sensory test may help to understand the attributes of a product that consumer view as critical to product acceptance. Having this in mind, as well as that the food industry frequently uses sensory analysis for different purposes such as product development, quality control, shelf life estimation, customer complaints, etc., it is often desirable to collect and consider sensory information from both a trained panel (especially descriptive one) and from consumers.

Consumer data can be analyzed alone, without taking other types of data into account, but by using sensory and consumer techniques in conjunction, a better understanding of the sensory attributes important to consumers can be gained. A perceptual mapping method that yields a graphical display of hedonic data is called preference mapping (Lawless and Heymann, 2010). Preference mapping is defined as methodology for investigating consumer liking for a series of products by the use of statistical mapping methods such as principal component analysis (PCA), principal component regression (PCR), partial least squares regression (PLS), or similar techniques (Næs *et al.,* 2010). Beside the information which products in the sample set are the most and least liked, the study of the relationship between

consumer and trained panel data can provide answers to the following questions *(Popper et al.,* 1997; Næs *et al.,* 2010):

- What sensory attributes, as measured by a trained panel, are important to how much a consumer likes or dislikes a product (what are the drivers of liking and disliking)?
- Which consumers prefer which samples (segmentation) and what characterize the different consumer segments (in terms of demographic, habits, attitudes)?
- How does one translate the terms consumers use to describe products into terms used by a trained descriptive panel?

Linear preference mapping methods are those based on a linear model for the relationship between the sensory and acceptance data for each consumer and in general can be split in two different methodologies based on the same underlying techniques - PCA and linear regression: *internal* (MDPREF) and *external* (PREFMAP) preference mapping (McEwan, 1996; Næs et al., 2010). MDPREF is based on first applying PCA on the consumer data, and then on applying the linear regression model to regress all the sensory attributes evaluated through descriptive analysis onto the estimated PCA scores from the consumer data. PREFMAP is based on first applying PCA on descriptive sensory data and then on applying the linear regression model to regress acceptance data for each consumer onto the estimated PCA scores from the descriptive data.

The aim of this study was to investigate what sensory attributes, measured by a sensory panel, are important to consumer acceptance of cherry pie filling in pastry products using external preference mapping technique.

MATERIAL AND METHODS

Samples

Six commercially available cherry pie fillings from different manufacturers were purchased at local market. All of the samples were packed in plastic jars and upon purchasing were stored at 5-8 °C. The fruit and dry matter contents of the samples were as follows (respectively): A: 35 % and 44 %; B: 40 % and 55 %; C: 40 % and 35 %; D: 40 % and 50 %; E: 50 % and 50 %; and F: 40 % and 35 %. These samples were used as objects in descriptive sensory analysis.

For the purpose of acceptance testing 12 different samples of cherry filled pastry rolls were prepared in a local bakery using the 6 cherry pie fillings and 2 types of dough: fermented and puff pastry dough. All of the pastry rolls were assessed within the first 6 hours after baking and subsequent tempering at room temperature.

Both type of samples, pie fillings and filled pastry rolls, were labelled with random 3-digit codes.

Descriptive sensory evaluation

Descriptive analysis was conducted by 8 University of Belgrade – Faculty of Agriculture staff and students. Four 2-hours training sessions were performed over a period of 2 weeks using different pie fillings and a word list of possible descriptors with definitions that could be used to describe the product. During the first 2 sessions panellists reached a consensus on 14 appearance, flavour, and texture attributes that somehow discriminated among the samples. The list of terms with their definitions is presented in Table 1.

The selected sensory attributes were scored by all of the panelists with respect to their intensities using 15 cm line scales within paper ballots. The scales had verbal anchors at both ends (Table 1) and the panelists were given free choice in using them. Evaluations were performed in sensory booths in the sensory testing laboratory at the University. All of the 6 pie filling samples had been presented to each panelist at the same time using the Latin Square design of order 6 which is produced by cyclic development of an initial row which is in the same order as the first column (A-B-C-D-E-F, B-C-D-E-F-A, etc.) (Hunter, 1996). The panelists evaluated the intensities of selected attributes by comparing the samples to each other. Low sodium bottled water was used for palate cleansing.

Table 1. Definitions of the attributes used in sensory evaluation of cherry pie filling

Attribute	Definition
APPEARANCE	
Colour description	The colour of the sample from *cherry-red* to *brown*.
Gel viscosity	The viscosity of the sample (*thin - thick*).
Amount of cherry fruit*	The amount of cherry fruit in the filling (*none - many*).
Degree of fruit disintegration	Degree to which the cherry fruit is disintegrated (*whole - disintegrated*).
ODOUR	
Cherry-odour	The intensity of cherry-odour (*none - intensive*).
Off-odour	The intensity of off-odour (*none - intensive*).
FLAVOR	
Cherry-flavour	The strength of all cherry flavours (*none - intensive*).
Off-flavour	The intensity of an off-flavour (*none - intensive*).
Sweetness*	The taste stimulated by sugars (*none - intensive*).
Sourness	The taste stimulated by acids (*none - intensive*).
TEXTURE	
Slipperiness*	The amount in which the product slides across the tong (*drag - slip*).
Fruit firmness	The force required to compress between tongue and palate (*soft - firm*).
Adhesiveness	Amount of force to remove sample from roof of the mouth (*no force - high force*).
RESIDUAL	
Mouth coating*	Amount of particles left on mouth surface (*none* to *much*).

* Excluded from further dimension reduction analysis.

Acceptance testing

One hundred students were tested with respect to the degree of acceptability of cherry pie fillings as an integral part of baked filled pastry rolls. Students were chosen if they were users of this kind of bakery products. One half of them (50 students) had been presented with 6 samples of filled rolls made of fermented dough, while the other half tasted the samples made of puff pastry dough. The samples of pastry rolls were evaluated for liking of product as a whole, cherry pie filling, and cherry flavour using the 9-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely), and also for intensity of sourness, sweetness, fruit content, and consistency of the cherry pie filling using 9-pint just-about-right scales (1 = too little, 5 = just about right, 9 = too much). Only the hedonic judgments of cherry pie filling will be considered within this manuscript.

Pairs of hedonic cherry pie filling data sets for fermented dough and puff pastry dough rolls filled with the same filling samples were initially subjected to paired-samples t-test. The results showed no statistically significant differences ($p > 0.01$) within all six pairs of pastry rolls, justifying the decision to treat all the cherry pie filling hedonic scores (100 in total per a cherry pie filling sample) as data obtained from the same population.

Statistical analysis

The distances from the left end of the scales to the marks made by the panelists, were measured manually. In order to perform multivariate (MANOVA) and univariate (ANOVA) analysis of variance raw data for each variable for each assessor were first standardized, i.e. scaled to unit variance and zero average (Romano *et al.,* 2008). One-way MANOVA with 'samples' as main effect (fixed factor) was applied to standardized data in order to test for the significance of multivariate effect for samples. To identify sensory attributes which significantly discriminate among samples, three-way analysis of variance (ANOVA) was applied to standardized data with main effects of 'samples', 'assessors' and 'replications' and all two-way interactions ('samples' = fixed factor; 'assessors' and 'replications' = random factors). When the main effect was significant Tukey honestly significant difference test (Tukey HSD) was used to separate the mean values for samples.

Sensory attributes which did not significantly discriminate among samples were excluded from subsequent statistical analysis. The rest of the attributes were subjected to Generalized Procrustes Analysis (GPA) and Principal Component Analysis (PCA). GPA was applied to original (raw) data divided into 16 personal construct grids (8 assessors x 2 replications). The consensus data matrix obtained by applying GPA, which was constructed of 6 rows (6 samples) and 10 columns (10 retained attributes), was subjected to PCA. Extracted principal components were used as explanatory variables (predictors) in further linear multiple regression analysis (the vector model) against the acceptance (hedonic) data for the cherry pie filling. This technique of mapping acceptability data for each consumer onto an existing perceptual map of the products is referred to as external preference mapping (PREFMAP) (McEwan, 1996; Næs *et al.,* 2010). The regression coefficients (i.e. consumer scores) obtained from the linear regression model were segmented using K-means cluster analysis. Data standardization, GPA and PCA were completed using Idiogrid software version 2.4/2008 (Grice, 2002). The rest of the statistical analyses were performed using SPSS Statistics 17.0. The level of statistical significance was set at 0.05.

RESULTS AND DISCUSSION

Standardized data of 14 sensory attributes were initially subjected to MANOVA which revealed a significant multivariate effect for 'samples'. Wilks' lambda was significant at $p < 0.001$. Subsequent 3-way ANOVA showed that 4 out of 14 attributes ('amount of cherry fruit', 'sweetness', 'slipperiness' and 'mouth coating') did not significantly discriminate among the samples ($p > 0.05$). These four attributes were excluded from further dimension reduction analysis. None of the attributes, left after removal of the four, showed a statistically significant difference between the assessors. The replication effect was significant ($p < 0.05$) only for 'gel viscosity'. The assessor*replication interaction was significant ($p < 0.05$) for 'degree of fruit disintegration', 'cherry-odour', 'off-odour, 'cherry-flavour' and 'sourness'.

GPA was performed on original sensory data divided into 16 personal construct grids (8 assessors x 2 replications). The results of the GPA yielded a consensus proportion of 0.91, which indicated strong agreement among the 16 individual replications. Randomization test (Grice and Assad, 2009) showed that the observed consensus proportion was statistically significant at the 0.05 level (observed $p < 0.01$). Individual isotropic scaling values (Grice and Assad, 2009) were relatively close to unity (ranged from 0.81 to 1.21), indicated that individual differences in overall variability of the grids were relatively small. The central figure of GPA – the consensus data matrix, obtained by applying GPA on raw data, was further subjected to PCA. Varimax rotation was chosen since it showed the best arrangement of the loading values in comparison with Quartimax and Equamax rotations, and urotated solution. The first three extracted principal components (PC) had eigenvalues larger than 1 (3.1, 2.9 and 2.5 respectively) and therefore, according to both Kaiser and Scree rule, were retained for describing objects in the new 3-dimensional PC-space. The first three PCs explained 85.1 % of the variance in the data matrix values. According to guidelines for inspection for significance of attribute loadings provided by Stevens (2009), an attribute was considered to load heavily on a given component if the loading value was equal to or greater than 0.72. A total of 9 attributes loaded heavily on the three PCs: PC-1 was strongly related to 'cherry-flavour', 'off-flavour' and 'sourness'; PC-2 to 'gel viscosity', 'degree of fruit disintegration' and 'cherry-odour'; and PC-3 to 'off-odour', 'fruit firmness' and 'adhesiveness'.

Figure 1 shows loadings and scores plots of the first three extracted PCs. Individual scores for the samples were first averaged and then plotted. Sample A is represented by cherry-odour, thin gel viscosity, lower degree of fruit disintegration, presence of off-odour, and low level of fruit firmness and adhesiveness. Sample B is represented by off-flavour, adhesiveness, fruit firmness, and low level of sourness and cherry-flavour. Samples C and E are represented by cherry-flavour, sourness, and absence of off-flavour. Sample D is represented by adhesiveness and fruit firmness, while sample F is represented by thick gel viscosity, higher degree of fruit disintegration, off-odour, off-flavour, and low levels of cherry-flavour, adhesiveness and fruit firmness.
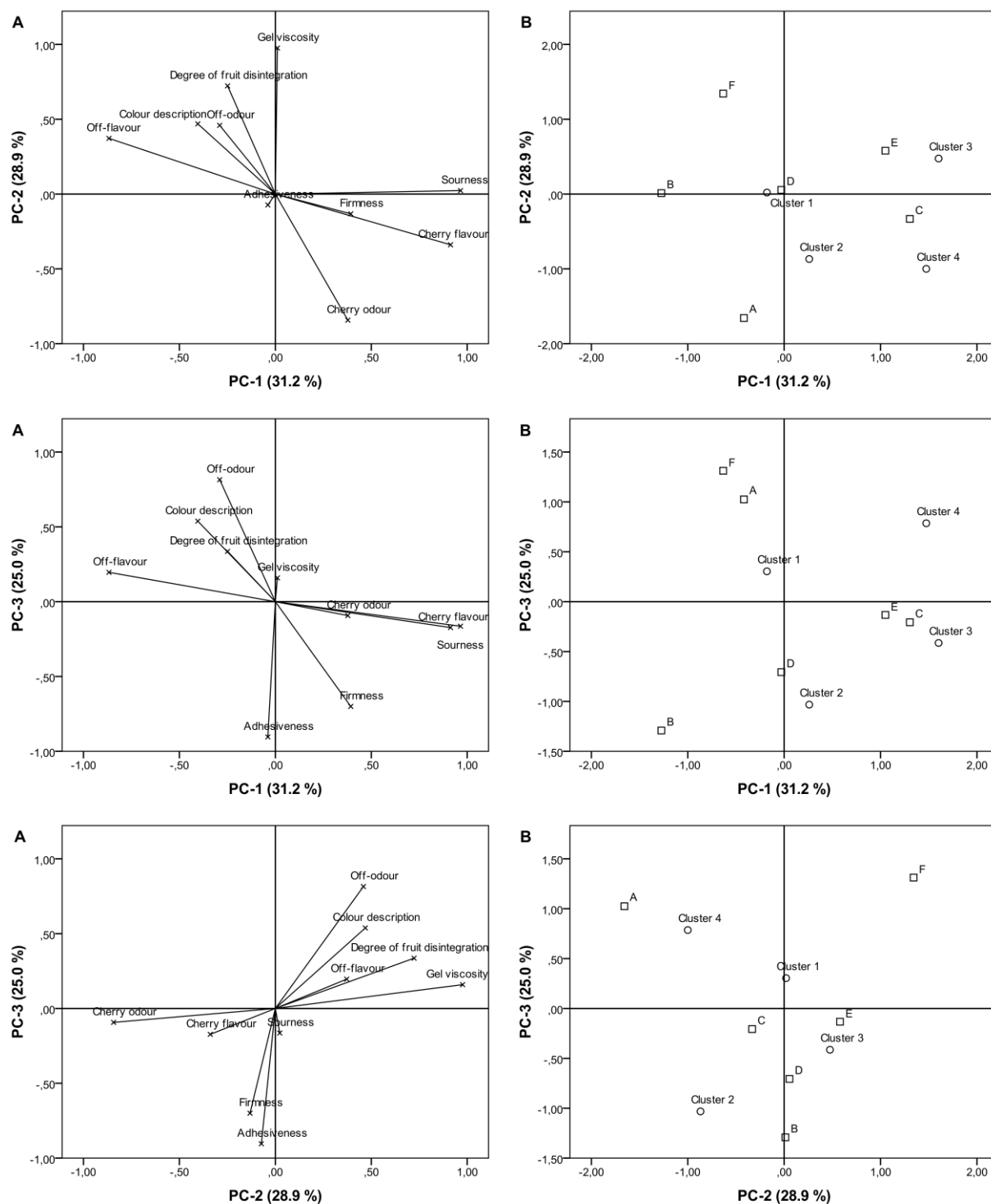
Figure 1. Attribute-loadings (A) and sample&consumer-scores (B) plots of the first three principal components extracted by applying PCA on consensus data matrix obtained by applying GPA on descriptive data of cherry pie filling samples. Rotation method: Varimax. The samples are marked with capital letters from A to F. Consumer clusters are marked with numbers from 1 to 4 (Cluster 1 = 31 %, Cluster 2 = 28 %, Cluster 3 = 20 %, and Cluster 4 = 21 %).

The mean hedonic scores for cherry pie filling of filled pastry rolls are shown in Table 2. Significant differences were found and two homogenous subsets of scores were distinguished. In general, the acceptance scores were lower for the samples characterized

by off-flavour and low levels of cherry-flavour (samples F and B). Considering overall acceptability scores (data not shown), none of the pastry samples did receive significant advantage against the others. According to the just-about-right scores (data not shown), all of the pastry samples were recognized as insufficiently sweet by consumers ($p < 0.05$; % consumers > 20 %).

Table 2. Mean consumer acceptance scores of cherry pie fillings

Samples	Mean hedonic scores ($n = 100$) Homogeneous subsets for $\alpha = 0.05$*		Sd
	1	2	
F	4.77		2.30
B	4.98		2.46
A		6.11	2.03
D		6.22	2.03
C		6.48	2.01
E		6.49	2.20

* The values within a homogenous subset are not significantly different

Individual consumer cherry pie filling hedonic scores were regressed against the first three extracted PCs of the descriptive analysis (linear regression). Preference mapping plots were produced using the regression coefficients as coordinates. Regression coefficients for individual consumers were clustered using K-means cluster analysis, averaged across clusters and plotted onto three-dimensional PC-space (Figure 1, B plots). Four clusters numbered from 1 to 4, with individual proportions of respondents ≥ 20 % (31 %, 28 %, 20 % and 21 %, respectively), were identified for consumer responses. The consumers in the clusters 3 and 4 liked the cherry pie fillings that were loaded positively on PC-1 (samples E and C), i.e. the samples characterized by cherry-flavour, sourness, and absence of off-flavour. The clusters 4 and 1 also showed a preference for the sample A, which was loaded positively on PC-3 and negatively on PC-2. This means that the most of the respondents like a cherry pie filling with distinctive cherry flavour and sourness, thin gel viscosity, lower degree of fruit disintegration, and low level of adhesiveness. The primary characteristic for preference of cherry pie filings for the cluster 2 was PC-3. This group of consumers preferred the product D characterized by fruit firmness, adhesiveness and absence of off-odour. As can be seen in preference mapping plots (Figure 1), none of the consumer clusters showed a preference for the sample F. In regard to evaluated sensory characteristics, the sample F was the one totally separated from the other samples. The sample F was the product with the highest degree of fruit disintegration, the presence of off-flavour, and with the lowest level of cherry flavour, sourness and fruit firmness, which was assessed by the consumers as unacceptable (Table 2).

CONCLUSIONS

Among the 10 sensory attributes of cherry pie filing samples measured by the sensory panel, that were subjected to dimension reduction analysis, distinctive cherry flavour, pronounced sourness, thin gel viscosity, lower degree of fruit disintegration, and low level of adhesiveness were the drivers of liking for the most of the tested consumers. The characteristics of cherry pie filings that appeared to be unacceptable for the consumers were high degree of fruit disintegration, presence of off-flavour, and low levels of cherry flavour, sourness and fruit firmness.

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ADVANTAGES AND SHORTCOMINGS OF LAB-ON-A-CHIP METHOD FOR INVESTIGATION OF HMW-GS OF WHEAT (*TRITICUM AESTIVUM*) CULTIVARS

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ABSTRACT

High molecular weight glutenin subunits (HMW-GS) are one of the most influential factors on wheat (*Triticum aestivum*) dough strength and elasticity. Therefore, their fast and reliable determinations are important for wheat scientists. In this study, promising Lab-on-a-Chip (LoaC) technique for the determination of three combinations of HMW-GS (2*, 7+9, 5+10; N, 7+9, 5+10; and N, 7+9, 2+12) of six wheat cultivars (Ljiljana, Dama, Pobeda, Bastijana, Simonida and Zvezdana) is examined. The advantages of this method are low coefficients of variation for molecular weight of HMW-GS and their percentage in total sum of subunits. However, the shortcomings were the impossibility to obtain x HMW-GS from Glu D1 locus in every analysis, especially in the first five lanes of chip, and the problem to obtain regular electropherogram without decline of baseline and expansions of HMW-GS peak. Generally, LoaC technique is good for qualitative and quantitative determination of HMW-GS subunits except in case quantitative determination of HMW-GS 2*.
Keywords: HMW-GS, LoaC, wheat cultivars

INTRODUCTION

High molecular weight glutenin subunits (HMW-GS) present one of the most influential genetically factors on wheat (*Triticum aestivum L.*) dough properties (Payne, 1987a). Therefore, their fast and reliable determinations are important for wheat scientists. The genes coding for HMW-GS are located on the long arms of chromosomes 1A, 1B and 1D at the Glu A1, Glu B1 and Glu D1 loci, respectively (Payne, 1987b). HMW glutenin subunits are classified on x-type and y-type (Shwery *et al.*, 1992). Subunits of x–type possess lower mobility on SDS–PAGE and have higher molecular weight than subunits of y-type. Theoretically, bread wheats should contain six different HMW-GS but, due to silencing of some of these genes, most common wheats possess from three to five subunits (Payne 1987b).

In the past decade, much attention was focused on developing microfluid or Lab-on-a-Chip (LoaC) technique and its application for sensitive biochemical analyses. This system has the potential for a fast, reliable, and automatable analysis in the separation and quantitation of proteins (Goetz *et al.*, 2004, Hey, 2007).

The LoaC method for protein analysis allows for the integration of electrophoretic separation, staining, destaining, and fluorescence detection into a single process and for it to be combined with data analysis.

The aim of this study was to examine advantages and shortcomings of LoaC technique for determination of HMW-GS of common wheat (*Triticum aestivum* L.) cultivars.

MATERIAL AND METHODS

Examinations were carried out on six common winter wheat (*Triticum aestivum* L.) cultivars ('Ljiljana', 'Dama', 'Pobeda', 'Bastijana', 'Simonida', and 'Zvezdana'), grown in Novi Sad (Serbia) in one production year (2008/2009). The chosen wheat cultivars possess three combination of HMW-GS (7+9 pair of subunits from Glu B1 locus, 5+10 pair of subunits from

Glu D1 locus; 2* subunits from Glu A1 locus, 7+9 pair of subunits from Glu B1 locus, 5+10 pair of subunits from Glu D1 locus and 7+9 pair of subunits from Glu B1 locus, 2+12 pair of subunits from Glu D1 locus) respectively, which are the most usual in Serbian wheat cultivars. Two of these HMW-GS combinations (2*, 7 + 9, 5 + 10 and N, 7 + 9, 5 + 10) are according to Denčić *et al.* (2008) and Tsenov *et al.* (2009) dominant in European winter wheat. All six cultivars were bred at the Institute of Field and Vegetable Crops in Novi Sad, Serbia.

Wheat samples were milled by MLU – 202 (Bühler, Uzwil, Switzerland) and the obtained flour was used for further analysis. The percentage of glutenin subunits was determined from 30 mg of flour after removing albumins, globulins and gliadins by three consecutive extraction processes, with three different solvents: deionisied water, 2% salt solution and 70% ethanol solution. The full range of glutenin subunits was subsequently extracted with a treatment solution (2% SDS solution containing 5% β-mercaptoethanol). A volume of 350 μL of the extract solution was added and afterwards heated for 5 minutes to 100 °C. Final solutions of glutenins were prepared by mixing a volume of 4 μL of the clarified sample extract with 2 μL of Agilent sample buffer and 84 μL of deionized water. Separation of proteins was performed using chip electrophoresis technique on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with Protein 230 Plus Lab-on-a-Chip kit. After analysis, every subunit was manually integrated and their percentage was calculated from the time corrected area.

The data were statistically analyzed by STATISTICA 12.0 software (StatSoft Inc., USA, 2013). Descriptive statistics was used to explore the MW and percentage gained HMW-GS of the examined wheat cultivars, and for that purpose mean, range and standard deviation as well as coefficient of variation (CV) were calculated.

RESULTS AND DISCUSSION

The reproducibility of molecular weight of HMW-GS on LoaC method was evaluated by examining at least 2 replicates of each sample. Coefficient of variation (CV) was calculated since it represents a relative measure of error because it weights the standard deviation for the size of the mean. The apparent sizes of glutenin subunits 2, 5, 2*, 7, 10, 9, and 12 & 9 were presented in Table 1. Samples containing the subunits 12 and 9 were barely resolved where only a shoulder peak was observed for subunit 9 in quite smaller area than subunit 12. The similar situation was observed when sample extracts containing the subunits 12 and 18, 16 and 10 or 1 and 4 (Rhazi *et al.,* 2009) were analysed. Furthemore it was not possible to determine the subunit 5 in three cultivars ('Bastijana','Pobeda' and 'Ljiljana') in duplication since it was overlapped with upper marker.

In general, the apparent sizes determined by LoaC method were on the same level as those obtained by Marchetti-Deschmann *et al.* (2011) using Protein 230 Plus Lab-on-a-Chip kit for glutenin subunits 2, 5, 7 and 10, whereas for 2*, 9 and 12 & 9 were quite higher. Moreover, determined ranges of subunits were even narrower than those obtained by Marchetti-Deschmann *et al.* (2011) except for subunit 2* (Table 1). The results were highly reproducible, since CVs of molecular weights were less than 2% for six determined subunits which is in accordance with results of other authors (Marchetti-Deschmann *et al.*, 2011; Rhazi *et al.*, 2009; Uthayakumaran *et al.,* 2006), except for glutenin subunit 2* whose CV was 3.64%.The cause of this could be the peak shape and fact that peak was quite low and wide. Similar or even better results were gained when HMW-GS of individual cultivars were determined (Table 2). These results showed that subunits 2* of 'Pobeda'represent the problem for determination.

The content of some HMW-GS (subunits 2 and 12 & 9) fluctuated slightly among genotypes (Table 3). Nevertheless, significant variations in the quantity of other HMW-GS (subunits 5, 2*, 7, 10 and 9) were detected (Table 3) and possible is to obtain different percentage in total sum of HMW-GS among cultivars. The determination of the amount of HMW-GS is important for prediction of wheat quality (MacRitchie *et al.*, 1991). Furthermore, according to Rhazi *et al.* (2009) the quantification of HMW-GS could be used to detect an over-expression of HMW-GS, for example, an overexpression of the subunit 7 is encountered in certain

cultivars. Also, subunits 7 possess the highest range of all examined cultivars (Table 3). Quantification of individual HMW-GS by the LoaC method of six Serbian bread wheat cultivars is represented in Table 4. These results showed that CVs obtained in presented study are higher than those obtained by Rhazi *et al.* (2009), but lower than those by Marchetti-Deschmann *et al.* (2011). The reason for this difference in the results of the individual HMW-GS quantification lies in the two facts. The first is that Rhazi *et al.* (2009) in their study showed results of only seven of 25 examined cultivars. The second is that Marchetti-Deschmann *et al.* (2011) in their paper expressed amount of individual HMW-GS by time corrected areas and not by percentage of individual HMW area to total HMW-GS area.

Table 1. Apparent sizes of HMW-GS determined using the LoaC method

Subunits	Mean (kDa)	Range (kDa)	Standard deviation (kDa)	Coefficient of variation (%)
2	230	5.30	2.43	1.06
5	230	0.60	0.42	0.18
2*	222	17.6	8.08	3.64
7	182	7.90	2.54	1.40
10	147	4.20	1.19	0.81
9	132	3.20	0.96	0.73
12 & 9	128	0.50	0.24	0.19

Table 2. Apparent sizes of HMW-GS of individual cultivars determined using the LoaC method

Cultivar	Subunits	Mean (kDa)	Range (kDa)	Standard deviation (kDa)	Coefficient of variation (%)
Simonida	2	229	2.10	1.48	0.65
	7	178	0.80	0.57	0.32
	12 & 9	128	0.30	0.21	0.17
Zvezdana	2	231	5.00	3.54	1.53
	7	178	1.20	0.85	0.48
	12 & 9	128	0.50	0.35	0.28
Bastijana	2*	216	1.20	0.85	0.39
	7	182	0.20	0.14	0.08
	10	148	0.60	0.42	0.29
	9	132	0.30	0.21	0.16
Pobeda	2*	227	13.1	9.26	4.08
	7	183	3.10	2.19	1.20
	10	148	1.90	1.34	0.91
	9	132	1.40	0.99	0.75
Ljiljana	7	183	3.20	1.41	0.77
	10	147	3.50	1.46	0.99
	9	132	3.10	1.27	0.96
Dama	5	230	0.60	0.42	0.18
	7	182	1.00	0.71	0.39
	10	146	0.50	0.35	0.24
	9	131	1.30	0.92	0.70

However, beside advantages for the determination of HMW-GS by the LoaC method with Protein 230 Plus Lab-on-a-Chip kit this method possesses few shortcomings. The first is the impossibility to obtain x HMW-GS from Glu D1 locus (subunit 5) in every analysis, especially in the first five lanes of chip (Figure 1). This is similar to results from the study of Marchetti-Deschmann *et al.* (2011) who showed that glutenin subunits 2 and 5 could have molecular

weight of 240 kDa. The second problem was to obtain regular electropherogram without decline of baseline and expansions of HMW-GS peak (Figure 2).

Table 3. Quantification of individual HMW-GS subunits examined Serbian bread wheat cultivars by the LoaC method

Subunits	Mean (%)*	Range (%)*	Standard deviation (%)*	Coefficient of variation (%)
2	4.72	1.46	0.63	13.36
5	10.93	4.18	2.95	27.01
2*	2.59	3.70	1.70	65.50
7	5.49	7.99	2.57	46.77
10	4.49	4.03	1.32	29.34
9	2.63	5.66	1.85	70.47
12 & 9	5.19	1.84	0.84	16.17

*Expressed as relative amount: percentage of individual HMW area to total HMW-GS area.

Table 4. Quantification of individual HMW-GS by the LoaC method of six Serbian bread wheat cultivars

Cultivar	Subunits	Mean (%)*	Range (%)*	Standard deviation (%)*	Coefficient of variation (%)
Simonida	2	4.89	0.18	0.13	2.59
	7	3.56	0.63	0.45	12.61
	12 & 9	4.57	1.04	0.73	16.04
Zvezdana	2	4.56	1.46	1.03	22.67
	7	5.03	0.55	0.39	7.695
	12 & 9	5.82	0.15	0.10	1.78
Bastijana	2*	1.20	1.05	0.74	61.88
	7	8.20	2.36	1.67	20.35
	10	5.35	0.85	0.60	11.17
	9	3.92	0.88	0.63	15.98
Pobeda	2*	3.98	0.8	0.59	14.72
	7	8.91	1.92	1.36	15.22
	10	5.28	0.88	0.62	11.73
	9	5.29	1.75	1.24	23.41
Ljiljana	7	5.28	4.20	2.01	38.13
	10	3.51	3.49	1.59	45.46
	9	1.16	1.25	0.53	45.32
Dama	5	10.93	4.18	2.95	27.01
	7	2.16	0.56	0.40	18.42
	10	4.82	0.95	0.67	13.98
	9	1.62	0.28	0.20	12.16

*Expressed as relative amount: percentage of individual HMW area to total HMW-GS area.
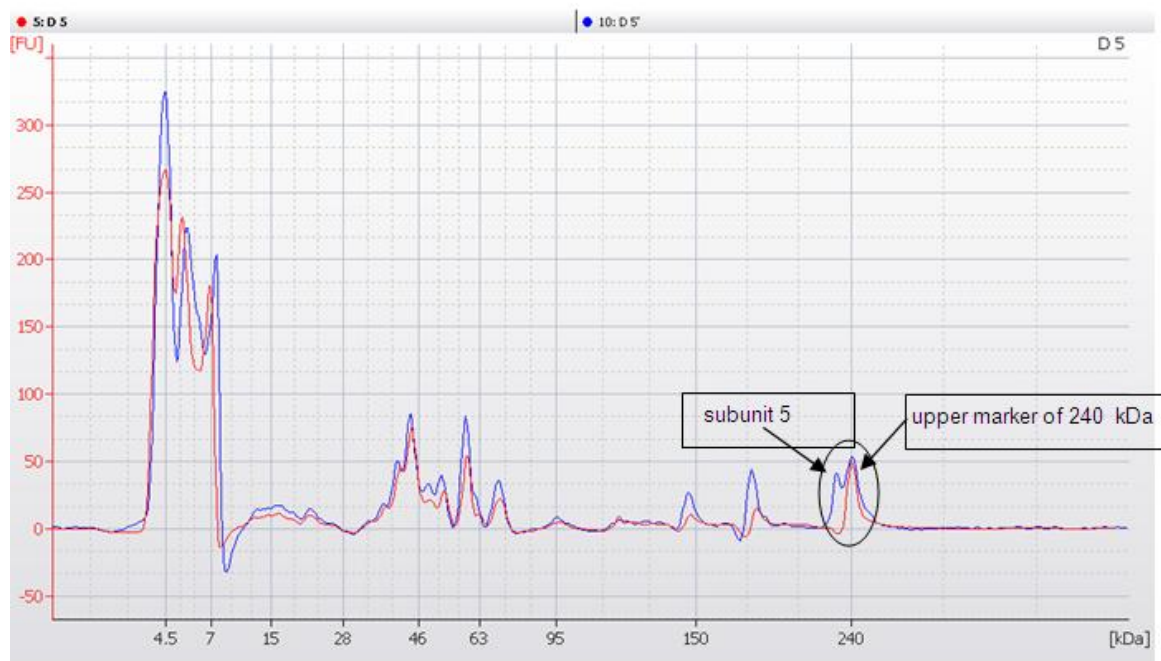
Figure 1. Electrophoregrams of 'Ljiljana' cultivar from lane 5 and 10 gained in the same chip analysis
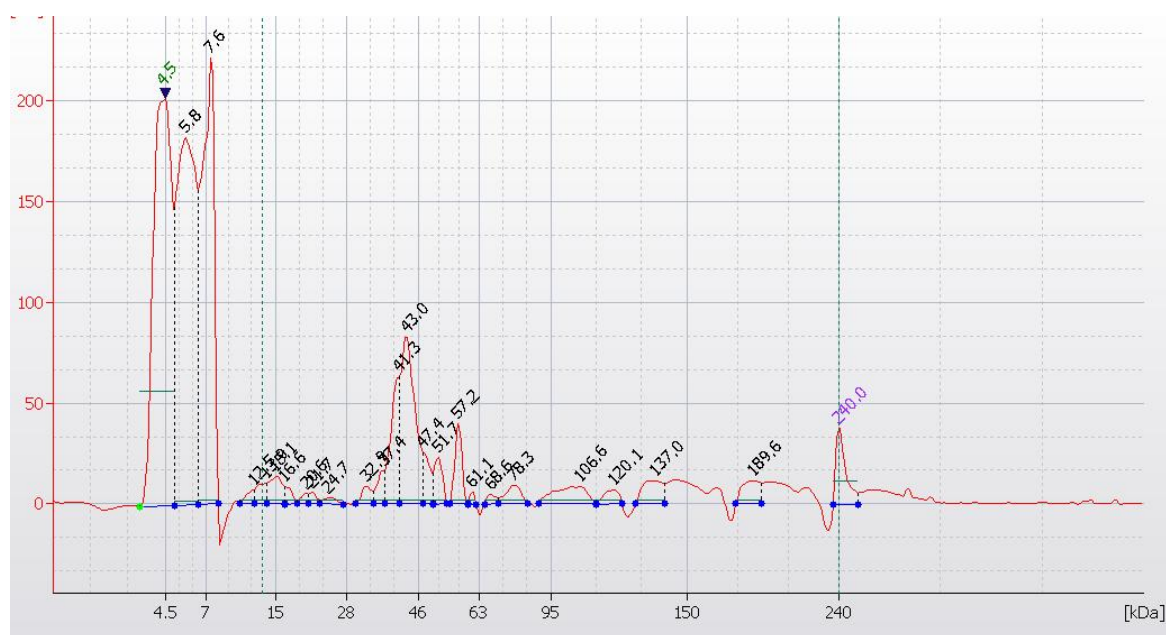
Figure 2. Electrophoregrams of 'Simonida' with decline of baseline and expansion of HMW-GS peak

CONCLUSIONS

The study confirmed that LoaC method possesses high reproducibility for molecular weight determination of HMW-GS, whereas reproducibility of their quantification was on lower level although it can be used to determine the variation of the relative amount of each individual HMW-GS.

However, the shortcomings were the impossibility to obtain x HMW-GS from Glu D1 locus (subunits) in every analysis, especially in the first five lanes of chip, and the problem to

obtain regular electropherogram without decline of baseline and expansions of HMW-GS peak.

Generally, LoaC technique with Protein 230 Plus Lab-on-a-Chip kit presents good tool for qualitative and quantitative determination of HMW-GS subunits, except in case quantitative determination of HMW-GS 2*.

ACKNOWLEDGEMENTS

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FATTY ACID PROFILE AND SENSORY EVALUATION OF BACON

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ABSTRACT

Smoked meat products present a significant part of the human diet in Serbia. They are important because of their good taste, high nutritional value, high level of production and large variety of products.

The aim of this study was to analyze and evaluate the fatty acid profile and sensory properties of the bacon with skin (B1) and of the bacon without skin (B2), which were smoked in traditional (TS) and industrial (IS) smokehouses. Sampling of both types of bacon has been performed before smoking and these samples were marked as raw material (RM). A total of 36 samples (TS: RM-6, B1-6, B2-6; IS: RM-6, B1-6, B2-6) were collected during the winter period on Zlatibor region. Fatty acid composition was determined by gas chromatograph equipped with a flame ionisation detector. Sensory evaluation of smoked bacon was performed according to the DLG-5-points-scheme by professional staff of the Federal Research Centre for Nutrition and Food, Kulmbach, Germany.

Twenty-four fatty acids (FA) were determined in the bacon and raw material. The contents of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in all analyzed samples were within the range of 37.04% - 40.48%, 45.64% - 50.27% and 12.38% - 15.62%, respectively. Statistical analysis showed that there were not significant differences ($p<0.05$) between the SFA, MUFA and PUFA contents in the raw material and two types of bacon. It could be concluded that the type of smoking and the presence of the skin had not influence on FA content of bacon.

According to DLG-5-points-scheme bacon with skin from IS was evaluated with DLG award in Gold, while other samples were evaluated with DLG award in Silver.

Keywords: fatty acids, sensory evaluation, smoked bacon

INTRODUCTION

Meat is considered a highly nutritious food and very important component for the human diet and vital function. Although some earlier investigations indicates that consumption of fat, especially from animal sources, increases risk for colon cancer (Armstrong and Doll, 1975; Boyle *et al.,* 1985), however, a growing back of evidence that healthy diet which includes lean red meat can produce positive change in lipid biochemistry (Watts *et al.,* 1988; Scott *et al.,* 1990). Lipid content in different food can be influenced by different factors such as thermal treatment of meat (Domínguez *et al.,* 2014), different animal nutritional treatments (Lopes *et al.,* 2014), etc.

Fatty acids are major components of lipids, which occur in practically all types of foods. Content of intramuscular fat and fatty acid composition, along with the biological factors, trace elements and vitamins, are key factors contributing to nutritional value of meat (Wyness, 2013). The World Health Organization, WHO, (2003) recommended that total fat, saturated fatty acids (SFA), n−6 polyunsaturated fatty acids (PUFA), n−3 PUFA and trans fatty acids should contribute <15–30, <10, <5–8, <1–2 and <1% of total energy intake, respectively. Some evidence suggests that 0.2-0.3% of the energy should be derived from preformed very long chain n-3 PUFAs (Rustan and Drevon, 2005). The benefit effect of n-3 PUFA in reducing the risk of cardiovascular disease, cancer, diabetes etc., are well documented (Simopoulos, 1991; Barceló-Coblijn and Murphy, 2009; Lopez-Huertas, 2010).

Considering the different amounts of long-chain n-3 PUFA in different types of food, He at al. (2004) indicate to have more benefit by eating fatty fish rich in long-chain n-3 PUFA if it is true that long-chain n-3 PUFA is solely responsible for any beneficial effect in reducing death from coronary heart disease.

Bacon is one of the popular traditional smoke meat products in south-west Serbia (Djinovic *et al.,* 2008; Saicic *at al.,* 2013). Typical bacon processing generally takes 1-1.5 months, usually starting in November and December. The aim of this study was to evaluate and compared the fatty acid composition of bacon with and without skin, smoked in traditional and industrial smokehouses. Furthermore, fatty acid composition of smoked bacons and raw material was compared in other to establish their possible changes during meat smoking. Sensory evaluation of smoked bacon was performed as well.

MATERIAL AND METHODS

Samples

A total of 36 samples of bacon with and without skin from traditional (TS) and industrial smokehouse (IS) were collected in February 2007. Bacon samples both from TS and IS were continuously exposed by smoke during 15 days. In traditional smokehouses smoking was not under controlled conditions. Traditional way of meat smoking is applied in practice only during the winter. Temperature and humidity were under outdoor conditions; the temperature of the smoke was between 18-20 °C. In industrial smokehouses samples were under controlled smoking program during 15 days. During those 15 days samples were smoked 64 h in four cycles. The temperature and humidity were between 16-24 °C and 78-88% respectively. Smoke was produced by Vemag glowing smoke generator H 504/C from beech wood. Industrial way of smoking is applied in practice during the whole year. Both types of bacon from IS and TS were collected before smoking and these samples were marked as raw material (RM). After process of smoking samples were collected from both smokehouses. After sampling, bacon were placed in sterile vacuum bags, vacuumed and placed in dark at -20 °C. Samples were transported on dry ice from Serbia to Germany.

Fatty acid analysis

Bacon samples were partially thawed at +4 °C one day before analysis. Intramuscular fat was extracted from samples with a solvent mixture of methanol/dichloromethane (1:2 v/v). Extracted fat was filtered through a fold filter tipped with anhydrous sodium sulphate. Fatty acid was esterified with trimethylsulphonium hydroxide according to the method of Schulte and Weber (Schulte & Weber, 1989). Analyses of fatty acid methyl esters (FAME) were performed on Hewlett Packard gas chromatograph (HP6890, Munich, Germany) equipped with a flame ionisation detector and a capillary column (Phenomenex, Zebron ZB-1).

Sensory properties

Sensory evaluation was carried out by an expert panel consisting of 5 members selected from the professional staff of the Federal Research Centre for Nutrition and Food, Kulmbach, Germany. Sensory evaluation of the bacon with and without skin was done according to the DLG-5-points-scheme (50. Auflage DLG, 2007). The bacon samples after process of smoking were evaluated on External appearance; Appearance, colour, colour maintenance, composition; consistency and odour and taste according to DLG-5-Award evaluation range. Evaluation range was done according the conditions for awards (at least one weighted must be achieved in the each test feature). DLG Award could be achieved as: DLG award in Gold (excellent, quality figures 5), DLG award in Silver (very good, quality figures 4.5-4.99), DLG award in Bronze (good, quality figures 4.00-4.49) and without DLG award (quality figures <4.00).

RESULTS AND DISCUSSION

The results of the individual fatty acids contents of analysed bacon with and without skin are shown in Tables 1 and 2 as mean value ± standard deviation. Results for sensory evaluation of the final products are shown in Table 3.

Table 1. Fatty acids contents, as % of total fatty acids, of raw material (RM) and intramuscular fat of bacon with skin from traditional (TS) and industrial (IS) smokehouses

| Fatty acid | Bacon with skin | | |
	RM (n=6)	TS (n=6)	IS (n=6)
C10:0	0.08 ± 0.02	0.08 ± 0.03	0.08 ± 0.02
C12:0	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.01
C14:0	1.13 ± 0.07	1.13 ± 0.42	1.15 ± 0.58
C14:1 n-5	0.37 ± 0.04	0.47 ± 0.03	0.29 ± 0.03
C15:0	0.05 ± 0.02	0.05 ± 0.02	0.07 ± 0.02
C16:0	22.93 ± 8.25	23.03 ± 7.22	22.37 ± 8.44
C16:1 *cis* n-9	0.26 ± 0.05	0.28 ± 0.03	0.29 ± 0.02
C16:1 *cis* n-7	2.23 ± 0.22	1.98 ± 0.25	1.64 ± 0.36
C17:0	0.31 ± 0.11	0.36 ± 0.07	0.54 ± 0.08
C17:1 n-8	0.28 ± 0.12	0.30 ± 0.07	0.41 ± 0.05
C18:0	12.28 ± 2.56	12.84 ± 2.95	13.82 ± 3.26
C18:1 *cis* n-9	41.80 ± 9.58	39.88 ± 6.18	39.69 ± 8.88
C18:1 *cis* n-7	2.93 ± 0.87	2.60 ± 1.10	2.44 ± 1.23
C18:2 *cis* n-6	12.06 ± 2.76	13.70 ± 2.35	13.32 ± 2.45
C18:3 n-3	0.58 ± 0.05	0.63 ± 0.06	0.67 ± 0.05
C18:2 *cis*9,*trans*11	0.10 ± 0.02	0.10 ± 0.02	0.18 ± 0.03
C20:0	0.20 ± 0.03	0.19 ± 0.04	0.23 ± 0.03
C20:1 n-9	0.91 ± 0.17	0.86 ± 0.03	0.88 ± 0.05
C20:2 n-6	0.62 ± 0.07	0.66 ± 0.05	0.64 ± 0.05
C20:3 *cis* 8,11,14 n-6	0.08 ± 0.03	0.09 ± 0.02	0.10 ± 0.02
C20:4 *cis* 5,8,11,14 n-6	0.22 ± 0.06	0.23 ± 0.05	0.34 ± 0.09
C20:3 *cis* 11,14,17 n-3	0.10 ± 0.02	0.10 ± 0.01	0.09 ± 0.03
C20:4 n-3	0.06 ± 0.01	0.04 ± 0.02	0.05 ± 0.01
C22:5 n-3	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01
FA	99.71 ± 0.55	99.74 ± 0.58	99.60 ± 0.66
SFA[1]	37.04 ± 1.75	37.75 ± 1.74	38.38 ± 1.25
MUFA[2]	48.78 ± 1.35	46.37 ± 1.59	45.64 ± 1.31
PUFA[3]	13.89 ± 0.58	15.62 ± 0.52	15.58 ± 0.38
Σn-3	0.81 ± 0.05	0.84 ± 0.04	0.95 ± 0.05
Σn-6	12.98 ± 0.45	14.68 ± 0.35	14.45 ± 0.45
Σn-6 / Σn-3	16.02 ± 0.95	17.48 ± 0.67	15.21 ± 0.82

[1]SFA - saturated fatty acids; [2]MUFA – monounsaturated fatty acids; [3]PUFA - polyunsaturated fatty acids

Twenty-four fatty acids (FA) were determined in samples of bacon with and without skin, as well as in raw material, both from traditional and industrial smokehouses. Their content as sum of saturated fatty acids, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids and Σn-6 / Σn-3 relationship are presented in Tables 1-2. The most common SFA was palmitic acid (C16:0) in all analysed bacon samples. Its content makes approximately 62% of the SFA content. In the case of MUFA and PUFA, oleic acid (C18:1 cis n-9) and linoleic acid (C18:2 cis n-6) were the predominant fatty acids and their content make approximately 87% and even 93% of the MUFA and PUFA content, respectively. The ratio of Σn-6 / Σn-3 in the all analyzed bacon samples were almost the same (Tables 1-2).

Statistical analysis of the data for fatty acid composition showed that there were not significant differences (p<0.05) between the SFA content of the raw material and bacon with skin and without of skin from both smokehouses. Also, the MUFA and PUFA contents of final bacon samples after process of smoking were not significantly different in comparison to raw material.

Table 2. Fatty acids contents, as % of total fatty acids, of raw material (RM) and intramuscular fat of bacon without skin from traditional (TS) and industrial (IS) smokehouses

Fatty acid	Bacon without skin					
	RM (n=6)		TS (n=6)		IS (n=6)	
C10:0	0.08	± 0.02	0.07	± 0.02	0.06	± 0.02
C12:0	0.07	± 0.03	0.06	± 0.02	0.06	± 0.01
C14:0	1.24	± 0.05	1.20	± 0.52	1.21	± 0.48
C14:1 n-5	0.74	± 0.04	0.45	± 0.04	0.54	± 0.05
C15:0	0.06	± 0.02	0.06	± 0.01	0.05	± 0.02
C16:0	23.39	± 9.25	24.56	± 8.32	23.74	± 7.56
C16:1 *cis* n-9	0.26	± 0.03	0.28	± 0.05	0.25	± 0.02
C16:1 *cis* n-7	2.46	± 0.18	2.01	± 0.15	2.09	± 0.26
C17:0	0.44	± 0.09	0.34	± 0.08	0.34	± 0.09
C17:1 n-8	0.45	± 0.08	0.26	± 0.07	0.30	± 0.05
C18:0	11.60	± 2.45	13.93	± 3.15	12.29	± 4.26
C18:1 *cis* n-9	42.26	± 8.55	39.25	± 7.18	40.57	± 8.32
C18:1 *cis* n-7	3.25	± 0.96	2.63	± 1.12	2.67	± 1.15
C18:2 *cis* n-6	10.65	± 2.36	11.71	± 2.55	12.35	± 2.65
C18:3 n-3	0.53	± 0.06	0.51	± 0.07	0.58	± 0.05
C18:2 *cis*9,*trans*11	0.10	± 0.02	0.16	± 0.03	0.16	± 0.03
C20:0	0.21	± 0.03	0.26	± 0.04	0.29	± 0.04
C20:1 n-9	0.85	± 0.15	0.86	± 0.03	0.92	± 0.06
C20:2 n-6	0.54	± 0.08	0.54	± 0.08	0.56	± 0.05
C20:3 *cis* 8,11,14 n-6	0.10	± 0.03	0.09	± 0.02	0.08	± 0.02
C20:4 *cis* 5,8,11,14 n-6	0.25	± 0.07	0.28	± 0.09	0.23	± 0.08
C20:3 *cis* 11,14,17 n-3	0.07	± 0.02	0.08	± 0.01	0.08	± 0.03
C20:4 n-3	0.06	± 0.01	0.07	± 0.02	0.05	± 0.01
C22:5 n-3	0.06	± 0.01	0.07	± 0.01	0.07	± 0.01
FA	99.77	± 0.45	99.73	± 0.55	99.71	± 0.56
SFA[1]	37.12	± 1.35	40.48	± 1.14	38.08	± 1.15
MUFA[2]	50.27	± 1.45	45.74	± 1.89	47.39	± 1.12
PUFA[3]	12.38	± 0.56	13.51	± 0.55	14.24	± 0.45
Σn-3	0.70	± 0.05	0.73	± 0.04	0.83	± 0.05
Σn-6	11.58	± 0.45	12.62	± 0.45	13.25	± 0.55
Σn-6 / Σn-3	16.54	± 0.85	17.29	± 0.87	15.96	± 0.92

[1]*SFA - saturated fatty acids;* [2]*MUFA – monounsaturated fatty acids;* [3]*PUFA - polyunsaturated fatty acids*

Table 3. Sensory evaluation of the final products according to the DLG-5-points-scheme

Product	Quality Evaluation	Evaluation (points)[1]	Quality Figures[2]
Bacon with skin			
TS	Too soft	4	4.80
IS	Without refusal	--	5.00
Bacon without skin			
TS	Reddish pork fat (not due to seasoning!)	4	4.70
IS	Too soft	4	4.80

[1] *Points (maximal point): 5*
[2] *Quality Figures (DLG award): 5.00 (DLG award in Gold); 4.50-4.99 (DLG award in Silver); 4.00-4.49 (DLG award in Bronze); <4.00 (without DLG award)*

Sensory evaluation according to DLG-5-points-scheme showed that sensory properties of bacon with skin, both from traditional and industrial smokehouses, were slightly better than sensory properties of bacon without skin. Nevertheless all samples were evaluated with DLG award. Bacon with skin from IS was evaluated with DLG award in Gold, while other samples were evaluated with DLG award in Silver (Table 3).

CONCLUSIONS

On the obtained data for fatty acids composition of bacon with skin and bacon without skin, that were smoking in industrial and traditional smokehouses, as well as in their raw material, it can be concluded that the type of smoking and the presence of the skin had not influence on fatty acid content as well as on sensory properties of bacon.

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SENSORY PROPERTIES OF CEVAPCICI PRODUCED WITH VARIOUS CHLORIDE SALTS

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ABSTRACT

Cevapcici are very popular barbeque product in Serbia, produced from pork, beef and lamb meat. As the other meat products, cevapcici are the important source of sodium originated from sodium chloride. Daily intake of sodium should be reduced because of bad effects of excessive sodium intake on human health (cardiovascular diseases, essential hypertension). In the aim to reduce sodium content, in this experiment were produced cevapcici with different amount of sodium chloride and potassium chloride (first group - 20 g of sodium chloride in 1000 g of meat; second group - 15 g of sodium chloride in 1000 g of meat; third group - 10 g of sodium chloride and 5 g of potassium chloride in 1000 g of meat). Products were sensory evaluated by quantitative descriptive analysis (ISO 6658:2005), using scales with 5 points (5 – the best expressed attribute; 1 – the worst expressed attribute). Sensory evaluation was performed by eight assessors, previously trained for detection and recognition of various tastes (ISO 3972:2011) and odours (ISO 5496:2006). Also, products were presented to twelve consumers and being evaluated by them. Both of them were performed ranking test for products according to ISO 8587:2006. The analysis of sodium and potassium content was performed by inductively-coupled plasma mass spectrometry (ICP-MS). Products from all experimental groups were acceptable for consumption. Cevapcici from the first group were evaluated as too salty and average evaluation was significantly different from averages from other two groups ($p \leq 0.05$). All products had acceptable taste, but overall acceptability for products from the first group was significantly different from overall acceptability for products in the second group ($0 \leq 0.01$). It can be concluded that the production of cevapcici with less amount of sodium chloride and with partially replacement of sodium chloride with potassium chloride is possible in the aim of reducing of sodium content in this kind of products.
Keywords: cevapcici, sensory properties, chloride salts, sodium, potassium

INTRODUCTION

Cevapcici are very popular barbeque product in Republic of Serbia as well in other Balkan countries, especially in Bosnia and Herzegovina. They are produced in whole country, mostly in similar way but from various raw materials. In Bosnia and Herzegovina, because of there are many Muslim people, cevapcici are produced exclusively from beef with large amount of tallow as are shoulder, neck and belly. In northern part of Republic of Serbia, cevapcici are produced from mixture of beef and pork and in southern parts from beef, pork and lamb meat. In the first two cases, common salt (sodium chloride) is only added (usually in the amount of 2%), but in the southern part of Serbia (Leskovac), it is added salt, garlic and paprika. However, cevapcici are a great part of fast food and they are consumed of major part of population, particularly of youth.

Due to great consumption of this product, it is clear that cevapcici represent an important source of sodium chloride in the human diet. Sodium chloride is the main source of sodium and in European countries more than 70% of the dietary salt intake is estimated to come from processed foods. Especially processed meat products contain relatively high amount of sodium (Doyle and Glass, 2010).

Intentional use of salt started around 5000-10000 years ago, at the beginning of agricultural development in the purpose to meat preservation achieving the low water activity that prevents microbial growth. Such salted meat could be transported to long distance. Salt in meat products causes saltiness (Ruusunen and Puolanne, 2005), and together with fats contributes to numerous sensory properties. Saltines is more expressed in products with higher moisture and fat content. One of the major functions of salt in meat products is solubilisation of miofibrilar proteins, which activates the proteins to increase the hydration and water holding capacity (Dötsch et al., 2009) that contributes to better textural characteristics of product. Increase of water holding capacity reduces cooking loss and increases the tenderness and softness of meat product.

In many developed countries the sodium intake exceeds the nutritional recommendations. A high sodium intake is main cause of essential hypertension and represents the potential risk for appear of cardiovascular diseases (WHO, 2007). Prehistoric man consumed less than 0.5 g of salt daily (Feng and MacGregor, 2010). Nowadays, daily intake of salt reached average value of 10 g, which is in evolutionary sense relatively recent. Intake of common salt is caused not only by physiological needs, but also by habits which are acquired in the early childhood, as well as tradition in diet (region, i.e. climatic conditions, preparation of food, live-stock resources, etc.). Of total daily amount of common salt introduced into organism by common amounts of food (dishes prepared in the households, bread, bakery products, cheese, etc.), and approximately 20% derives from meat products (Wirth, 1991).

Decrease of sodium content in the food is an important task for the food industry in the 21st century. There is growing focus on a healthier diet among consumers in the society (Desmond, 2006). It is very difficult to achieve an adequate saltiness in the sodium-reduced products, because only sodium chloride has typical and real salty taste

The goal of this study was to examine the possibility to reduce sodium chloride content in cevapcici as well as to replace part of sodium chloride with potassium chloride in the sodium-reduced product.

MATERIAL AND METHODS

Three different groups of cevapcic were produced in this trial, with different amount of salts. Composition and amount of salts are presented in the Table 1. It was used ground beef and pork (shoulder) and tallow adding sodium chloride and potassium chloride according to table. After mixing, mass for cevapcici were stored at 4 °C for 24 hours. After storage, mixture of meat and salts was formed into suitable shape for roasting, approximately 7 cm in the length. Cevapcici were roasted on electric grill and after that presented to eight trained assessors and twelve consumers.

Table 1. Composition of products, g

	Material		
	Group 1	**Group 2**	**Group 3**
Beef (shoulder)	600	600	600
Fat (beef tallow)	200	200	200
Pork (shoulder)	200	200	200
Sodium chloride	20	15	10
Potassium chloride	-	-	5

Products were sensory evaluated by quantitative descriptive analysis (ISO 6658:2005), using scales with 5 points (5 – the best expressed attribute; 1 – the worst expressed attribute). Sensory evaluation was performed by eight assessors, previously trained for detection and recognition of various tastes (ISO 3972:2011) and odours (ISO 5496:2006). Also, products were presented to twelve consumers and being evaluated by them. Both of them were performed ranking test for products according to ISO 8587:2006.

In roasted cevapcici were determined sodium and potassium content. Homogenized sample (approximately 0.3 g) was digested in microwave oven with nitric acid (p.a. SIGMA) and hydrogen peroxide (30%, p.a., MERCK). The analysis was performed by inductively-coupled plasma mass spectrometry (ICP-MS) using the instrument "iCap Q" (Thermo Scientific, Bremen, Germany), equipped with collision cell and operating in kinetic energy discrimination (KED) mode. The following isotopes were measured: ^{39}K and ^{23}Na. The quality of the analytical process was controlled by the analysis of the standard reference material (NIST SRM 1577c). Measured concentrations were within the range of the certified values for all isotopes.

Statistical analysis was performed using Microsoft Excel 2010 by method of descriptive statistics, analysis of variance and student t-test with expression of differences between arithmetic means of each group at the two levels of significance ($p \leq 0.05$ and $p \leq 0.01$). Different letters in superscript of average values presents the significant statistical difference.

RESULTS AND DISCUSSION

Products from all of three groups had acceptable taste, according to both assessors and consumers. There was significant difference between average value for taste acceptability for products from the first and from the second group ($p \leq 0.05$) according to assessors (Table 2). Most of them had perception that cevapcici from the first group was too salty in the comparison with other two groups. Because of that it was determined statistical significance ($p \leq 0.05$) between the first and the second and the third group in saltiness, respectively. Also, products from the first group got the evaluation for overall impression that significantly less ($p \leq 0.01$) than evaluation for products in the second group. Products from the second group were best evaluated and they were in overall impression statistically different from the third group of products ($p \leq 0.05$). The main reason was the a bit bitter after taste in the products from the third group. Consumers recognized higher level of saltiness in the products from the first group, but not in the statistically significant level. Between average values for consistency it was not determined significant differences ($p \geq 0.05$) according to both assessors and consumers.

Table 2. Sensory evaluation of cevapcici ($^{a, b}$ p ≤ 0.05; $^{x, y}$ p ≤ 0.01)

Group	M	Sx	Sd	Cv	Group	M	Sx	Sd	Cv
Taste acceptability									
Assessors, n = 8					**Consumers**, n = 12				
1st	3.38 ^{a}	0.41	0.99	29.40	1st	4.33	0.51	0.85	19.61
2nd	4.63 ^{b}	0.47	0.48	10.47	2nd	4.08	0.23	0.76	18.59
3rd	4.13	0.63	0.60	14.53	3rd	3.92	0.61	0.86	22.01
Saltiness									
Assessors, n = 8					**Consumers**, n = 12				
1st	3.25 ^{a}	0.38	0.66	20.35	1st	3.83	0.45	0.80	20.85
2nd	4.50 ^{b}	0.47	1.00	22.22	2nd	4.42	0.27	0.86	19.52
3rd	4.50 ^{b}	0.68	0.71	15.71	3rd	4.08	0.67	1.32	32.33
Consistency									
Assessors, n = 8					**Consumers**, n = 12				
1st	3.88	0.45	0.78	20.15	1st	4.08	0.46	0.28	6.77
2nd	4.25	0.44	0.66	15.56	2nd	4.17	0.24	0.37	8.94
3rd	4.00	0.61	0.71	17.68	3rd	4.08	0.61	0.28	6.77
Overall impression									
Assessors, n = 8					**Consumers**, n = 12				
1st	3.25 ^{x}	0.37	0.43	13.32	1st	4.00	0.47	0.71	17.68
2nd	4.63 ^{a,y}	0.47	0.48	10.47	2nd	3.67	0.26	1.18	32.14
3rd	4.13 ^{b}	0.63	0.60	14.53	3rd	3.75	0.60	1.01	26.94

Six of eight assessors ranked cevapcici with reduced sodium chloride content (second group) at the first place, while two assessors gave the first place to the products from the third group. Due to bitter after taste, products from the third group (with added potassium chloride) got the last place in the ranking (three assessors) as well as products from the first group because of over saltiness (five assessors). The difference between the first and the second group was very high (p ≤ 0.01), also the difference between the first and the second group was significant (p ≤ 0.05).
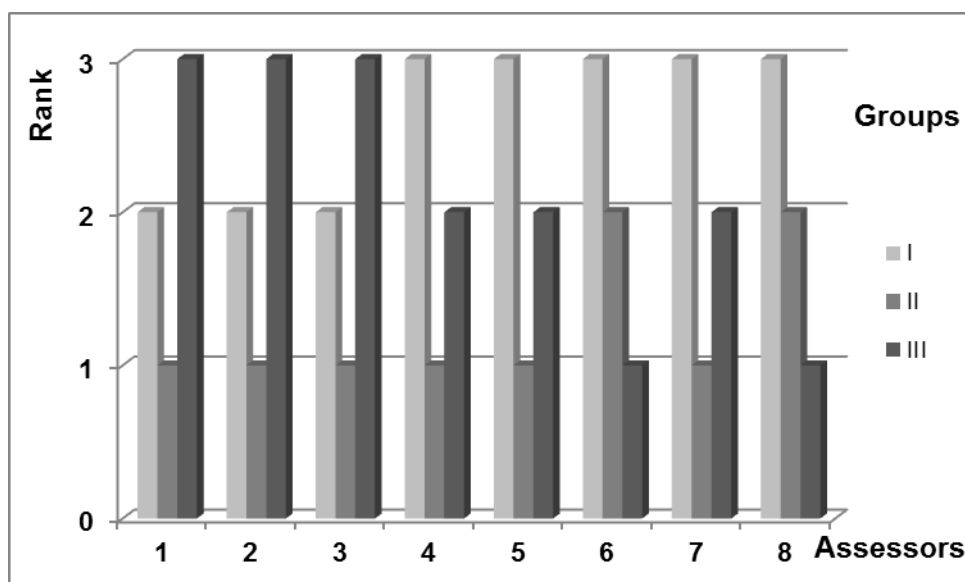
Figure 1. Ranking – assessors, n = 8

Consumers were not so consistent in their ranking. Five consumers gave the first place to the products from the first group, one to the products from the second group, while six consumers gave the first place to the products from the third group. The last place got the products from the first group (with the largest amount of sodium chloride) from five consumers, and three consumers gave the last place to the products from the second and the third group, respectively. Due to that the consumers were not consistent in the ranking, the significant statistically differences between averages did not determined (p ≥ 0.05).
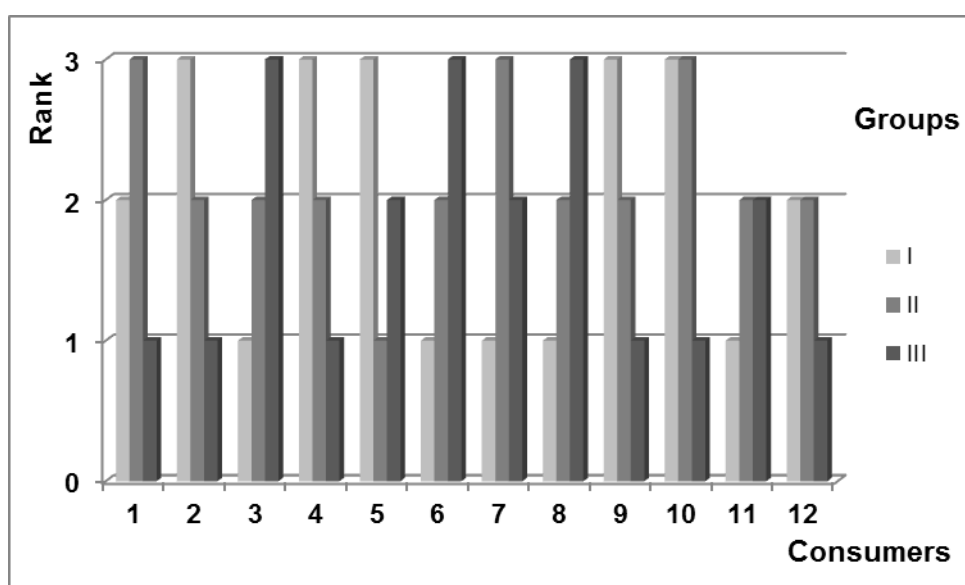
Figure 1. Ranking – consumers, n = 12

In the Table 3 are presented the content of sodium and potassium in 100 g of product. The content of sodium of 1156 mg/100 g determined in the cevapcici from the first group was the largest, consequently to the largest amount of added sodium chloride (20 g/1000 g of meat). In the products from the second group, sodium content is still high (940 mg/100 g). Both values present approximately the half of recommended amount of daily sodium intake that is 2000 mg daily, according to recommendation of World Health Organization (WHO, 2007). In accordance with the added potassium chloride, potassium content was the highest in the products from the third group (743 mg/100 g) and sodium content was the lowest (660 mg/100 g).

Table 3. Sodium and potassium content in 100 g of products, mg

	Group		
	I	**II**	**III**
Sodium	1156	940	660
Potassium	443	437	743

According to new guidelines issued by WHO (2007), adults should consume less than 2000 mg of sodium or 5 grams of salt, and at least 3510 mg of potassium per day. A person with either elevated sodium levels or low potassium levels could be at risk of raised blood pressure which increases the risk of heart disease and stroke. Most people in the western countries have a potassium-to-sodium ratio of less than 1:2. That means that modern man ingests twice much sodium as potassium. Some researchers recommend a dietary potassium-sodium ration greater than 5:1 to maintain optimal health. Common strategies for the sodium reduction in meat products are replacing parts of sodium chloride with other chloride salts as potassium chloride, calcium chloride and magnesium chloride, or non-chloride salts e.g. phosphates and lactate salts either separately or in combination (Ruusunen and Puolanne, 2005). Among the chloride salts, potassium chloride is the most commonly used alternative (Dötsch *et al.,* 2009). It contributes to some saltiness and textural characteristics but in most cases imparts off-flavour such as bitterness and metallic flavor (Doyle and Glass, 2010). Reduction of salt/sodium content in food presents a challenge in food industry in 21st century to improve human health and decreasing a risk of cardio-vascular diseases which are common cause of human death.

CONCLUSIONS

Products from all of three groups had acceptable taste, according to both assessors and consumers. Most of them had perception that cevapcici from the first group was too salty in the comparison with other two groups (p ≤ 0.05).
Products from the first group got the evaluation for overall impression that significantly less (p ≤ 0.01) than evaluation for products in the second group. Products from the second group were best evaluated and they were in overall impression statistically different from the third group of products (p ≤ 0.05).
Consumers recognized higher level of saltiness in the products from the first group, but not in the statistically significant level (p ≥ 0.05). Six of eight assessors ranked cevapcici with reduced sodium chloride content (second group) at the first place, while two assessors gave the first place to the products from the third group.
Using potassium chloride as a partial replacer for sodium chloride, it can be reduced sodium content in cevapciti from 1156 mg/100 g (first group) and 940 mg/100 g (second group) to 660 mg/100 g of products.

ACKNOWLEDGEMENTS

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ANTIOXIDANT POTENTIAL OF FERMENTED DAIRY PRODUCTS DURING STORAGE

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ABSTRACT

Development of new functional products is essential to satisfy increasing consumer's requires in term of health benefits in dairy industry. It has been found that several bioactive peptides sequences are encrypted in milk proteins which have the potential to provide health benefits to consumers. Among many different bioactive peptides, the antioxidant and antihypertensive peptides are the most widely studied. The final dairy products could be enriched by vast amount of these peptides using different microorganisms in milk fermentation. Proteolysis of milk proteins contributes to yoghurt flavor and quality, via peptides and free amino acids and indirectly through their precursors.

Therefore, the aim of this research was to compare the antioxidant potential: DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radicals scavenger activity and sensory characteristics of fermented dairy products obtained by yoghurt, probiotic and kombucha starter culture, during 21 days of storage.

The trend of changes in DPPH activity differs among the samples, but the samples produced by kombucha have the highest DPPH antioxidative potential after production and 21 days of storage. All analyzed samples showed significant scavenging ability against ABTS. After production, the highest activity was determined in the yoghurt (TEAC value 6.74 mmolmg^{-1}), followed by probiotic (6.34 mmolmg^{-1}) and kombucha (5.40 mmolmg^{-1}) products.

No significant differences were observed in appearance, flavour, taste, and overall preference scores between the three groups of fermented dairy products.

Keywords: kombucha, probiotic, yoghurt, antioxidative activity, sensory characteristics

INTRODUCTION

From the distant past, fermented dairy products have been consumed as valuable food sources in recommended daily intake.They have also been recognized as foods with health effect. Although there is no significant difference between the composition of fermented milk and unfermented milk, the various investigations show that these changes affect the nutritional value and bioavailability (Takano and Yamamoto, 2011). Quality of fermented dairy products is influenced by metabolic activities of the starter culture during the fermentation and storage period. Type and ratio of microorganisms in the starter culture contribute to different physico-chemical and sensory characteristics of fermented dairy products (Tamime *et al.,* 2007).The use of non-conventional, functional starter cultures could provide manufacture various products with desirable technological and nutritional advantages. Kombucha holds great opportunity as an innovative starter culture in dairy industry (Malbaša *et al.,* 2009; Iličić *et al.,* 2013; Hrnjez *et al.,* 2014a, Hrnjez *et al.,* 2014b,). As the result of milk fermentation by kombucha inoculums, products similar to yoghurt or kefir are produced (Milanović *et al.,* 2008; Milanović *et al.,* 2012; Iličić *et al.,* 2013). Up to now, kombucha has been considered as a mixture of yeasts (*Pichia, Zygosaccharomyces, Saccharomyces, Schizosaccharomyces, Saccharomycodes, Brettanomyces, Torulaspora*and *Candida*) and acetic acid bacteria (*Acetobacter* and *Gluconobacter*) which traditionally has been applied for the fermentation of sweetened black or green tea (*Camellia sinensis*) so far (Dufresne and Farnworth, 2000; Teoh *et al.*, 2004). Novel researches showed the significant presence of lactic acid bacteria (LAB) (Wu *et al.,* 2004; Zhang *et al.,* 2011; Marsh *et al.,*

2014). The *Lactobacilli* genera are more prevalent in kombucha than was previously understood, particularly at the later stages of fermentation (Marsh *et al.,* 2014).

Kombucha has been recognized as beneficial beverage in cases of: digestive aliments, kidney stones, gall bladder problems, diabetes, arteriosclerosis, high level cholesterol, high blood pressure, angina, gouty eczema, arthritis, rheumatism, atherosclerosis, irritability, anxiety, headaches, dizziness, fatigue, tiredness, in combating stress and cancer as well as vitalizes the physical body, etc. (Thummala *et al.,* 2013; Semantee *et al.,* 2013; Houda *et al.,* 2012; Guttapadu *et al.,* 2000;Jayabalan *et al.,* 2008). The ability of kombucha fermented milk product to scavenge different radicals: hydroxyl radical, superoxide anions and reducing power were previously attributed to the phenolics, flavonoids, vitamin C and vitamins B presented in the kombucha inoculums (Jayabalan *et al.,* 2008).

The aim of this research was to compare the antioxidant potential: DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radicals scavenger activity and sensory characteristics of fermented dairy products obtained by yoghurt, probiotic and kombucha starter culture, during 21 days of storage.

MATERIAL AND METHODS

Sample production
Fermented milk products were manufactured in laboratory conditions, from pasteurized and homogenized milk with 2.8% fat (Dairy »AD Mlekara« Subotica).The following starter cultures were used for milk fermentation:

1) Probiotic starter culture – ABT-7 – probiotic culture-Probio-Tek® contains LA-5®, *Lactobacillus acidophilus,* BB-12®, *Bifidobacterium*, *Streptococcus thermophilus*, (CHR Hansen, Denmark);
2) yoghurt starter culture – YF-L812 – contains *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus,* (Chr. Hansen, Denmark), and
3) kombucha inoculums–total number of viable cells was as follows: approximately 5×10^4 of yeast cells per cm^3 and approximately 2×10^5 of bacteria cells per cm^3 of the mentioned mixture (Loncar *et al.,*2013).

Kombucha was cultivated on a black tea (*Camellia sinensis*– oxidized, 1.5 g/L) with saccharose concentration of 70g/L. The tea was cooled at the room temperature, after which inoculums from a previous fermentation was added in concentration of 10%. Incubation was performed at 25±2 °C for 7 days. Kombucha inoculum in concentration of 10% (30 mL) was added for milk fermentation. Commercial starters were added according to manufacturer's specification – 0.005 g/100g. All samples were produced in triplets at 42°C. Fermentation was continued until pH=4.6 were reached. Then samples has cooled to 4ºC, stirred and packed in polypropylene glasses and stored in refrigerator at 4ºC.Depending on the used starter culture different samples were produced. The samples were labeled as K0, K7, K14, K21, P0, P7, P14, P21, Y0, Y7, Y14, Y21. Letters K, Y, P indicate the type of starter culture (K- kombucha, P-probiotics, Y-yoghurt) and numbers 0, 7, 14, 21 indicate the day of storage.

Preparation of water-soluble extracts (WSEs) of fermented dairy products
The WSEs were prepared according to Shori and Baba (2013). Each sample (10 g) was mixed with 2.5 mL distilled water and the pH was adjusted to 4.0 using 1 M HCl. The yoghurt was then incubated in water bath (45°C) for 10 min and the precipitated proteins were removed by centrifugation at 10 000 g for 10 min, 4°C. The supernatant was collected and the pH adjusted to 7.0 using NaOH (0.5 M), followed by another centrifugation (10 000 g, 10 min, 4°C). The supernatant was collected and refrigerated until further analysis.

ABTS^{+}radical cation decolourization assay (TEAC)
The radical scavenging activity was determined by the ABTS^{+} decolourization assay as described by Re *et al.* (1999). The bleaching rate of ABTS^{+} solution was monitored in the

presence of the prepared water-soluble extracts at 734 nm, using T80/T80+ UV-Vis spectrophotometer (PG instruments LTD). Briefly, 0.2 mL of sample (containing 0.5, 0.75, 1.5 and 1 mgmL^{-1} proteins) was added to 2 mL of diluted ABTS$^{+\cdot}$solution (A$_{734nm}$=0.7±0.02) and the absorbance reading was taken up to 10 min. Appropriate solvent blanks were run in each assay. In the same way, a standard Trolox curve was prepared with known Trolox concentrations. The percentage decrease in absorbance at 734 nm at 10 min was calculated and plotted as function of potential antioxidant or Trolox concentrations. The Trolox equivalent antioxidant coefficient (TEAC) was calculated by dividing the absorbance percentage inhibition versus antioxidant concentration slope by the Trolox plot slope and was expressed as mmol TEAC per mg of proteins in hydrolysates (mmolTEACmg^{-1}).

DPPH$^{\cdot}$ (2.2-Diphenyl-1-picrylhydrazyl radical) scavenging activity assay

The free radical scavenging activity (RSA) of protein extracts was evaluated using the DPPH$^{\cdot}$ scavenging activity assay as described by Morales and Jimenez-Perez (2001). An aliquot of sample (200 μL) was added to 1mL of a daily-prepared solution of DPPH$^{\cdot}$ (74 mgL^{-1}) in ethanol. The mixtures were shaken for 5 min at room temperature, centrifuged at 12x4g (Eppendorf Mini Spin plus) for 5 min and the absorption was measured at 520 nm (T80/T80+ UV-Vis Spectrophotometer PG instruments LTD). The concentration of DPPH$^{\cdot}$ in the reaction mixture was calculated from the calibration curve. The DPPH$^{\cdot}$ scavenging activity, expressed as antiradical activity (RSA) was calculated as follows (Eq. 1):

$$\% \ RSA = \left(\frac{[DPPH_b] - [DPPH_t]}{[DPPH_b]} \right) x100 \ (1)$$

where [DPPH$_b$] is the concentration of DPPH$^{\cdot}$ in blank (in the presence of buffer instead of protein extract) and [DPPH$_b$] is the concentration of DPPH$^{\cdot}$ in the sample (in the presence protein extract), after 10 min of reaction.

Sensory analysis

Sensory analysis of the samples was carried out by panelists selected from University staff members. The five points system and the descriptive method with assessing the scoring range from 1 to 5, was used. Sensory evaluations determined by the coefficient of importance for appearance – 1, colour – 2, flavour – 3, consistency – 4 and taste – 10. The single complex indicator which reflects the overall sensory quality expressed as % of the maximum quality was obtained by adding the single score. Dividing this value by the sum of coefficients of interest (20) a weighted average score is obtained, which expresses the overall quality of the product.

Statistics

All chemical analyses and assays were performed in triplicate for all produced samples (n) and values were expressed as average value. Univariate treatments of the data were performed by analysis of variance (ANOVA), using "Statistica9" software program. Differences among analysed parameters of produced samples with different starter cultures were considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Antioxidative activity of fermented milk products

The radical scavenger activity (RSA) of the water-soluble extracts of the fermented milk products was evaluated by the ABTS$^{+\cdot}$ radical cation and DPPH radical assays. Both these assays indicate the ability of substances to act as electron donors or H atom donors in free radical reactions.

At the end of the milk fermentation, the ABTS scavenger activity in the products varied from 6.73 to 5.23 TEAC mmolmg^{-1} in Y and K products, respectively. During the first week of storage all samples showed slight increase of ABTS RSA but significant reduce of the activity was observed in a second week. It was significantly lower compared to the first day.

Even more all samples had slight increase of ABTS activity during last week of storage compared to the second and the highest TEAC value (8.92 mmolmg^{-1}) was observed in the sample Y21 (Fig.1a).

The DPPH RSA in K sample (Fig. 1b) was significantly higher ($p > 0.05$) after production (17.88±0.17%) compared to Y and P samples (9.46±0.3% and 13.23±0.04% respectively). The trends of changes in DPPH activity were different among the samples, but as well as the ABTS RSA, it was reduced during the second and again raised during the last week of storage. Kombucha starter used for milk fermentation improved DPPH RSA during refrigerated period due to its own antioxidative capacity which was shown by others authors (Jayabalan *et al.,* 2008; Vitas *et al.,* 2013). The kombucha samples showed the highest DPPH RSA ($p < 0.05$) after 21 days of storage (8.24±0.40%) compared to Y21 and P21 samples (6.72±0.21% and 7.05±0.25%, respectively).

The difference between the DPPH and ABTS RSA could be due to previously reported disadvantage of DPPH radicals which could be dissolved only in organic media. If it is used for hydrophilic antioxidants, such as protein hydrolysates, it may lead to precipitation of proteins whose activity is analysed (Chang *et al.,* 2007; Tang *et al.,* 2010). In contrary, ABTS could be solubilized in both aqueous and organic media; thus, radical scavenging activities of hydrophilic and lipophilic compounds can be measured (Tang *et al.,* 2010). Our results also suggest that the ABTS method was more appropriate than the DPPH assay for the measurement of antioxidant activity of fermented milk.
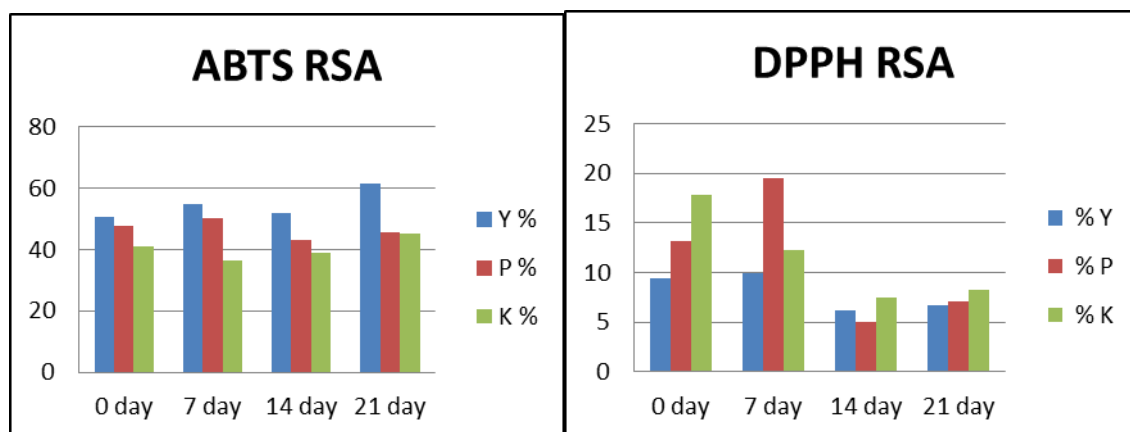
Figure 1. The ABTS and DPPH scavenger activity of fermented dairy products during storage

Sensory analysis

The sensory evaluations of fermented dairy products during 21 day of storage are shown in the Fig. 2. No significant differences were observed in appearance, flavour, taste, and overall preference scores among the three groups of fermented dairy products during this period. Kombucha fermented dairy product had a characteristic, distinctive mild sour, refreshing taste and conspicuous aroma. The consistency and taste score was greater in Y0 and P0 samples than in the K0 sample. However, prolonged storage period up to 14 days showed that kombucha inoculums affected the consistency and flavour which were reduced in Y and P samples, but increased in K sample. During the third week of storage, significant reduction in overall quality of all produced samples were observed and all type of products were not safe for consumption.
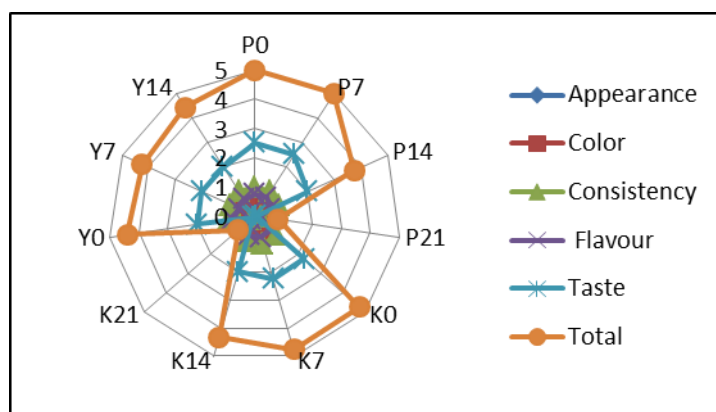
Figure 2. Sensory caracteristics

CONCLUSIONS

Fermented dairy product obtained by komucha inoculum may be a convenient new product which satisfies consumer`s interest in the functional food. In all samples, higher ABTS than DPPH radical scavenging activity was established, while both slightly decreased during the second and again increased during the third week of storage. During the storage, the milk product fermented by kombucha (K) had similar trend of changes in sensory properties as products with probiotic (P) and yoghurt (Y) starters. Based on these results, the kombucha fermented dairy product had distinctive sensory and pronounced antioxidative activity characteristics as conventional yoghurt starter cultures and thus could have promising application in dairy industry.

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SOCIO-DEMOGRAPHIC AND LIFESTYLE CHARACTERISTICS AND ATTITUDES OF CONSUMERS IN VOJVODINA TO FUNCTIONAL FOOD

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ABSTRACT

Functional food differs from conventional food in several ways. Within functional food, special components are directly connected with well-defined physiological effects and health benefits associated with a certain product. Known as a food for specific healthy consumption, this food includes functional ingredients which influence structure or function of the body. A number of factors influence consumer attitudes toward foods and reshape food supply trends. These include age of the population, consumers 'desire to enhance personal health, change in consumer awareness and expectations, advancing scientific evidence that diet can alter disease prevalence and progression, advances in food science and technology and changes in food regulations, etc. In order to determine consumers' socio-demographic and lifestyle characteristics as well as their attitudes towards buying functional food in Vojvodina, the research was conducted in cities such as Novi Sad, Subotica, Zrenjanin, Vršac, Ruma and Inđija. A survey involved 400 consumers who filled in questionnaires mainlyin supermarkets offering functional food. The questionnaire included 3 groups of questions. The first group of questions focused on socio-demographic characteristics of respondents; the second group was designed to gather information about consumers' life style (health condition, type of health care, and type of nutrition), while the third group included questions about functional food, consumers' attitudes towards it, their awareness, needs for buying, etc. The survey results were analysed by program package software SPSS 19 using non-parametric tests and presented by certain descriptive statistics. Mann-Whitney U test and Kruskall-Wallis test showed that there were no significant differences in attitudes toward functional foods between different socio-demographic groups. Although 81% of the consumers claimed that they had no previous knowledge of the functional food terminology, after clarification of the terms used, 58% of respondents realised that they had already consumed functional food products before. Considering the aforementioned, the lack of knowledge about functional food implies that it is very important to create effective marketing campaigns for such products.

Keywords: *functional food, consumers' attitudes, Vojvodina*

INTRODUCTION

During the last few decades the interest and demand for both healthy food and different beverages has increased and it is expected to continue increasing in the future. Raising people's awareness about taking care of their health and development of the food industry, medicine, and those branches of science that deal with the relationship between nutrition and health has led to the popularization of the foods for which special health effects are proven. Moreover, functional foods and/or supplements may be used in the context of a healthy lifestyle or as a means to compensate for an unhealthy lifestyle. Thus the recent industry of functional foods has become significantly popular as marketers aim to sell food products that can benefit a consumer's health beyond a healthy of organic food products ability (Somehagen*et et al.*, 2013).

A number of factors influence consumer attitudes toward foods and reshape food supply trends. These include age of the population, consumers 'desire to enhance personal health, change in consumer awareness and expectations, advancing scientific evidence that diet can alter disease prevalence and progression, advances in food science and technology and changes in food regulations, etc. In order to make adequate marketing actions it is essential to understand behaviour of consumers as well as their attitudes towards functional food.

There are many researches concerning consumers' attitudes toward functional food such as Jong *et al.,* (2003), Ares and Gámbaro (2007), Annunzaiata and Vecchio (2010), Annunzaiata and Vecchio (2011), Vukelić *et al.,* (2012),Vukelić *et al.,* (2013), Somehagen *et al.,* (2013) etc.

Considering the aforementioned, the aim of this research is to analyse socio-demographic and lifestyle characteristics as well as their attitudes towards buying functional food products and give suggestions how to enhance competitiveness of functional food production in Vojvodina and in Serbia.

MATERIAL AND METHODS

In order to determine consumers' socio-demographic and lifestyle characteristics as well as their attitudes towards buying functional food in Vojvodina, the research was conducted in cities such as Novi Sad, Subotica, Zrenjanin, Vršac, Ruma and Inđija. A survey involved 400 consumers who filled in questionnaires mainly in supermarkets offering functional food. The questionnaire included 3 groups of questions. The first group focused on socio-demographic characteristics of respondents; the second group was designed to gather information about consumers' life style (health condition, type of health care, and type of nutrition), while the third group included questions about functional food, consumers' attitudes towards it, their awareness, needs for buying, etc. The survey results were analysed by program package software SPSS 19 using non-parametric tests (Mann-Whitney U test and Kruskall-Wallis test) and presented by certain descriptive statistics.

RESULTS AND DISCUSSION

Functional food differs from conventional food in several ways. Within functional food, special components are directly connected with well-defined physiological effects and health benefits associated with a certain product. Known as a food for specific healthy consumption, this food includes functional ingredients which influence structure or function of the body. Foddai *et al.* (2012) define functional food as "the synergy created when health and diet is combined together". That is relatively new concept which has emerged as a result of the recent increasing focus on – and awareness of – the influence of diet on overall health (Poulsen, 1999). There are numerous scientific evidences supporting the fact that the nutrition enriched with certain groceries is directly connected with reduced risk of chronic, non-infectious diseases. According to these acknowledgements, the concept of functional food was developed whose market has grown considerably and was worth in 2011, 24 billion dollars worldwide and it is expected to continue increasing in the range of 8 – 14% annually (Somehagen *et al.*, 2013). In order to increase the chances of success in this market, a food firm cannot afford not to broaden its knowledge on functional food consumer's perception and social motivations under which the consumer behaves (Urala and Lahteenmaki, 2003).

Considering the aforementioned, to make adequate marketing actions and to improve competitiveness of functional food production in Vojvodina and in Serbia, it is very important to analyse socio-demographic and lifestyle characteristics of consumers and to understand their behaviour as well as their attitudes towards functional foods.

In table 1 demographic and lifestyle characteristics of the study participants are presented. Analysis of the main socio-demographic variables shows that respondents are predominantly women, more living in urban area and the majority was between 26 and 45 years old. About 57% have reported university degree education and 39% finished secondary school. Also, 45% of respondents have net monthly income of the household between 30000 and 75000 RSD and 36% between 75000 and 150000 RSD. Similar results when it comes to gender, age and level of education were obtained by Annunyiata and Vecchio (2011). When it comes to lifestyle characteristics, majority of respondents claim that they are healthy and satisfy (54%), 33% have minor health problems and only 13% claim that have serious health

problems. According to the result obtain, it can be concluded that people in Vojvodina believe that their nutrition is healthy (48%) and common (46%).

Table 1.Socio-demographic and lifestyle characteristics of the study participants

Socio-demographic and lifestyle characteristics		Percents
Gender	male	39%
	female	61%
Region of living	urban	60%
	rural	40%
Age, years	18 – 25	8%
	26 – 35	35%
	36 – 45	22%
	46 – 55	17%
	more than 55	18%
Level of education	Primary school	4%
	Secondary school	39%
	University degree	57%
Net monthly income of the household in RDS	Up to 15000	1%
	15000 – 30000	14%
	30000 – 75000	45%
	75000 – 150000	36%
	More than 150000	4%
Participants health	Healthy and satisfied	54%
	Have minor health problems	33%
	Have serious health problems	13%
Type of diet (nutrition)	Healthy	48%
	Common	46%
	Vegetarian	3%
	Dietary	3%
Way of taking care about health	Do not take care	31%
	Trying to have healthy nutrition	38%
	Exercising regularly	13%
	Eating healthy and regularly exercising	18%

Source: Research results

In order to analyse consumers' attitude toward functional food products, respondents were asked about their knowledge of the concept functional food and about consumption of functional food. In Table 2 obtained results regarding socio-demographic characteristics of respondents are presented.

According to the presented results in table 2, it can be concluded that only 19% of respondents answered to have the knowledge of the concept functional food. Most of them were women from the urban area, in the age 26-35, with higher and high education and monthly income between 30000 – 75000 RSD. Similar results were obtained by Menrad (2006), who implies that 20.7% of respondents in Germany heard about concept functional food. The percentage for other countries is following: 19, 1% in Poland, 33% in Spain, 10, 7% in UK. However, after clarification of the terms used, 58% respondents confirmed that they have consumed functional food products and most of them (89%) claimed that they have tried yogurt with added probiotic as a functional food product. Also, those who have used functional food (58%) were asked to explain why they decided to buy it, regarding their health condition, the way how they take care about their health and their nutrition regime (table 3).

Presented results in table 3 show that most of respondents (64%) believed that functional food products could have a positive influence on human health. Consumers who stated that

were mostly satisfied with their health condition or had minor health problems. 24% of respondents bought functional food because they found it more tasteful, 6% of respondents out of curiosity, while 6% of respondents, of which 2% had minor health problems and 4% had serious health problems, bought this kind of products since doctor or nutritionist had recommended it. Menedar (2006) obtained similar results. He concluded that consumers in Germany, Poland, Spain and UK stated that "very important" and "important" factors for buying functional food products were "to stay healthy" and "to do myself good". Factors for buying functional food products, "recommended by medical doctor or nutritional consultant" were noted as "less important" in Germany, while as "neutral" in Poland, UK and Spain.

Table 2.Knowledge of the concept and consumption of functional food products regarding socio-demographic characteristics of respondents

SOCIO-DEMOGRAPHIC CHARACTERISTICS OF RESPONDENTS	Knowledge of the concept functional food		Consumption of functional food products	
	Yes (%)	No (%)	Yes (%)	No (%)
Gender				
Male	8	31	22	17
Female	11	50	36	25
Region				
Urban	11	49	32	28
Rural	8	32	26	14
Age				
18-25 years	0	8	5	3
26-35 years	13	22	18	17
36-45 years	4	18	16	6
46-55 years	2	15	10	7
More than 55 years	4	14	9	9
Level of education				
Primary school	0	4	0	4
Secondary school	4	35	25	14
University degree	15	42	33	24
Net monthly income of the household in RDS	0	1	1	0
Up to 15000	2	12	5	9
15000 – 30000	9	36	26	19
30000 – 75000	6	30	22	14
75000 – 150000	2	2	4	0
More than 150000				
Total	19	81	58	42

Source: Research results

Using non-parametric tests, Mann-Whitney U test and Kruskall-Wallis test (chi-square tests) showed that there were no significant differences in attitudes toward functional foods between different socio-demographic groups. All null hypotheses were defined as that there were no differences in attitudes toward functional foods between different socio – demographic groups (such as between gender, between region of living, between different age group, between different level of education and different net monthly income of the household) and were accepted as $p > 0.05$.

Gained results are consistent with studies of Verbeke (2006) and Annunyiata and Vecchio (2011) where gender and age where not significantly associated with the knowledge of functional food and the reason and the frequencies of functional food consumption. However, level of education as well as existence of an ill family member are variable that were

significantly associated with knowledge about functional food products (p<0.05) and consumption frequency (p<0.01).

Table 3.Motivation to buy functional food regarding health condition, way of nutrition and treatment to health

HEALTH CONDITION AND NUTRITION OF RESPONDENTS	Motivation to buy functional food			
	Favourable influence on health (%)	Better taste (%)	Recommended by doctor or nutritionist (%)	Curiosity (%)
Description of respondents' health condition **Healthy and satisfied** **Minor health problems** **Serious health problems**	23 29 12	6 10 8	0 2 4	5 1 0
Type of diet (nutrition) **Healthy** **Common** **Vegetarian** **Dietary**	38 20 3 3	11 13 0 0	1 0 0 5	3 3 0 0
Way of taking care about health **Do not take care** **Trying to have healthy nutrition** **Exercising regularly** **Eating healthy and regularly exercising**	14 22 8 20	4 16 4 0	0 4 0 2	2 1 1 2
Total	64	24	6	6

Source: Research results

CONCLUSIONS

Consumers are getting more aware of the importance of healthy nutrition and its influence on their health. Therefore, functional foods are increasingly becoming popular among consumers. The results obtained in this research implied that respondents are confused of what functional food products are. Although 81% of the consumers claimed that they had no previous knowledge of the functional food terminology, after clarification of the terms used, 58% of respondents realised that they have already consumed functional food products before. Majority of respondents (64%) believe that functional food products can have a positive influence on human health and they are mostly satisfied with their health condition or have minor health problems. 24% of respondents buy functional food because they find it more tasteful, 6% of respondents buy functional food products out of curiosity, while 6% of respondents, of which 2% have minor health problems and 4% have serious health problems, buy this kind of food since doctor or nutritionist have recommended it.

Considering the aforementioned, the lack of knowledge about functional food implies that it is very important to create effective marketing campaigns for such products, make them more visible and recognizable to consumers, inform consumers about their benefits in regard to conventional food. Furthermore, it is necessary to define market of functional food products in Vojvodina and Serbia, adopt certain regulations which can be helpful for increasing of consumers' confidence about functional food and make larger consumption of this kind of products where the role of labelling should be strengthened. All mention above, can contribute to better health conditions of the nation.

ACKNOWLEDGEMENTS

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CHARACTERIZATION AND POSSIBILITY OF APPLICATION OF SOME POLYMER PACKAGING MATERIALS FOR PACKAGING FERMENTED DAIRY BEVERAGES

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ABSTRACT

Packaging materials and packaging conditions are very important factors with great influence on the quality of dairy product during it's shelf life. Polymers have very vast application in food industry regarding their's chemical inertness. Qualitative characteristics of packaging materials: tensile strenght, elongation at break and uniform thickness are very important for adequate forming pakaging units during production, manipulation and transport. Barrier characteristics – permeability of gasses, vapour and lights, have direct influence on quality of the beverage during the storage.

The aim of this work is to analyse physico-mechanical, barrier and structural properties of choosen polymer packaging materials such as polyethylen (PE), polyamid-polyethylen (PA/PE) and polyprophylen (PP) for packaging fermented dairy beverages. Also, it was investigated are those materials suitable for application in modified atmosphere packaging's (MAP) technique.

On the basis of the results that were obtained it can be concluded that polyamid-polyethylen coextruded foil can be used in MAP conditions thanks to vary good barrier properties. Polyprophylen has better phisico-mechanical properties then the other two materials so it could be used in different manipulative operations as a strong protection. Polyethylen has a high value of tensile strenght and according to it's properties it is best to combine this material with the other polymer in order to obtain the material properties of advanced indications of primary one.

Keywords: packaging materials, polymers, fermented dairy beverages

INTRODUCTION

Packaging materlas and packaging conditions are very important factors which influence the quality of the product during it's shelf life.

Materials which are in direct contact with product have to meet the prescribed requirements for safe food: that their components do not migrate in the content, or migrate within acceptable limits, and do not enter into reactions with the product.

The basic requirement of application packaging materials for packaging food products is chemical and health correctness. The phenomena of migration of packaging material's constituents into the food exists, but migration must be within the limits of legislation (Arvanitoyannis and Bosnea, 2004; Lazić and Novaković, 2010). Due to its inertness, polymeric packaging materials are now widely implemented for packaging food products.

The qualitative properties of the packaging material such as uniformity of thickness, tensile strength and elongation at break are important for the successful formation of the packaging during the manipulation and storage (Pejić *et al.,* 2012). Packaging materials must have good physical and mechanical properties – resistance on pressure, shock, drop, friction, temperature treatment in a variety of procedural and manipulative operations, which may be achieved by a combination of primary, secondary and tertiary packaging.

Barrier properties: permeability to gases, water vapour, aromatic substances and light, have a direct impact on the quality and shelf-life of packaged content (Steinka *et al.*, 2006; Pajin *et al.*, 2006). Barrier properties of packaging material with phenomena of different permeability affecting the preservation, qualitative properties and viability of packaged products. By studying various properties of these polymers, both synthetic, and biopolymers obtained by modern technology, packaging for specific food products can be chosen. However, the

choice of packaging materials for a specific food product depends on many other factors, primarily the product attributes, applied technological processes and existing equipment, economic aspects, and lately pointed out the necessity of analyzing the environmental aspects (Lazić and Novaković, 2010).

Good performance, practicality of implementation and lower cost per packaging unit resulted in rapidly penetrating of polymer packaging materials in all the important activities of modern life, including technological processes of production and packaging of food and beverages (Lazić and Novaković, 2010; Ahvenainen, 2003).

Nowdays, one of the contemporary ways of packaging is in modified atmosphere, where changes the composition of the gas mixture (headspace) in packaging over product directly depend on the permeability of packaging materials, sealing formed packaging and biochemistry changes in the product during the storage (Pejić, 2013). Changes in the gas mixture above the packed content, if the package is hermetically formed, in this case are directly influenced to permeability materials to gases, as well as the biochemical processes that had occurred during the storage (Lazić and Novaković, 2010; Pejić, 2013). Polymeric materials are more or less permeable to gases and within adequate physical and mechanical characteristics they can be used for packaging of fermented dairy products (Tamime and Robinson, 2004).

The aim of this work is analyzing selected polymeric packaging materials by investigating their physico-mechanical, barrier and structural properties, in order to examine their application for packaging fermented dairy beverages. Also, it is examined whether choosen materials are siutable for application for packaging fermented dairy beverages in MAP.

MATERIAL AND METHODS

Selected packaging materials are:
• polyethylene (PE) film 70 µm,
• coextruded polyamide / polyethylene (PA30/PE70) film of 100 µm,
  and packaging:
• polypropylene (PP) cup 0.18 L.

Physico-chemical and barrier characteristics of the packaging materials
Surface mass was determined by the method described in the standard SRPS G.S2.702.
Film thickness was determined by the method described in the standard SRPS G.S2.733.
Tensile strength and elongation at break were determined by the method described in SRPS G.S2.734 standards. Testing was conducted on an INSTRON 4301 device. Examined test-tube had dimensions 15 x 80 mm. The distance between the clamps was 50 mm, and the speed 100 mm/min. Measurements were was repeated 5 times for each film (A0, A1, A2, A3 and A4).
Permeability to gases CO_2, O_2 and N_2 was measured by the Lessee method, according to DIN 53 380 on the device Lyssy GPM-200, using a GASUKURO Kogyo GC-320 gas chromatograph and HP 3396 integrator, and air permeability was calculated by computer.
Determination of the structural characteristics was done by infrared spectroscopy (FTIR) to SRPS GS511-2011 on IR spectrophotometer in NICOLET iS 10 Thermo Scientific (Massachusetts, USA) in addition to the attenuation of total reflection (ATR).

RESULTS AND DISCUSSION

Physical-mechanical and barrier properties of packaging materials
Physical and mechanical properties of packaging materials were analysed by determining: thickness, surface mass, tensile strength and elongation at break, and the barrier properties were investigated by determining the permeability of gases and air.
The results shown in Table 1 confirm the declared compositions of polymer materials and the surface deviations and the thickness of the masses are in the range of standard values.

Table 1. Characteristics of the packaging materials

Samples / characterisctics		PE	PA/PE		PP
Thickness (μm)		68.95±1.69	100±0.89		$(1.54±0.005)10^{3}$*
Surface mass (g/m^2)		69.79±1.32	PA	29.12±0.52	1412.81±39.81*
			PE	67.59±1.01	
			Total	96.71±0.74	
Tensile strenght (N/15mm)	Longitudinal	19.42±1.54	44.0±3.92		-
	Cross-sectional	16.88±1.22	39.8±0.59		108.21±11.17**
Elongation at break (%)	Longitudinal	449.72±50.38	429.0±18.99		-
	Cross-sectional	718.5±17.13	481.2±9.62		44.86±1.64**
Gas permeability (ml/m^2/24h, 1bar)	CO_2	6214.2±2.45	148.4±8.02		31.2±0.44*
	O_2	2330.1±4.81	23.5±2.44		31.4±2.84*
	N_2	663.2±1.21	3.7±0.92		25.6±1.75*
	Air	1017.1±2.37	7.9±1.51		26.9±3.66*

* the data relating to coextruded foil
**the data relating to cup

Mechanical properties - tensile strength and elongation at break are important because they show the benefits of a material suitable for the application, behavior during filling and closing as well as the resistance during transport, handling and storage. Based on the results showed in the Table 1 it can be concluded that PP has a higher mechanical properties than PE and PA/PE (value of tensile strength cross-sectional) and that PE has the highest values of elongation at break (%), which is in accordance with a literature data (Kirwan and Strawbridge, 2003). Results of the mechanical properties' determination showed very good physical and mechanical properties of coextrusion polyamide/polyethylene and polypropylene.

On the bases of the results obtained by investigation of the permeability of gases it can be concluded that analysed samples are of various properties. For polyethylene values of permeability for CO_2 and O_2 are high: 6214.2 ml/m^2 24 h, 1 bar and 2330.1 ml/m^2 24 h, 1 bar, respectively. PA/PE foil has multiple lower permeability values of all three gases and air and it could be used for packaging with MAP application. The lowest permeability to CO_2 had PP tape which is expected since the thickness of the sample. The values obtained are consistent with the nature of investigated materials and agree with published statements (Kirwan and Strawbridge, 2003; Lazić, 1994).

Structural characteristic of packaging materials

Figures 1 to 5 show the IR spectrum recorded in the ATR technique for polymeric material declared as PE foil, PA/PE coextruded foil and PP cups, respectively.
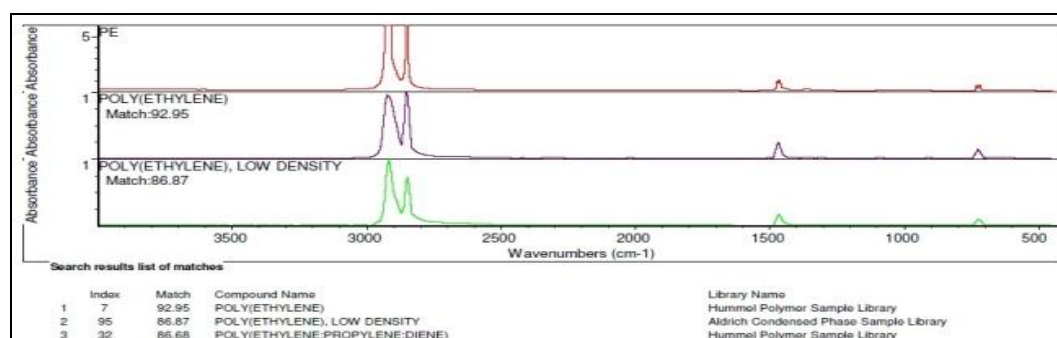
Figure 1. IR spectrum in ATR technique PE foil sample
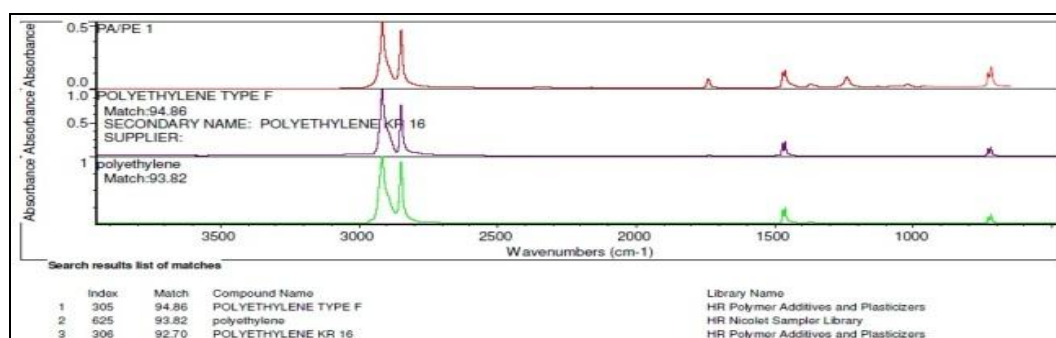
Figure 2. IR spectrum in ATR technique PA/PE sample, PE – side
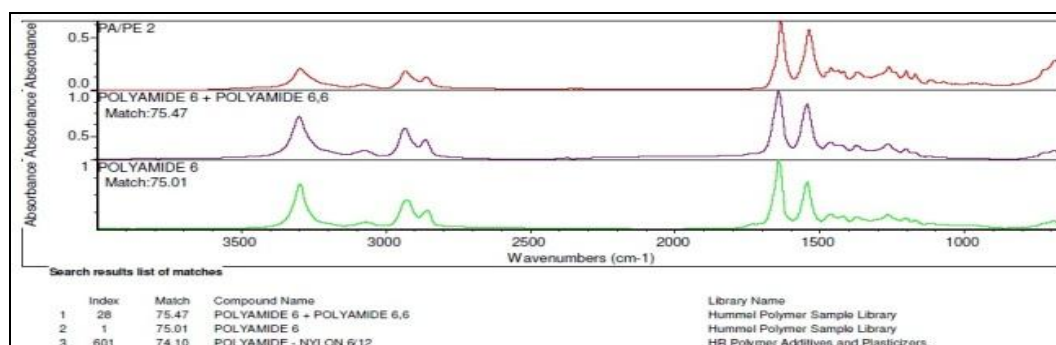
Figure 3. IR spectrum in ATR technique PA/PE sample, PA – side
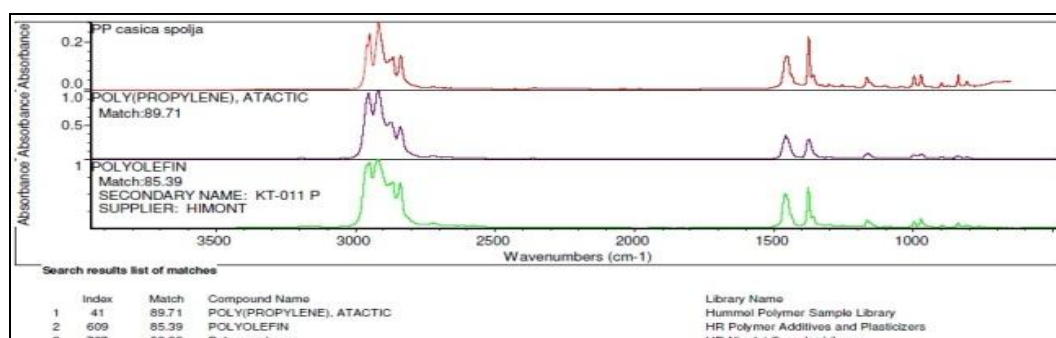
Figure 4. IR spectrum in ATR technique PP cups, external side
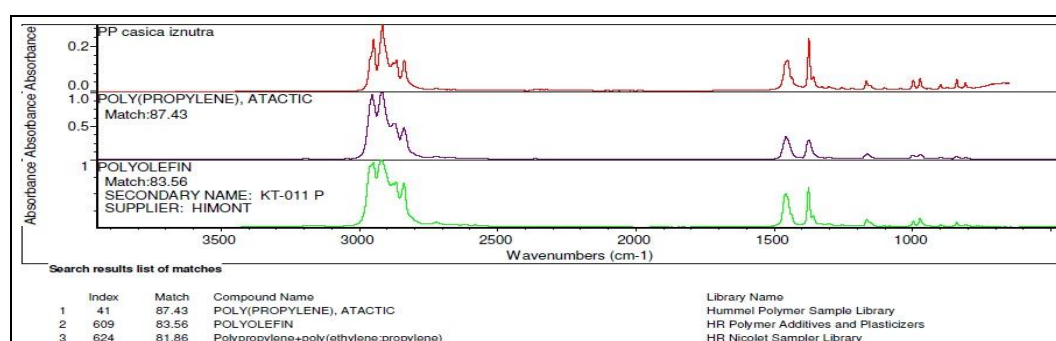
Figure 5. IR spectrum in ATR technique PP cups, internal side

Based on the results recorded IR spectrum of packaging materials, it can be concluded that the composition of the investigated materials are appropriate.

CONCLUSIONS

On the basis of the obtained results it can be concluded that the coextruded polyethylene-polyamide foil can be applied for the packaging of fermented dairy products in terms of modified atmosphere thanks to very good barrier properties. In view of the high value of elongation at break in the transverse and longitudinal directions, the polyethylene may find its application in a combination with other polymers for the packaging of fermented dairy products, applied alone, however, does not provide adequate protection to the product of the various environmental factors. Polypropylene has the best mechanical properties in comparison with the other two packaging materials, and as such can be used as a good protection of fermented dairy products in the various handling operations.

ACKNOWLEDGEMENTS

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MALONDIALDEHYDE AS THE MARKER OF LIPID OXIDATION OF RICE-BUCKWHEAT GLUTEN-FREE COOKIES DURING STORAGE

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ABSTRACT

Rice-buckwheat gluten-free cookies (20% RF/LBF) were produced using light buckwheat flour (LBF) and rice flour (RF) (20:80) in gluten-free cookie formulation. Although cookies are known for their long shelf-life, 20% RF/LB possessed a high amount of vegetable fat (28% on flour weight basis) which made them susceptible to oxidative changes. The progress of lipid oxidation of 20% RF/LB was monitored by measuring malondialdehyde (MDA) content in unpacked and packed (polypropylene bags) cookies kept at elevated temperature ($40 \pm 1°C$) during 9 months.
The obtained results indicated that MDA content can be used as the marker of lipid oxidation of the gluten-free cookies kept at elevated temperature ($40 \pm 1°C$) during storage, i.e. MDA content can be used for predicting their shelf-life. Monitoring of MDA revealed that the unpacked cookies kept at elevated temperature were acceptable until the 3. month of storage while the packed cookies underwent the oxidative changes at the end of the 4. month.
Keywords: gluten-free cookies, lipid oxidation, MDA, shelf-life

INTRODUCTION

Due to a permanent intolerance to gluten proteins of many common cereals, celiac patients must be on a strict long-life gluten-free diet, which usually lacks in certain essential nutrients (Thompson *et al.*, 2005). Therefore, one of the main goals in creation of gluten-free products is their fortification to achieve a balanced diet, i.e. to obtain added value products. Many attempts were done referring sweet bakery products, such as cookies (Torbica, Hadnađev, Dapčević-Hadnađev, 2012), muffins (Matos, Sanz, Rosell, 2013) and buscuits (Schoenlechner *et al.*, 2006).
Cookies are very popular because they are ready-to-eat food which is easy to store and handle. Although they are known for their long shelf-life, they possess a high amount of vegetable fat (20-30% on flour weight basis) which makes them susceptible to oxidative changes (Zieliński *et al.*, 2012).
Lipid oxidation, as one of the most remarkable changes in foods, leads to the development of rancidity followed by unpleasant odours and flavours which contribute to unacceptability of the products.
The progress of lipid oxidation can be followed by measuring the content of marker compounds, among which some are volatile compounds, such as aldehydes. These secondary lipid oxidation products are generated from a wide range of hydroperoxides formed during the initiation stage of the reaction (Laguerre, Lecomte, Villeneuve, 2007) and strongly contribute to the aroma at trace amounts (Sun *et al.*, 2010).
Althought hexanal is usually measured as the marker for lipid oxidation of cookies (Pastorelli *et al.*, 2007; Berenzon, Saguy, 1998), their oxidative deterioration was also monitored by measuring 2,4-decadienal and 2,4-heptadienal in shortcake biscuits (Yang *et al.*, 2013).
One of the possibilities to follow the lipid oxidation is using malondialdehyde (MDA) as the marker for lipid deterioration. Malondialdehyde is known to be produced from the decomposition of hydroperoxides derived from the oxidation of both ω-3 and ω-6 polyunsaturated fatty acids and represents an indicator for the early oxidation appearance (Frankel, 2005). The most frequent analytical approach is to measure thiobarbituric acid

(TBA) value which corresponds with the content of MDA-TBA complex formed between malondialdehyde and TBA. This method lacks specificity as other carbonyl compounds present or formed in the food during the process may also react with TBA (Shahidi, Zhong, 2005; Ameur, Trystram, Birlouez-Aragon, 2006). Therefore, HPLC techniques are more advisable for measuring MDA as more accurate results are obtained by their application.

In our previous work, gluten-free cookies based on rice-light buckwheat flour mixture (RF:LBF – 80:20) (20% RF/LBF) with enhanced mineral content and antioxidant capacity were created and assessed as ones with the most acceptable sensory properties compared to control cookies (rice cookies) and others based on rice-light buckwheat flour mixture (RF:LBF - 90:10 and 70:30). Having in mind the mentioned cookie susceptibility to lipid oxidation, the objective of this work was to evaluate the end-point of shelf-life of the unpacked and packed (polypropylene bags) rice-buckwheat gluten-free cookies (20% RF/LBF) kept at elevated (40 ± 1°C) temperature during storage measuring MDA content using modified HPLC method described by Karatas, Karatepe and Baysar (2002).

MATERIAL AND METHODS

Materials
Rice flour – RF (moisture 11.67%, protein (N×5.7), 7.96%, fat 0.27%, ash 0.25%, reducing sugars 1.37%, and starch 88.58%) and light buckwheat flour – LBF (moisture 11.24%, protein (N×5.7) 8.68%, fat 1.47, ash 1.08%, reducing sugars 1.77%, and starch 85.38%) were obtained from Hemija Komerc, Novi Sad, Serbia. Vegetable fat A.P 34-36 originated from refined palm and sunflower oil was obtained from Puratos, Belgium. Sodium hydrogen carbonate – $NaHCO_3$ (≥ 99.5%, p.a) was purchased from Carl Roth, Germany, carboxymethyl cellulose sodium salt – CMC from Alfa Aesar, Germany, diacetyl tartaric acid esters of distilled monoglycerides – DATEM (Pantex DW90) from InCoPa, Germany, while the other ingredients (salt, sugar and honey) were purchased at a local market.

Preparation of cookies
The formulation of rice-buckwheat gluten-free cookies (20% RF/LBF) was made according to Torbica, Hadnađev and Dapčević-Hadnađev (2012). Mixture of RF and LBF was prepared using 80:20 proportions. Dough mixing, processing and baking were performed on a laboratory-scale equipment as described by the mentioned authors (2012). The ingredients were weighed as follows: flour (270.0 g of RF + 60.0 g of LBF), deionized water 75.0 g, vegetable fat 85.0 g, granulated sugar 70.0 g, honey 45.0 g, $NaHCO_3$ 9.0 g, DATEM 9.0 g, CMC 4.5 g, and salt 2.1 g.

Packaging and storage of cookies
The rice-buckwheat gluten-free cookies were packed into polypropylene bags. Every cookie was packed separately. The packed cookies were investigated in comparison with those in the bulk form (unpacked cookies).

Both packed and unpacked cookies were stored in a dark place at elevated temperature (40 ± 1°C) during 9 months. The cookies were analysed monthly.

Malondialdehyde (MDA) analysis
Preparation of MDA standards
Stock solution was prepared by mixing of MDA with 1 mL hydrolyzed acetal (10 µL of 1,1,3,3-tetraethoxypropane (TEP) was diluted to 10 mL with 0.1 M HCl in a screw-capped test tube, placed in a boiling water bath for 5 min and rapidly cooled under tap water producing a hydrolyzed acetal) in 100 mL flask followed by diluting with water to achieve 2.92 g MDA/mL. Standard solutions were prepared daily by diluting the stock solution with water to obtain concentration ratio of 0.08–3.33 nmol/mL for preparing the calibration curve.

Sample preparation

The sample preparation was carried out following the procedure described by Tsaknis *et al.* (1998). Two grams of cookie powder were measured into a 250 mL boiling-flask, 90 mL of water was added and pH was adjusted to 1.5–1.8 with 2 M of HCl. The flask was connected to a standard micro-Kjeldahl unit and the content was distilled. The distillation was conducted as rapidly as possible and terminated when 250 mL of distillate had been collected in a 250 mL calibrated flask and ready to use for further HPLC-DAD analysis.

HPLC-DAD analysis

The chromatographic separation and quantification of MDA was performed using the HPLC method described by Karatas, Karatepe and Baysar (2002) with some modifications.

Liquid chromatograph (Agilent 1200 series), equipped with a DAD detector and an Eclipse XDB-C18, 1.8 µm, 4.6 × 50 mm column (Agilent) was used for quantification of malondialdehyde in obtained distillates. Separation of the analyte was achieved with a column temperature of 30 °C and sample injection volume of 20 µL. The mobile phase consisted of two eluents, 30 mM KH_2PO_4 (A) and methanol (B), delivered at a flow rate of 0.6 mL/min. The isocratic elution was employed with the ratio A:B (90:10, v/v). The DAD wavelength was set at 260 nm. The total run time of the analysis was 8 min.

RESULTS AND DISCUSSION

Malondialdehyde is a reactive aldehyde found in foods and it is formed due to the decomposition of both ω-3 and ω-6 fatty acid hydroperoxides during processing and storage of foods (Frankel, 2005; Belitz, Grosh, Schieberle, 2009; Shahidi, Hong, 1991). Also, it is used as a marker of lipid oxidation and consequently can be employed for predicting the end-point of shelf-life of some fat-containing foods. Therefore, the unpacked and packed (polypropylene bags) rice-buckwheat gluten-free cookies (20% RF/LBF), which are characterized by high amount of fat (approximately 20%), kept at elevated temperature (40 ± 1°C) were monitored to assess lipid oxidation during 9-month storage period measuring their MDA content. The determined MDA content in the examined stored cookies were shown in Figure 1.
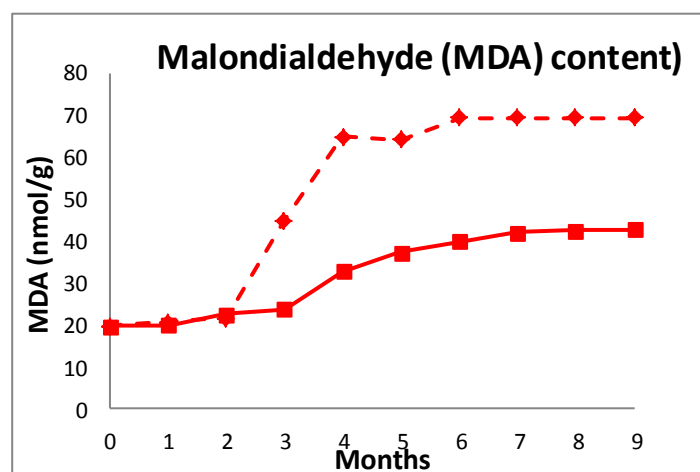
Figure 1. *Changes in malondialdehyde (MDA) content in packed (-♦-) and unpacked (-■-) gluten-free cookies kept at elevated temperature (40 ± 1°C) during 9 months of storage*

MDA content in freshly baked rice-buckwheat gluten-free cookies was 1427 μg/kg (19.8 nmol/g, Fig. 1) and this value is in line with previously determined data obtained by Papastergiadis *et al.* (2014) who found out that cookie samples contained more than 1300 μg/kg MDA (min 1344 μg/kg MDA and max 4175 μg/kg MDA). According to the published and obtained data, cookies can be positioned in a category with a high MDA content

suggesting their high contribution to the intake of MDA. Consumption of foods characterized by high MDA contents is known to be dangerous for biological systems due to its potential toxicity to humans which is attributed to their high reactivity with proteins and DNA (Esterbauer, 1982; Uchida, 2003; Guillen, Goicoechea, 2008).

During the storage of rice-buckwheat gluten-free cookies kept at elevated temperature MDA content increased with storage time to a maximum after which it became constant (Figure 1). Monitoring of MDA revealed that significant accumulation of MDA in the unpacked cookies kept at elevated temperature happened in the 3. month (3243 μg/kg MDA) being followed by one more increase in the 4. month (4677 μg/kg MDA) and reached the value of 5005 μg/kg MDA at the end of 9-month storage period.

The packed cookies kept at elevated temperature underwent the oxidative changes at the end of the 4. month (2364 μg/kg MDA) after which the MDA content slightly increased to the value of 3077 μg/kg MDA at the end of the storage period.

Being focused on MDA values, it can be concluded that the 3. and 4. month were critical for the unpacked and packed cookies, respectively, and therefore may be accepted as the end-points of cookie shelf-life when they are exposed to high temperature (40 ± 1°C) which could be reached under inadequate storage conditions during summer period.

The difference between susceptibility of the unpacked and packed cookies kept at 40 ± 1°C to lipid oxidation was due to packaging since the unpacked cookies were directly exposed to oxygen while the packed cookies were in contact with slowly increasing amount of O_2 inside the polypropylene bags. The obtained results indicate a positive effect of packaging on prolonging the shelf-life of the examined cookies.

Since that hexanal is often used as a marker of lipid oxidation, it worth to be mentioned that the same end-points of cookie shelf-life were assessed using hexanal and MDA as markers of lipid deterioration in the examined cookies (data not shown for hexanal). It indicates the various possibilities in determining cookie shelf-life depending on the available techniques for the determination of a lipid oxidation marker.

CONCLUSIONS

- Monitoring of MDA revealed the unpacked cookies kept at elevated temperature (40 ± 1°C) were acceptable until the 3. month of storage while the packed cookies underwent the oxidative changes at the end of the 4. month.
- MDA content can be used for predicting the end-point of rice-buckwheat gluten-free cookie shelf-life.

ACKNOWLEDGEMENTS

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THE EFFECTS OF ALUMINUM ON CALCIUM AND PHOSPHORUS CONTENT IN WHEAT

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ABSTRACT

To study the effects of the soluble Al-forms on the plants is very important since increasing acidity of the soils leading to the mobilization of the aluminium deposit, and Al-containing food packaging cause serious environmental pollution as well. The toxicity of the trivalent Al form is a factor reducing plant growth and limiting crop productivity, moreover the Al content of edible plant parts could be dangerous for animals and for the people, as well.

The effects of aluminium at acidic pH on the growth and on the element content of winter wheat (*Triticum aestivum* L. cv. Martonvásári-8) seedlings was investigated for two weeks. Plants were grown in a complete nutrient solution with 0.1 mM Al addition at pH 4.5, and control plants were grown without aluminium. The growth of the plants and Al content in the different plant parts were followed.

The weight of the roots was decreased (82 % of the control) by Al stress, but in the case of the shoots no significant changes were found after two weeks. In the Al-treated plants Al-content of the roots was one magnitude higher than in the shoots.

No significant differences were found in the potassium and nitrogen content of the control and Al-treated plants, it varied between 95-104 % of the control, both in the case of the roots and the shoots.

Significant difference was found in the phosphorus content of the roots of the aluminium stressed plants, it was increased by 35%. The P translocation to the shoots was inhibited by Al. A decrease of the Ca^{2+} content of the roots was detected in the case of Al-treated plants.

The effects of the 0.1 mM Al-stress caused changes mainly in the roots of winter wheat.

Keywords: *acidic pH, Al-stress, Ca and phosphorus content, winter wheat*

INTRODUCTION

Aluminum is a light metal, it is the most frequent metal of the earth crust, occurs mainly as biologically inactive, insoluble deposit. Environmental factors, such as industrial contaminations, acid rains increase the soil acidity, leading to the mobilization of Al. Half of the world's potential arable lands are acidic, therefore Al-toxicity decrease crop productivity.

Food packaging and post-use disposal cause environmental pollution, recently aluminium foils are priority toxins in the United States and Germany. Al is generally harmless to plant growth in pH-neutral soils, but in acid soils the concentration of toxic Al^{3+} cations increases (Yang *et al*., 2013.). Approximately over 50% of the world's potential arable lands are acidic, therefore Al toxicity is a major factor reducing plant growth and limiting crop productivity. The trivalent Al^{3+} ion is toxic to all living cells (Driscoll, 1985). In humans Al^{3+} ion may cause a variety of neurological disorders, it has significant role in Alzheimer's disease (AD). In plants, ionic Al rapidly inhibits root elongation and subsequently the uptake of nutrients and water (Ligaba *et al.,* 2004.), resulting in poor growth. Al toxicity is a major limiting factor of crop production on acid soils, (Kochian *et al.,* 2004). Some plants have evolved mechanisms to detoxify Al^{3+}, both externally and internally (Ma and Furukawa, 2003.)

In higher plants the Al-induced secretion of organic acid (OA) from the roots and the role of phosphorus (binding to the aluminium) is a known mechanism of Al tolerance (Ma *et al.,* 2001).

In this study the in vivo effect of soluble Al^{3+}form on winter wheat was followed for two weeks, seedlings were grown hydroponically. The changes caused by Al-stress (0.1 mM) at acidic pH in the growth and in some important element content were measured.

MATERIAL AND METHODS

Plant growth and element analysis

A nutrient solution (modified Hoagland) at pH= 4.5 containing 0.1 mM $AlCl_3$ was used to examine the effects of Al-stress on wheat (*Triticum aestivum* L. cv. Martonvásári-8).

Control plants were grown under Al free condition. The nutrient solution contained: mM: 2 $Ca(NO_3)_2$, 1 $NaNO_3$, 1,0 $MgSO_4$, 0,05 KH_2PO_4, 0,05 K_2HPO_4, uM: 10 Fe (FeEDTA), 10 B, 1,0 Mn, 0,7 Mo, 0,5 Zn, 0,5 Cu, 0,5 Si, 0,1 Co. Fifty seeds were then placed on plastic nets over plastic beakers, each containing 4 l of nutrient solution, and 4 replicate beakers were cultivated in the case of each treatment. The seedlings were grown hydroponically under controlled condition for two weeks with a 12 h day-time illumination of 60 W m-2, day/ night temperatures were 23/18 $^{\circ}$C, the relative humidity of the air was 85%. The fresh and the dry weight of the root and shoot were measured, and N, P, K, Ca and Al content were determined after wet digestion.

Nitrogen content was analyzed by Kjedahl method, phosphorus was determined colorimetrically and the content of K, Ca and Al was measured by atomic absorption spectrofotometry described by Szabó-Nagy *et al.* 1987.

Statistical analysis

The fresh and the dry weight of the root and shoot were measured as averages of 5 plants. The element concentrations are given as the mean of three replicate determination, replicates differed by less than 10%. For statistical method ANOVA (Excel) was used.

RESULTS AND DISCUSSION

The solubility of the packaging aluminium foil was studied at acidic (pH 4.5) and a quite high amount of Al^{3+} ions, near 2500 ppm was found in the solution on the surface of the foil. The measurement of Al content was made by inductively coupled plasma–mass spectrometry (ICP-MS).

The toxicity of the trivalent Al form is a factor reducing plant growth and limiting crop productivity**.** In this study the in vivo effects of ionic form of Al (0.1 mM, at pH 4.5) on wheat growth and on the element contents were followed (Figure 1.) control plants were grown under Al free condition.

The weight of the roots was decreased (82 % of the control) by Al stress, but in the case of the shoots no significant changes were found after two weeks.

The Al content of the roots was near one magnitude higher than that of the shoots in the Al treated plants, suggesting that the translocation of Al is hindered.

No significant differences were found in the Potassium and Nitrogen content of the control and Al-treated plants, it varied between 95-104 % of the control, both in the case of the roots and the shoots. In the P and Ca content of the roots significant differences were found, caused by 0.1 mM Al-stress. P content was increased by 35%, while Ca content was decreased by 26%. The precipitation of aluminium by phosphorus in the apoplast, and the accumulation of Al-P complex in the root, could explain the measured increased P level. The Al-induced inhibition of Ca^{2+} uptake was found by Huang *et al.,* (1992) in the Al-sensitive wheat. Changes in ionic composition of wheat root indicate that the ionic uptake processes were affected by Al-stress seriously.
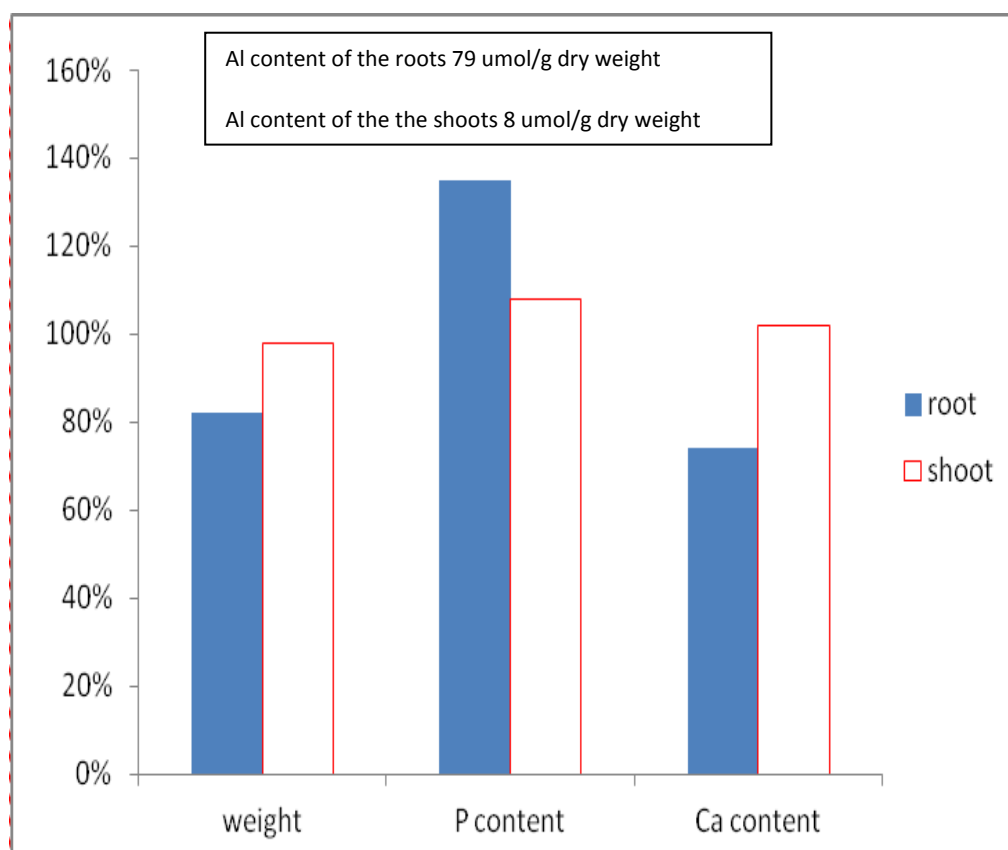
Figure 1. Comparison of the growth (Fresh weight) and some important element content of the control and Al-treated wheat plants, data presented as percentages of the control. Similar results were obtained in three independent series of plants.

Al-induced reduction in growth can be the result of a depletion of organic-P pool in the plants. On the contrary, higher concentrations will just be the result of impaired growth. Al-stress and P deficiency on nutrient uptake processes have some similarities (Keltjens and Loenen, 1990), Szabó-Nagy *et al.,* (1987) found growth retardation caused by P deficiency. Suppressed ionic (Ca^{2+}) net uptake might be the result of enhanced leakage of plasma membranes due to a shortage of phospholipids as induced by deficient P supply, or malfunctioning of Al-saturated phospholipids in the presence of Al^{3+}. Aluminum (Al) toxicity and phosphorus (P) deficiency often coexist in acid soils, and severely limit crop growth and production. P addition significantly increased Al tolerance in four soybean genotypes differing in P efficiency. Some genotypes may be able to enhance Al tolerance not only through direct Al-P interactions but also through indirect interactions (Liao *et al.,* 2006). Generally Al toxicity and P deficiency are studied separately as independent factors. Al toxicity and P deficiency often coexist in acid soils, and these two factors may strongly interact.

P deficiency and Al stress has some similar effects on plants, for example significantly reduced root weight. However, interaction of Al and the metabolism of other important elements, such as P needs more studies.

A general mechanism of Al tolerance in plants is the release of organic acid anions from the roots in response to Al stress (Kochian *et al.,* 2005), these anions chelate Al and form nonphytotoxic Al forms (Ma, 2007).

CONCLUSIONS

Food packaging and post-use disposal cause environmental pollution, recently aluminium foils are priority toxins. From the aluminium foil 2500 ppm Al was released at acidic pH, and it can cause damaging effects on plants. The Al-stress is a very serious and growing problem

all over the world, decreasing crop production in the last decades since the acidity of the soil and the wast of the aluminium foil is increasing. Some plant species and cultivars depending on their genetical inheritances have evolved some mechanisms for detoxifying Al both internally and externally. Some plants have high Al contrentrations in the shoots, while in the case of winter wheat, we found increased aluminium content in the roots. The shortage of the available P level in the presence of aluminium could be leading to P deficiency symptoms. Under aluminium-stress an increased P-content, a decreased Ca^{2+} content of the wheat roots were found, while in case of N and K^+ contents were no significant differences.

The growth (quantity) of plants and the quality (element content, toxic compounds) of the plant material are important factors for crop production and they seriously affects the food and feed quality.

ACKNOWLEDGEMENTS

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OPTIMIZATION OF WHEAT STARCH SUSPENSIONS MICROFILTRATION PROCESS

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ABSTRACT

The objective of this study was to optimize the wheat starch suspensions microfiltration process in order to determine the best operation conditions (transmembrane pressure, suspension flow rate and suspension concentration) in the presence of Kenics static mixer, as a turbulence promoter. Beside the influence of process parameters, the effect of pore size diameter was also taken into account. Used membranes in all experiments were single channel ceramic membranes with 200 nm and 500 nm pore size. The goal of optimization process was maximization of individual responses permeate flux in presence of Kenics static mixer (J_{SM}) as well as reduction of specific energy consumption (ER). Reduction of specific energy consumption was a limiting factor for the use of static mixer and its value significantly decreased with the flow rate, because the permeate flux was not high enough to compensate the pressure drop increase along the membrane channel.
Keywords: microfiltration, starch, static mixer, optimization

INTRODUCTION

Starch processing industries are faced with very strict legal regulations related to environmental protection, as well as the increasing costs of wastewater treatment and their storage (Ikonić *et al.*, 2011a). One possible solution in the wastewater treatment process is the application of membrane separation processes. The membranes have the ability to reach an adequate degree of separation without the use of heat energy and thus improve the quality of the products and reduce processing costs. Cross-flow microfiltration is one of the most common membrane separation process used in the field of the starch wastewater treatment.

The term microfiltration (MF) denotes pressure-driven flow through a microporous membrane to separate and recover micron or submicron sized particles from fluids. In MF applications, purification is accomplished by appropriate choice of membrane pore size whereby all solutes larger than the membrane pore size (e.g. cells or inclusion bodies) are completely rejected (Venkiteshwaran and Belfort, 2010).

Besides a number of advantages, such as providing higher-quality products, easy operational control, and lower maintenance costs compared to conventional separation methods, the main disadvantage of membrane separation processes is a significant initial permeate flux decline, governed by concentration polarization and cake formation on the membrane surface (Ahmad *et al.*, 2012). Membrane fouling cannot be completely avoided, but can be reduced by using different techniques. In order to increase the permeate flux, a number of methods, including a hydrodynamic method for inducing and increasing the turbulence, as well as causing the instability and non-stationary flow through the module, are used (Gupta *et al.*, 1992; Al-Bastaki and Abbas, 2001; Zhen *et al.*, 2006; Gabrus and Szaniawska, 2009). Irrespective of the use of various techniques to control the membrane fouling, in most cases, the flux decreases to unacceptably low values and it is necessary to clean the membrane in order to regenerate the permeate flux. This work aims to study the optimization of starch suspensions crossflow microfiltration with a Kenics static mixer as a turbulence promoter. The main operating variables in membrane technology are the transmembrane pressure, suspension flow rate and suspension concentration and their

influence on microfiltration process was examined. Also, the effect of pore size on microfiltration process was not neglected.

MATERIAL AND METHODS

Starch suspensions were prepared using dry wheat starch (Fidelinka-Skrob; Fidelinka, Subotica, Serbia) suspended in distilled water. The chosen concentrations were in the range of wastewater concentrations, after several hours of precipitation. The experiments were carried out using a conventional cross-flow microfiltration unit (Figure 1). The feed was circulated by a centrifugal pump. The microfiltration was done in the retentate recycling mode while the permeate was collected in the vessel until the required volumetric concentration factor (VCF) was obtained. The transmembrane pressure difference was adjusted by the regulation valve. The inlet and outlet pressures of the membrane module were measured by two pressure gauges. The average of these two pressure values gave the value of the transmembrane pressure as the outside of the membrane is vented to the atmosphere. The feed flow rate was regulated by a valve and measured with a rotameter. The single-channel ceramic membranes used had a nominal pore size of 200 nm (M200) and 500 nm (M500) (GEA, Germany). The lengths of the membranes were 250mm, with a useful membrane surface of 0.005m^2. All experiments were carried out at constant temperature (25 ± 2 °C).

All measurements in this study were carried out in triplicate and the results were averaged. The static turbulence promoter used during experiments was the stainless steel Kenics static mixer. The static turbulence promoter was inserted inside the whole membrane tube and was fixed properly to avoid any movement due to the fluid flow.
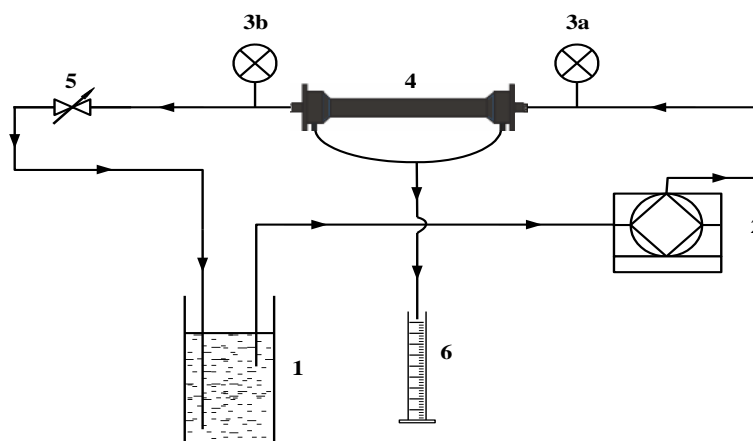
Figure 1. Schematic representation of experimental set-up for cross-flow microfiltration.
1-feed tank, 2-pump, 3a, 3b-manometers, 4-membrane module, 5-regulation valve, 6-permeate outlet

The operational conditions were planned according to Box-Behnken experimental design. The three independent variables, transmembrane pressure, suspension flow rate, and suspension concentration, varied as follows:

- transmembrane pressure (TMP): 0,3; 0,6 and 0,9 bar
- suspension flow rate (Q): 80, 130 and 180 L/h
- suspension concentration (C): 5, 10 and 15 g/L.

The initial volume of the wheat starch suspension was microfiltrated until VCF reached the value of 2. The volume concentration ratio (VCR) is defined as VCR = $V_0/(V_0-V_P)$, being V_0 the initial volume in the feed tank and V_P the volume of permeate. This information is essential for correct industrial design (Alvarez *et al.*, 1996).

The efficiency of the static turbulence promoter was checked through determination the reduction of specific energy consumption (*ER*). The calculations were defined as follows:

$$P = Q \cdot \Delta P$$

(1)

where *P* is the hydraulic dissipated power (W), *Q* suspension flow rate (L/h), *ΔP* pressure drop (bar).

$$E = \frac{P}{J_P A}$$

(2)

where *E* is specific energy consumption (kWh/m^3), J_P the permeate flux (L/m^{2}h), *A* membrane surface (m^2).

$$ER = \frac{E_{NSM} - E_{SM}}{E_{NSM}} \times 100$$

(3)

where *ER* is reduction of specific energy consumption (%), E_{NSM} specific energy consumption without static mixer (kWh/m^3), E_{SM} specific energy consumption with static mixer (kWh/m^3).

Optimization of the observed process is preceded by the determination of a mathematical model that describes the influence of defined process parameters and their interactions on the observed responses. For this purpose, the response surface methodology (RSM) is used and it may be defined as an empirical statistical technique applied for the regression analysis of the data obtained from the adequately planned experiments, simultaneously solving the system of equations (Allen, 2006; Myers and Montgomery, 1995).

Statistical analysis of experimental data was performed using STATISTICA 9. software (STATISTICA, 2010), while the optimization of microfiltration process, applying the method of the desirability function, were performed using Design Expert 8.1. (DESIGN-EXPERT, 2010).

RESULTS AND DISCUSSION

The influences of transmembrane pressure, suspension flow rate and concentration on the observed responses (J_{NSM}-permeate flux in the system without static mixer, J_{SM}-permeate flux in the system with static mixer, ER-reduction of specific energy consumption) were described by polynomial model of the second degree. The results of the statistical analysis according to the experimental plan are presented, in detail, in Ikonić (2011b). These experiments were carried out in order to select the best operation conditions to obtain as high a permeate flux as possible.

The equations which describe the impact of process parameters on the mentioned responses are as follows:

M200

$J_{NSM} = 143.322 + 65.813 * TMP - 0.090 * Q - 11.489 * C - 33.256 * TMP^2 + 0.001 * Q^2 + 0.468 * C^2 + 0.058 * TMP * Q - 1.030 * TMP * C - 0.007 * Q * C$

$J_{SM} = 118.511 + 109.090 * TMP - 0.220 * Q + 1.035 * C - 42.212 * TMP^2 + 0.001 * Q^2 - 0.127 * C^2 + 0.163 * TMP * Q - 1.875 * TMP * C - 0.007 * Q * C$

$ER = -193.728 - 42.143 * TMP + 3.662 * Q + 15.856 * C + 34.212 * TMP^2 - 0.025 * Q^2 - 0.839 * C^2 + 0.242 * TMP * Q - 0.844 * TMP * C + 0.024 * Q * C$

M500

$J_{NSM} = 79.720 + 104.384 * TMP - 0.060 * Q - 1.749 * C - 74.709 * TMP^2 + 0.001 * Q^2 + 0.039 * C^2 + 0.079 * TMP * Q - 0.601 * TMP * C - 0.011 * Q * C$

$J_{SM} = 54.120 + 161.335 * TMP - 0.028 * Q + 5.783 * C - 95.806 * TMP^2 + 0.001 * Q^2 - 0.320 * C^2 + 0.039 * TMP * Q - 1.225 * TMP * C - 0.010 * Q * C$

$ER = -202.393 - 13.140 * TMP + 4.010 * Q + 10.785 * C + 59.440 * TMP^2 - 0.025 * Q^2 - 0.638 * C^2 - 0.373 * TMP * Q - 0.581 * TMP * C + 0.030 * Q * C$

Examination of the wheat starch suspensions microfiltration on a ceramic membrane with different mean pore diameter (200 and 500 nm), in terms of the feed suspensions concentration, showed that the permeate flux decreased (up to 25%) with the increase of the membrane pore size. This behavior is a consequence of increasing internal fouling of the membrane with a larger pore size, even though the proportion of particles below 1µm is almost negligible, and the dominant resistance to the permeate flow is formation of the filter cake on the membrane surface.

In order to increase permeate flux during the experimental work, static mixer is used, as a turbulence promoter. It was observed that insertion of a static mixer into the membrane channel results in the increase of permeate flux, regardless to the value of the process parameters, and achieved increase in permeate flux was from 20% to 80% on the membrane M200 and from 20 to 50% on the membrane M500. The increase in permeate flux is

conditioned by the establishment of turbulent flow regimes and the characteristic movement of fluid along the membrane channel, which is a consequence of the characteristic helical connecting elements of Kenics static mixer.

The use of Kenics static mixer have to be examined from an economic point of view, and the most important parameter is the specific energy consumption, defined as hydraulic dissipated power per unit volume of permeate (Krstić *et al.*, 2002). Inserting a static turbulence promoter into a tubular membrane causes an increase in flow velocity and pressure drop for the same flow rate. The hydraulic dissipated power (P) or power required for the fluid circulation, increases due to the pressure drop increase along the membrane, leading to large energy consumption. Therefore, the improved performances should be checked by consideration of the energy consumption (Krstić *et al.*, 2004; Vatai *et al.*, 2007). If the increase of the permeate flux, caused by inserting the static mixer into the membrane channel, is not large enough to compensate the increased hydraulic dissipated power, then its use is not justified from an economic point of view (Ikonić *et al.*, 2012). The results show that the reduction of specific energy consumption depends almost exclusively on the suspensions flow rate.

The finale goal of the response surface methodology application is optimization of the observed process, so the developed models can be used for simulation and optimization. In this paper, optimization of experimental conditions was done by the method of simultaneous maximization of two responses, namely permeate flux in systems with static mixer (J_{SM}) and the reduction of the specific energy consumption (ER).

The general approach of the concept of desirability function is to convert the individual response, Y_n, in individual preferred d_n functions which values are ranged from 0 to 1. The value "0" of an individual function represents the worst value, while the value "1" represents the best value of the observed response. The overall desired function (D) is equal to the geometrical center of individual desired functions (Cojocaru *et al.*, 2009). High values of D show the best values in the system functions that correspond to the optimal solution of a system. Optimal values of the experimental parameters are determined from the value of individual desired functions that optimize the overall desired function (Jokić *et al.*, 2010).

The objective of the optimization was the selection of the transmembrane pressure, flow rate and concentration of suspension which provide maximum values for the selected responses, i.e. their individual desired functions will be as high as posible, ideal "1". The optimal values of transmembrane pressure, flow and concentration depend on the importance of individual responses, which are ranked from 1 to 5. Tables 1 and 2 provide an overview of process parameters optimal values depending on the significance of the observed responses, as well as the optimized value of the responses.

Table 1. Results of optimization of microfiltration process (M200)

Importance		TMP (bar)	Q (L/h)	C (g/L)	J_{SM} (L/m^{2}h)	ER (%)	Overall desirability
J_{SM}	ER						
2	5	0.9	84.59	6.73	171.31	22,81	0.925
3	3	0.9	90.26	5.00	176.60	8.10	0.913
5	2	0.9	112.68	5.00	179.87	-15.90	0.922

TMP – transmembrane pressure, Q – suspension flow rate, C – suspension concentration, J_{SM} – permeate flux in the system with static mixer, ER – reduction of the specific energy consumption

The optimal conditions of the wheat starch suspensions microfiltration process on the membrane M200 indicates that the process should be conducted at the maximum value of the transmembrane pressure of 0.9 bar, flow rates between 85 and 100 L/h and a concentration from 5 to 7 g/L, while the optimal conditions for microfiltration process on membrane M500: transmembrane pressure of 0.85 bar, flow rates between 85 and 100 L/h and the concentration of 7 g/l. With the increase of the flow rate, the permeate flux in the system with a static mixer does not significantly change, but the reduction of the specific

energy consumption significantly decreases and for the flow rates above 100 L/h, the use of the static mixer is not justified from an economic point of view.

Table 2. Results of optimization of microfiltration process (M500)

Importance		TMP (bar)	Q (L/h)	C (g/L)	J_{SM} (L/m^{2}h)	ER (%)	Overall desirability
J_{SM}	ER						
2	5	0.85	84.80	7.04	139.39	19.19	0.935
3	3	0.85	85.96	6.97	139.53	18.23	0.889
5	2	0.84	107.80	6.35	141.63	-7.55	0.855

TMP – transmembrane pressure, Q – suspension flow rate, C – suspension concentration, J_{SM} – permeate flux in the system with static mixer, ER – reduction of the specific energy consumption

CONCLUSIONS

The experimental results clearly show that pore size diameter had influence on permeate flux during microfiltration process and with the increase of the membrane pore size (200 nm and 500 nm) permeate flux decreased (up to 25%). The improved performances of starch suspension cross-flow microfiltration can be obtained by using Kenics static mixer. Compared to the process without turbulence promoter, the permeate flux improvement is ranged from 20% to 80% on the membrane M200 and from 20 to 50% on the membrane M500. However, the reduction of specific energy consumption decreases with the flow rate, because the permeate flux is not high enough to compensate the increase in pressure drop along the membrane channel. Optimal values of process parameters were determined using desirability function concept simultaneously maximizing the permeate flux in systems with static mixer (J_{SM}) and the reduction of the specific energy consumption (ER). The most important parameter that influences the choice of the optimal solution is the flow rate. The flow rates, ranged from 80 to 100 L/h, provided positive values of reduction of specific energy consumption and the use of static mixers is justified from an economic point of view. This conclusion is made for both examined membranes. Optimal conditions for the wheat starch suspension microfiltration process on the membrane M200 are: the maximum value of transmembrane pressure of 0.9 bar, flow rates from 85 to 100 L/h and the minimum concentration of 5 to 6 g/L, while the optimal conditions on the membrane M500 are: transmembrane pressure of 0.85 bar, flow rates between 85 and 100 L/h and the concentration of 7 g/l.

ACKNOWLEDGEMENTS

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MOLASSES AS A SUPPLEMENT FOR BAKED PRODUCTS

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ABSTRACT

Taking into consideration the global trends in food research oriented towards obtaining food with high nutritive and biological value, extensive study has been conducted to investigate the quality of molasses, a by-product of sugar industry, in order to estimate its suitability as an ingredient in food industry.

Although it is best to use sugar beet molasses in a traditional way, increasing demands after food products with enhanced nutritional value conjointed with specific composition of molasses has lead to the idea that sugar beet molasses is an ingredient with high potential to complete the array of useful ingredients for the production of functional and fortified food. Molasses is a concentrate of biogenic elements (potassium, calcium, magnesium, sodium) and many bioactive substances (proteins, betaine, glutamic acid, purine and pirimidine bases, organic acids, melanoidines).

The results of the study showed that sugar beet molasses can be used as an ingredient in food industry offering new commercial possibilities from the aspect of consumers' health benefits.

Keywords: sugar beet molasses application, bread, tea biscuits.

INTRODUCTION

Molasses is concentrated liquid extract of the sugar refining process, applicable for use in bakery and confectionery industry. Molasses has high content of solids (approximately 80%), sucrose (51% in average), 1% rafinose, 0.25% glucose and fructose, 5% proteins, 6% betain, 1.5% nucleosides, 1.5% purine and pirimidine bases, organic acids and pectins (Šušić *et al.,* 1989). Important constituents of molasses are also minerals and vitamins. Minerals are nutrients necessary for growth and normal physiological functioning of body and are part of many enzymes and hormones (Bíró *et al.,* 1988). They must be supplied by food since the body cannot synthesize them. In molasses, calcium, potassium and iron are present in substantial amounts although their contents vary over wide ranges. It is especially important to note that minerals in molasses are dissolved and that potassium is dominant with a share of 75% (by weight) of total cations (Šušić *et al.,* 1989). Regarding potassium intake, it has been known that vegetable sources of potassium are more advantageous having more favourable potassium to sodium ratio, potassium content almost double to that of sodium (Bíró *et al.,* 1988). Molasses also contains B group vitamins and does not contain fats and fibres. In addition, molasses shows humectancy, antioxidant and water activity lowering properties which are important for the shelf-life of the products. Dark colour of molasses comes from melanoidines and caramelization products. Molasses is an effective colorant in whole wheat, cracked wheat and bran items to mask their unappetizing colour (Hickenbottom, 1996).

Flour based confectionery products such as biscuits and related products hold an important position in total production and consumption of confectionery products in Serbia (Pajin *et al.,* 2005). The range of ingredients used for the production of tea biscuits present on our market is uniform and differences are mainly due to variations in flavourings and final production steps. However, incorporation of nutritive important ingredients can contribute to their diversification regarding appearance, flavour, texture and nutritional quality (Willbrandt, 1989). One such ingredient, high in nutritional and biological value, is sugar beet molasses, a by-product of sugar beet processing. Molasses is a promising ingredient for use in

confectionery products having been a natural sweetener, nutritious (rich in minerals and vitamins) and as such an alternative to highly refined sugars (Lević *et al.,* 2005).

Expanding the ranges of bakery products in terms of producing supplemented or dietetic products has been an increasingly important trend in contemporary baking. Bakery products as basic and popular food, could be used for the prevention of nutritive deficiencies of many important nutrients, supplementing the products with biologically valuable ingredients. Such an ingredient is a by-product of sugar beet processing - molasses.

In the paper, the effect of different sugar beet molasses content on the quality of wheat bread type 850 and tea biscuits is discussed.

MATERIALS AND METHODS

In this paper, investigations were performed on the samples of sugar beet molasses which were taken directly from the production lines of Serbian sugar factories.

Basic quality parameters for molasses (Table 1) were determined according to methods described in handbooks for the laboratory control of sugar processing (Milić et al., 1992). The methods are harmonized with the regulations guided by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA, 2003).

Table 1. Physicochemical properties of molasses

Sucrose content, %	50.50
Moisture content, %	17.20
Ash content, %/d.b.	13.92
Total nitrogen, %/d.b.	2.21
Invert sugar content, %	1.45
pH	7.20
Aw	0.63
Colour, I.U.	16830

Application of sugar beet molasses in the tea biscuits

Ingredients

Commercially available wheat flour type 500 (ash content 0.46-0.55g/100 g dry matter) was used. The flour had characteristics that complied with special requisites of biscuit flours (Gavrilović, 1981, Nikolić *et al.,* 1988): low protein (9.5% d.b.) and wet gluten content (20%), quality class B_2 by faringram. Rheological parameters were also adequate: extensigraph area 32 cm^2, resistance 160 B.U., extensibility 127 mm, amylograph peak viscosity 340 B.U.

Preparation of biscuits

Basic biscuit formulation includes wheat flour, margarine and sugar in ratio 100:40:35, respectively. As leavening agent, baking powder was used at 1% dose (flour basis). Sugar beet molasses was added at four doses: 5, 10, 15 and 20% (flour basis). The amount of sugar in the formulation was reduced with increasing doses of molasses: from 35% in control to 13.6% in biscuit made with 20% molasses. The amount of water added varied to obtain cohesive dough suitable for the production of mould-cut biscuits.

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 1 hour at +4 ºC. After resting, dough was sheeted to uniform thickness (10 mm) and cut out using a round cutter.

Colour parameters of biscuits
Colour of biscuits was measured by a photoelectric tristimulus colorimeter MOMColor 100. Colour parameters were recorded as CIE (dominant wavelength DW (nm), colour purity P (%) and average reflectance Y (%) and CIELab values (lightness-L*; red and green hue-a*, yellow and blue hue-b*) (Robert, 1977).

Textural parameters of biscuits
Hardness of tea cookies was measured by cutting on a TA.XTplus Texture Analyzer (Stable Micro Systems, England, UK) with a reversible blade, using its knife edge. The analyzer was set to measure force in compression. Each cookie was precisely centred on the platform and the blade was lowered to deform and break (snap) the biscuits at a travel distance of 15 mm and test speed 2.0 mm/s. The trigger force was set at 25 g. The peak force was measured and represented the index of cookie hardness.
Shear force was determined on Warner Bratzler device (Standardized Warner-Bratzler Shear Force Procedures).

Application of sugar beet molasses in the semi-white wheat bread T-850
Baking tests were performed under laboratory conditions using rapid mixing procedure. Breads with 5%, 10% and 15% of molasses (calculated on flour) were tested in order to determine the optimum dosage of molasses that provides acceptable bread quality with extended shelf-life in comparison to standard semi-white wheat bread type 850.

Table 2. Bread formulations

Ingredients	Wheat bread type 850
Wheat flour T-500	-
Wheat flour T-850	100
Beorustik mixture	-
Baker's yeast	2.5
Salt	2
Improver	0.3
Molasses	5/10/15
Water	depending on water absorption

Textural parameters of bread
Each bread sample was evaluated on the basis of the following characteristics: specific volume, penetrometer number, crumb attributes (elasticity, crumb grain structure), and flavour. Investigation of chemical composition included water, ash, protein, reducing sugar content and acidity number determination according to official regulations (Pravilnik No 74/1988). Penetrometer number was determined by PNR6 SUR Berlin (Sommer & Runge KG) penetrometer and was used to evaluate crumb resilience instrumentally. Water activity (A_w) measurements were performed with material taken from the central part of the loaf and included crumb and crust (aw Wert Messer Lufft model 5803 Lufft Stutgart) on 21°C.

Data analysis
Data were analyzed by ANOVA test using Stat-graphics Plus V 7.1 (Statistical Graphics Corporation, UK). Mean separation was accomplished by Tukey's test.

RESULTS AND DISCUSSION

Effect of molasses addition on the quality of tea biscuits

Results of analysis of physical properties of tea biscuits with molasses are displayed in the Table 3. The addition of molasses increased biscuit diameter in comparison to the control. The lowest increase of 6.1% was recorded for 20% molasses level and the highest increase of 8.9% for 10% molasses. The highest spread ratio was recorded for higher molasses doses (15 and 20%). Density of biscuits increased with increased content of molasses which also reflected to the biscuit structure (shear force and hardness in Table 1).

Table 3. Physical attributes of tea biscuits with molasses

Quality attributes	Molasses content, %				
	0	5	10	15	20
Average diameter, cm	6.40±0.11[a]	6.83±0.13[ab]	6.97±0.11[b]	6.95±0.21[b]	6.79±0.23[ab]
Spread ratio, %	-	21.6±5.21[ab]	14.7±3.23[a]	28.6±4.84[b]	27.3±4.31[b]
Height, cm	1.30±0.31[b]	1.15±0.52[ab]	1.24±0.432[ab]	1.10±0.14[a]	1.09±0.15[a]
Volume, cm^3	41,80±1.2[b]	42.06±1.1[b]	47.36±1.3[a]	41.88±1.4[b]	39.54±1.1[c]
Density, kg/cm^{3}x 10^{-6}	514±3.45[a]	537±3.68[ab]	564±4.21[b]	606±4.53[c]	564±3.85[bc]
Shear force, kg	1.62±0.22[a]	1.80±0.19[b]	1.46±0.16[a]	2.63±0.22[c]	3.64±0.23[d]
Hardness, kg	10.65±1.8[ac]	12.45±2.4[b]	9.82±2.0[a]	13.38±3.8[bc]	15.4±4.5[c]

The biscuits made with molasses were intensively coloured (dark bloomy and brown). Dark colour of biscuits comes from melanoidines and caramelization products of molasses. Molasses can be used as an effective natural colorant in a wide range of products. The lower and upper surfaces of the supplemented biscuits were smoother as compared to the control. The addition of molasses contributed to the formation of more regular, even and brittle fracture and better mastication. The most regular structure was observed with the samples containing 10 and 15% molasses. The addition of molasses in doses 5 to 15% positively affected taste and odour (full, aromatic, pleasant, caramel-like, and ginger cake-like).

Sensory estimations of biscuit colour are in agreement with objective colour measurements. Indicator of yellow colour (b* value) and lightness (L* value) decreased with increased molasses concentration in dough. There was distinctive difference in the colour and lightness between upper and lower surfaces of biscuits – the lower surface was darker in all samples (Table 4).

Table 4. Sensory and objective colour parameters for tea biscuits cookies supplemented with molasses

Colour parameter		Molasses content, %								
		0		5		10		15		20	
		Surface								
		upper	lower	upper	lower	upper	lower	upper	lower	upper	lower
CIE LAB	a*	1.75	-4.24	0.64	10.45	4.36	4.44	-0.27	8.48	11.99	5.43
	b*	28.04	33.40	30.53	32.41	27.64	31.54	24.18	27.46	11.32	21.52
	L*	79.65	74.30	62.74	57.09	53.57	53.81	51.22	46.68	43.41	40.29
CIE	DW (nm)	579	574.5	578	582.5	580.5	579	576.5	582.5	582.5	582
	Y(%)	56.08	47.18	31.28	25.02	21.58	21.80	19.46	15.78	13.44	11.43
	P(%)	29.3	40.2	41.5	47.1	57.3	48.8	39.0	47.1	23.7	40.2

Effect of molasses addition on the quality of bread

In Tables 5 and 6 results of bread-making tests are presented.

According to the obtained results, the addition of molasses to bread accomplished:

- increased bread yield (5.3-17.3 g);
- reduction of bake losses (2.7-5.8%);

Table 5. Baking tests of semi-white breads with different molasses content

Molasses content, %	Water absorption, %	Dough consistency	Bread yield, 24 h, g/100g	Bake loss, %	Bread volume		Penetrometer number
					ml	ml/g	
Control	58.0	elastic	134.7	17.2	1700	5.13	84.7
5	54.0	little firm	140.0	14.5	1370	4.00	61.2
10	53.0	little firm	146.5	12.7	1170	3.34	48.2
15	52.0	plastic	152.0	11.4	1010	2.85	42.2

Table 6. Sensory evaluation of semi-white bread with different molasses content

Molasses content, %	Crumb properties		Crumb grain structure	Porosity according to Dallmann
	Firmness, g	Resilience, %		
Control	560	36.2	almost spongy	6
5	550	29.8	almost fine	7
10	726	29.4	almost coarse	7/8
15	1200	23.7	coarse	8

Water absorption decreased by 4-6% with the addition of molasses. Bread volume and crumb quality decreased (crumb became less porous and slightly firmer) but these adverse effects were less pronounced with the addition of 5% molasses. Addition of 5% of molasses successfully masked the yellow-greyish colour of bread crumb observed in the control sample. Addition of 15% molasses to bread dough adversely affected dough consistency (plastic dough), crumb structure and texture and taste forming too sweet crumb and bitter crust.

Taking into consideration the content of minerals in used molasses (Table 7) and an average content of minerals in bread from tables of food composition (Kaić-Rak, 1990), the estimated increment of mineral content in semi-white bread supplemented with 5% flour basis of molasses (3.4 g molasses/100 g of bread) would be as follows: higher content of potassium by 49.7%, calcium by 28.0%, sodium by 4.3% and magnesium intake 2.60 mg/100 g of bread.

Table 7. Content of minerals in molasses (sample 82.8% solids)

Content of minerals	% dry basis	Content of minerals' ions per 1 kg of molasses, g	
K_2O	4.15	K^+	28.40
CaO	0.39	Ca^{2+}	1.36
Na_2O	1.15	Na^+	6.09
MgO	0.15	Mg^{2+}	0.74

CONCLUSIONS

Molasses is an interesting ingredient because of the following attributes:

- it is very nutritious because of high levels of vitamins and minerals,
- it provides an alternative to highly refined and unpopular sugars,
- it is ideal for use in many foods.

Sugar beet molasses can be directly added to dough for machine cut and deposited biscuits in dose up to 20% flour basis contributing to improved colour (chocolate/caramel-like) and softer consistency.

Biscuits prepared with 10 and 15% molasses had better physical and sensory properties (structure, odour, taste) comparing to the control.

The addition of molasses to bread and bakery products extends shelf-life of the products and improves their taste but it deteriorates the structure and texture of the bread crumb. Bread with 5% of molasses (based on flour) is characterized with deep golden brown crust and is sensory evaluated as very acceptable. Bread supplemented with 5% of molasses was estimated to have higher content of potassium by 49.7%, calcium by 28.0 % and sodium by 4.3% in comparison to standard semi-white bread.

ACKNOWLEDGEMENTS

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EXTRUSION PROCESS OPTIMIZATION OF CORN GRITS ON SINGLE SCREW LABORATORY EXTRUDER

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ABSTRACT

The properties of corn grits extrudates were investigated as a result of different extrusion conditions including extrusion temperature, screw speed and moisture content. The experiment was conducted on the Brabender® single screw laboratory extruder. Box-Benhken design method was successfully used in this experiment and the selected process responses were expansion index (EI), water absorption index (WAI) and water solubility index (WSI). Statistical regression analysis and desirability function were applied in order to develop a predictive model, which revealed that moisture had the strongest influence on the parameters of investigation. The temperature had weak effect on the WSI and WAI, while a stronger influence was noticed on expansion index. The purpose of this study was to determine optimal operating extrusion conditions of corn grits for improvement of the process. Optimal levels of the tested parameters were determined by the desirability function approach. It was found that the optimal parameters are: screw speed: 175 r. p. m., moisture: 16% and temperature: 190°C.
Keywords: *extrusion, optimization, EI, WAI, WSI*

INTRODUCTION

Extrusion is a highly versatile and efficient operation which can be applied in production of food ingredients and food such as various types of snack foods, breakfast cereals, mainly from corn meal, rice and wheat flour in many shapes and variety of textures (Ding *et al.*, 2006; Lazou and Krokida, 2010). Food extrusion is a process in which food material is forced to flow under conditions of mixing, heating and shear. It reperesent low cost, and very efficient technology in food processing (Riaz, 2000).

During extrusion the material is subjected to intense mechanical shear and before being forced through the die, it undergo many chemical and structural transformations, such as starch gelatinization, protein denaturation, complex formation between amylose and lipids, and degradation reactions of vitamins, pigments, etc (Ilo and Berghofer, 1999).

Variations in processing conditions (screw speed, extrusion temperature, die profile, extruder type, etc.) affect process variables as well as the product quality. The appropriateness of extruded foods depends on their functional properties like water absorption, water solubility and oil absorption indexes and expansion index (Ali *et al.*, 1996; Hernandez-Diaz *et al.*, 2007).

The aim of this study was to investigate functional properties (water absorption and water solubility indexes) as a function of extrusion conditions (screw speed and extrusion temperature) and material characteristic (moisture content). Furthermore, in order to obtain optimal levels of the tested parameters desirability function approach was applied.

MATERIAL AND METHODS

Whole corn was ground using hammer mill (ABC Inžinjering, Pančevo, Serbia) equipped with sieve with openings diameter of 1mm, afterwards material was sieved to obtained fraction of particles smaller than 1000 μm which was used for processing. Extrusion cooking of conditioned samples was carried out in a single screw extruder (Brabender 20DN, Germany)

with 1.9 cm barrel diameter, 20:1 barrel length and diameter ratio, maximum screw speed 275 min^{-1}, compression ratio 1:3 and die diameter 3 mm.

The experimental design and statistical analysis were performed using Stat-Ease software (Design-Expert 7.0.0 Trial, Minneapolis, MN, USA). Experiments with three independent variables: screw speed (X_1), moisture (X_2) and temperature (X_3), were carried out by Box-Behnken experimental design. Response parameters were expansion index (EI), water absorption index (WAI) and water solubility index (WSI). The levels of independent variables and design matrix are shown in Tables 1 and 2, respectively. Mean values of triplicate determinations were analysed to fit the following first (1) and second-order (2) polynomial models which are used to calculate predicted responses:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3 \qquad (1)$$
$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \qquad (2)$$

where Y is the predicted response, X_1, X_2 and X_3 correspond to the independent variables, b_0 is intercept, b_1, b_2 and b_3 are linear effects, b_{11}, b_{22} and b_{33} are squared effects and b_{12}, b_{23} and b_{13} are interaction effects of the factors. The goodness of fitting and the significances of all terms in the polynomial equations were determined through appropriate statistical methods (coefficient of determination (R^2), F-value at a probability (P) of 0.05).

Table1. Values of factors in Box-Benhken design

Factor	Name	Low value	High value
X_1	Screw speed (rpm)	175	200
X_2	Moisture (%)	16	20
X_3	Temperature (°C)	170	190

Table 2. Box-Benhken matrix and responses

Screw speed(rpm)	Moisture (%)	Temperature (°C)	EI	WAI (g/g)	WSI (%)
X_1	X_2	X_3	Y_1	Y_2	Y_3
175	16	180	2.01	5.98	16.31
175	18	170	1.31	5.42	14.18
175	18	190	1.57	5.75	15.07
175	20	180	1.40	6.31	11.35
187.5	16	190	2.3	5.63	17.52
187.5	16	180	2.2	5.32	17.0
187.5	18	180	1.50	5.90	14.95
187.5	18	180	1.50	5.91	14.92
187.5	18	180	1.50	5.91	14.92
187.5	20	170	1.17	5.20	11.78
187.5	20	190	1.5	6.03	12.58
200	16	180	2.34	5.02	17.65
200	18	170	1.50	5.06	15.24
200	18	190	1.66	5.65	16.08
200	20	180	1.50	5.30	13.50

Expansion index, the ratio of diameter extrudate and the diameter of die, was used to express the expansion of extrudate (Alvarez-Martinez *et al.*, 1988; Fan *et al.*, 1996). Six samples were used for each extrudate to calculate the mean.

The water absorption index (WAI) and water solubility index were determined according to the method of Anderson et al. (1970). A 0.2 g ground sample was dispersed in 5 ml of distilled water in weighed 15 ml glass centrifuge tube. The tube was stirred on a Vortex mixer for 2 min and then centrifuged for 20 min. The supernatant was decanted into a tarred evaporating dish for determination of its solids content. The remaining gel obtained after removal of supernatant was weighed and the WAI was calculated as:

$$WAI = m_g/m_s$$

where m_g is the weight of the hydrated gel (g) and m_s is the weight of sample (g).

The water solubility index (WSI) is the weight of dry solids from the supernatant m_{ds} (g) expressed as a percentage of the weight of the sample m_s (g) (Lazou *et al.*, 2010).

$$WSI = m_{ds}/m_s*100$$

RESULTS AND DISCUSSION

In order to ensure the best quality characteristics of extrudates it necessary to investigate and optimize extrusion parameters. The effects of screw speed, moisture and extrusion temperature on EI, WAI and WSI are shown in Table 2. Multiple regression analysis was performed to fit the response functions (Y1-3), and polynomial equations (Table 3) have been obtained. The analysis of variance (ANOVA) is used to determine the adequacy and the significance of the obtained models. The analyses were done by means of Fisher's F test, and the results are shown in Table 4. The regression models were significant ($P < 0.05$) with a satisfactory value of determination coefficients.

Table 3. Second, first-order polynomial models for investigated responses (Y_{1-3})

Parameter	Equation	R^2
EI	$Y_1 = 10.2287 + 0.008303X_1 - 2.4372X_2 + 0.1377X_3 - -0.0023X_1X_2 - 0.0002X_1X_3$ $0.0009X_2X_3 + 0.0002X_1^2 + 0.0699X_2^2 - 0.0003X_3^2$	0.9930
WAI	$Y_2 = 3923 - 0.0243X_1 + 0.0727X_2 + 0.02739X_3$	0.6359
WSI	$Y_3 = 18.31201 + 0.0556X_1 - 1.1789X_2 + 0.0407X_3$	0.9783

From the regression model (Y_1) of expansion index, the value of determination coefficient ($R^2 = 0.9930$) indicates that only 0.7% of the total variance could not be explained by the model. Among the model (Y_1) terms X_1, X_2, X_3, X_2^2, are significant with the probability of 95%. The interactions between X_1X_2, X_1X_3 X_2X_3, as well as quadratic terms X_1^2 X_3^2 however, did not have significant influence on expansion index (Table 4). EI was positively affected by the linear effects of temperature and moisture. The influence of screw speed is less significant compared to the influence of temperature and moisture (Table 3).
Similar results were obtained by Singh *et al.* (2007) who reported that the low moisture content of the material limit flow, increasing the shear rate and residence time, which increases the degree of starch gelatinization and, also the expansion. The increase in screw speed induced a significant rise in the longitudinal expansion of extrudates (Mezreb *et al.*, 2003). The increase in expansion of starch with temperature was attributed to its higher degree of gelatinization.
The WAI measures the international amount of water absorbed by starch and can be used as an index of gelatinization (Anderson *et al.*, 1970, Ding *et al.*, 2006). This parameter indicates the ability of a macromolecule to interact with water and as well as WAI. WAI depends on the

availability of hydrophilic groups to be bonded with water molecules and gel-forming capacity of macromolecules (Rocha-Guzman *et al.*, 2008).

Table 4. Analysis of variance (ANOVA) for the experimental results

	F-value			P-value		
Source	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Model	78.29	6.40	165.27	<0.0001*	0.0091*	<0.0001*
X_1	23.81	10.52	37.27	0.0046*	0.0078*	<0.0001*
X_2	389.87	2.37	421.21	<0.0001*	0.1520	<0.0001*
X_3	33.94	7.28	10.88	0.0021*	0.0207	0.0071*
X_1X_2	4.99	-	-	0.0756	-	-
X_1X_3	0.94	-	-	0.3757	-	-
X_2X_3	0.24	-	-	0.6448	-	-
X_1^2	1.32	-	-	0.3027	-	-
X_2^2	98.13	-	-	0.0002*	-	-
X_3^2	0.93	-	-	0.3794	-	-

X_1-Screw speed (rpm); X_2-Moisture (%); Temperature (°C); EI; WAI (g/g); WSI (%);
* significant at P < 0.05

The obtained model (Y_2), with a determination coefficient R^2=0.64 is proved as significant (P<0.05).The model terms X_1 and X_3 are significant at 0.05 level (P<0.05) while term X_2 doesn't have a significant effect on WAI (Table 4). WAI was positively affected by the temperature and moisture, and negatively by screw speed.
The present study confirms findings that WAI of extruded maize grits increased with increased temperature and increased feed moisture (Lazou, *et al.*, 2010; Gomez and Aguilera, 1983; Ding *et al.*, 2006) (Table 2).
Our stady is in agreement with Mezreb *et al.* (2003) who claimed that the water absorption index decreased with increasing screw speed. The results of this research support the idea that faster screw speed tends to increase shear rate but decrease residence time leading to less gelatinized product.
WSI, often used as an indicator of degradation of molecular components (Kirby *et al.*, 1988), measures the amount of small molecules solubilised in water released from the starch after extrusion (Mezreb *et al.*, 2003). The WSI is indeed related to the degree of starch transformation. WSI decreased with increased feed moisture content, but temperature increase had an inverse effect (Table 3). According to the determination coefficient of WSI R^2=0.9783 it can be concluded that only 2.17 % of the total variance could not be explained by the model Y_3, which is proved to be significant (P<0.05). The significance at 0.05 level (P<0.05) is associated with following model terms: X_1, X_2, X_3. It can be seen that the WSI was positively affected by screw speed and temperature and negatively by moisture (Table 3).
The increase of screw speed induced a sharp increase of specific mechanical energy. The high mechanical shear degraded macromolecules, so the molecular weight of starch granules decreased. Consequently, the WSI increased because starch granules were then more soluble in water (Mezreb *et al.*, 2003).
The optimisation was made by use of desirability function concept which combines multiple responses into one response by assigning a value from 0 (one or more characteristics are unacceptable) to 1 (all process characteristics are on target). After the transformation of estimated responses into individual desirability values (from 0 to 1), the overall desirability of the process is calculated as geometric mean of the individual desirability functions (Miljić and Puškaš, 2014). All the processing variables were kept within range while the responses were

maximized (EI and WAI), or kept in range (WSI).The final optimised extrusion parametars, obtained with RSM, were 175 rpm, 190 ⁰C and moisture 16% which shoud ensure following levels of investigated responses: EI-2,17; WAI-6,04 g/g; WSI-16,91%. Under these conditions high value of desirability function (0.821) was obtained.

CONCLUSION

Water solubility and absorption parameters characterize the extruded product and are often important in predicting how the extruded material may behave if further processed (Hill & Norton, 1995). We concluded that moisture have the strongest influence on the expansion index, WAI and WSI. The temperature had weak effect on the WSI and WAI, while a stronger influence was noticed on expansion index ($p < 0.05$). The screw speed has the strongest effect on WSI. Optimal levels of the tested parameters were determined by the desirability function approach. It was found that the optimal parameters are: screw speed: 175 r. p. m., moisture 16% and temperature 190 °C.

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ANTIMICROBIAL ACTIVITY OF SELECTED ESSENTIAL OILS AGAINST *Staphylococcus aureus* COMPARED WITH ANTIMICROBIAL DRUGS

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ABSTRACT

In recent years, antimicrobials have become the most widely prescribed drugs in the world. Often and excessively prescribing of antimicrobial drugs is one of the main reasons for the development of resistant microorganisms. European Centre for Disease Control (CDC) and the World Health Organization (WHO) have launched a large-scale action aimed at raising awareness of antimicrobial substances and their rational use. In this paper we have investigated antimicrobial activity of essential oils and referent antimicrobial agents on the growth of *Staphylococcus aureus*. Antimicrobial effects of oil on the growth of *Staphylococcus aureus* was determined by disk diffusion method, and different concentrations of essential oils were prepared by dissolution in 96% alcohol (1:1 and 2:1). Essential oils of cinnamon and thyme showed very good inhibitory effect on the growth of *Staphylococcus aureus*. The essential oil of cinnamon in combination with alcohol in a 2:1 ratio showed highest inhibition of the growth of *Staphylococcus aureus*, even higher than any of tested antimicrobial agents
Keywords: *antimicrobial substances, antimicrobial activity, essential oils*

INTRODUCTION

Food products can be carriers of various pathogenic bacteria due to food over raw materials during the manufacturing process or subsequent contamination. Their presence or toxins can cause poisoning of people, sometimes with lethal ending.

Staphylococcus aureus is a pathogen gram-positive, facultative anaerobic, aspororogenous, non-motile highly resistant to external influences, so it can be held for months in withered or frozen food. It grows at temperatures up to ie 46 ^{0}C and resist half an hour at 60 ^{0}C (Weisglass 1989). Also, this bacteria resists in nutrient medium with high concentrations of sodium chloride (up to about 15%) and sugar. Strains of this bacterium are resistant to the effects of many disinfectants and antimicrobial substances (Weisglass (1989). Its synthesize a large number of toxins and enzymes that contribute to pathogenicity (Karakasevic, 1987).

Best known toxins that are involved in the pathogenesis of gastroenteritis caused by *Staphylococcus aureus* infection are enterotoxins. They are formed very rapidly during the multiplication of bacteria in contaminated food. Staphylococcal enterotoxin is very stable at high temperatures and therefore is often the cause of intoxication by food. The first symptoms of staphylococcal toxin poisoning occur 2-6 hours after consuming of contaminated food. Symptoms of poisoning are severe vomiting, cramps and abdominal pain, and often diarrhea, with general weakness and headache. The disease usually lasts two days.

Staphylococcal food poisoning occurs periodically, but usually in the warmest period of the year. The most common sources of origin of these poisonings are school canteens, nurseries, but also during the celebration or gathering a large number of people.

Staphylococci can quickly multiply in food with the addition of raw milk and cream (dairy ice cream, cakes with cream, mashed potatoes etc.). Hams may be a product of high risk to human health when it comes to staphylococcal poisoning, if their contamination occurs

subsequent manipulation, because the salt content and residual nitrite level are not an enough for the prevention of staphylococci multiplication. Also, all types minced meat, chicken, tuna, sandwiches, etc. food represents a very favorable environment for bacterial growth, and can be the cause of the poisoning with this bacteria.Food keeping at ambient temperature, out of the fridge, inadequate hand hygiene and instruments of labor, work surfaces, accelerates rapidly multiplication of staphylococci (Weisglass 1989).

The natural reservoirs of of staphylococci are humans and domestic animals. It is located on the skin and mucous membranes, choking and nose in the about 50% of healthy population. *Staphylococcus aureus* causes a wide range of diseases that involve various skin infections, pneumonia, osteomyelitis, endocarditis, toxic shock syndrome and septicemia (blood poisoning).

These diseases are usually treated with appropriate antimicrobial substances, but such treatment is often not sufficiently effective due to resistant strains. Strains of *Staphylococcus aureus* (more often than other bacteria) become resistant to antibiotics (Burnie *et al.*, 2009; Mahboubi and Kazempour, 2009; Nostro *et al.,* 2004).

In the early fifties, the first use of broad spectrum antibiotics such as chloramphenicol, tetracycline and streptomycin, was rapidly followed by the development of resistance to these antibiotics to staphylococci and gram-negative bacteria. Their resistance to penicillin from 5% in the 1947. climbed up to 50% in the 1961. (Karakasevic, 1987).

Because of the resistance of bacteria to the large number of antibiotics, and because of the ability of plants to synthesize biologically active substances, importance of the application of some plant origin preparations in the control and eradication of bacteria was increased.

Different parts of the plant (root, leaf, flower, fruit, stems, bark) have been used successfully to treat many diseases. They can with their antioxidant and antimicrobial activities affect many physiological processes in the body, and thus protect against free radicals and the development of undesirable microorganisms.

Essential oils of plants often have a double effect in the body: directly - in direct contact destroy or inhibit various forms of microorganisms and indirectly introduced into the body act as immunomodulators. Also, essential oils as natural, biologically active substances are of great interest to the pharmaceutical industry in the control of human diseases that are caused by microorganisms (Kitic, 2010, Koacevic, 2004).

The use of plants in the food industry, instead of synthetic preservatives, antioxidants or other food additives has increased significantly in recent years (GA Al-Bakri, UF Afifi 2007).

Consumers and producers are showing interest in the application of essential oils from aromatic plants with antimicrobial activity for the control and management of pathogenic and/ or toxigenic microorganisms in food (Valero *et al.,* 2003; Soliman *et al.* 2002).

Such interest is justified given that there are estimated that 30% of the population each year in the industrialized countries suffer from diseases transmitted with food, and in 2000. according to the World Health Organization (WHO), at least 2 million people died from diarrhea worldwide.

As one of the ways overcome this problem is the the constant development of new application available natural antimicrobial substances (Skocibusic *et al*., 2006).

Essential oils are aromatic, light volatiles substances that are present in varying amounts in each plant with strong fragrance. They can be found in all parts of plants, but they are not evenly distributed. They may be concentrated in the one plant part (flowers, leaves, seeds, roots, bark) (Kitic, 2010, Kovacevic, 2004.).

The amount of essential oils in the herbs varies within a wide range. Herbs or their essential oils as a food additive are used to achieve appropriate sensorial characteristics, but also can provide adequate stability and quality of food. (Rabsch *et al.,* 2001, Fasseas MK *et al.*, 2008, O'Brien, 2002, Gutierrez *et al.,* 2009)

The aim of this study was to determine the antimicrobial activity of various antimicrobial substances or antibiotics against *Staphylococcus aureus*, as well as to determine the effect of the essential oils of different plant species used in the food and pharmaceutical industries on the growth of this pathogen.

MATERIAL AND METHODS

Essential oils
For the purpose of the study essential oils of fennel, thyme, cinnamon, cloves (BEOLAB, Serbia) eucalyptus, mint, (PHARMAMED, Bosnia and Herzegovina) rosemary (MEILAB, Serbia), anise (TIMKA, Germany) lemon (BLAGOLEKS, Bosnia and Herzegovina) and orange (PRIMAVERA, Germany) were used. All essential oils were purchased from the local pharmacies. Essential oil is used as a pure, mixed with 96% alcohol in the proportion 1:1, and mixed with alcohol in the ratio of 2:1.

Antibiotics used for the study
For the testing of antibiotic sensitivity of the test microorganism, commercial paper discs impregnated with penicillin, tetracycline, amikacin, ampicillin, amoxicillin, streptomycin, ciprofloxacin, cefuroxime, lincomicin, oxacilin, fucidic acid, clindamycin, ofloxacin, erythromycin, vancomycin and bacitracin were used (LIOFILCHEM s.r.l., Italy).

Test microorganism
Reference culture cultivated from the reference strain of *Staphylococcus aureus* subsp aureus 1884. ATCC 25923, batch No. 03/10 (BCCM™/LMG BACTERIA COLLECTION, Belgium) was used for the test. From this culture appropriate working culture was made by inoculation of nutrient broth followed by incubation at 37 ^{0}C during 18 h. Total volume of 0.1 ml of the broth culture, having 10^9 cfu/ml, was inoculated onto the test plate of nutrient agar.

Culture media
Media used for the test were nutrient broth and nutrient agar (code No. 1216 and 1117, respectivelly, Laboratorios CONDA S.A., Spain).

Test methods
To investigate possible inhibiting effect of the essential oils on the growth of *Staphylococcus aureus,* diffusion method was used. The oils were poured into metal cylinders with a diameter of 9 mm placed on the solid medium surface (nutrient agar) inoculated with reference culture of *Staphylococcus aureus*.

The surface of Petri plates is inoculated by broth culture of the test microorganism as described above. The nutrient medium was left still for 15 minutes. Immediately after that, the sterile metal cylinders were put onto the surface of the agar. Volume of 10µl of each essential oil and their alcohol mixtures were poured into the cylinders by micropipette. As a control, one cylinder with 10 µl 96% alcohol was used. For each essential oil were performed three repetitions.

In addition to the essential oil tests, commercial antimicrobial discs were used to get results for a comparison.

All Petri plates are incubated for 24 hours at 37ºC. After incubation reading of the results were performed by measuring inhibition zone diameters. The mean value was calculated for each type of essential oil.

RESULTS AND DISCUSSION

The results obtained by examining the influence of different antimicrobial compounds on the growth of *Staphylococcus aureus* are shown in Table 1.

Ciprofloxacin is the antimicrobial substance which showed most prominent zone of inhibition of *Staphylococcus aureus* growth. Zones of inhibition of other antimicrobial compounds have shown great diversity, and are diameter ranged from 9.5 mm in the case of lincomycin and vancomycin, to 32.5 mm in the the case of ciprofloxacin. *Staphylococcus aureus* was resistant to the effects of bacitracin.

The effect of ciprofloxacin, as antimicrobial substance which showed zone of inhibition, was used to compare the activities of essential oils.

Table 1. The inhibition zones (mm) of antibiotics for the growth of Staphylococcus aureus

Antibiotic	The inhibition zone (mm) *	Antibiotic	The inhibition zone (mm) *
Penicillin	10,0	**Lincomycin**	**9,5**
Tetracycline	25,0	Oxacillin	10,0
Amikacin	23,6	Fucidic acid	18,0
Ampicillin	25,0	Clindamicyn	10,0
Amoksicilin	31,3	Ofloksacin	23,3
Streptomycin	28,5	Erythromycin	24,0
Ciprofloxacin	**32,5**	Vancomycin	**9,5**
Cefuroxim	20,0	Bacitracin	-

*The values are given in mm and the mean value of the zone of inhibition for the three measurements

The results obtained by testing the effect of various essential oils on the growth of *S. aureus* are shown in Table 2.

Table 2. The inhibition zone (mm) of etheric oils for the growth of Staphylococcus aureus

Plant	Etheric oil	Etheric oil:ethanol 1:1	Etheric oil:ethanol 1:2
Cinnamomum zeylanicum	33,3	36,6	**37,6**
Thimus vulgaris	**33,3**	21,6	20,3
Rosmarinus officinalis	21,6	26,6	**27,6**
Eugenia aromatica	**21,5**	**21,5**	15,0
Foeniculum vulgare	5,0	**6,5**	**6,5**
Mentha piperita	**15,0**	12,0	**15,0**
Citrus limonum	-	-	-
Eucalyptus meliodora	-	-	-
Citrus sinensis	-	-	-
Pimpinela anisus	-	-	-

*The values are given in mm and the mean value of the zone of inhibition for the three measurements

From the table 2, it can be seen that a noticeable difference in the activity of pure essential oils and alcoholic solutions of essential oils. Essential oils of cinnamon and rosemary, showed the highest activity against *Staphylococcus aureus* when was applied essential oil: alcohol in the proportion of 2:1.
In clove, same effect had the same essential oil and essential oil: alcohol in the proportion of 1:1, while the mint same effect achieved combined pure essential oils and essential oil: alcohol in the proportion of 2:1.
The essential oil of fennel has shown highest activity against *Staphylococcus aureus* in alcoholic solution of essential oils, regardless of proportions.
Essential oils of lemon, eucalyptus, orange and anise showed no effect on the growth of *Staphylococcus aureus.*
Figure 1 shows the results of comparing the maximum inhibition zones of certain essential oils, regardless of whether they are in the pure state or alcoholic solution with inhibitory zone of most effective antimicrobial substance – ciprofloxacin.
As is evident, cinnamon and thyme have more prominent antimicrobial activity against *Staphylococcus aureus* than ciprofloxacin. Because of these characteristics of etheric oil of cinnamon and thyme are extracted in relation to other tested oils, however, it is necessary to

point out that the essential oils of clove and rosemary has shown good results in the inhibition of growth of test bacteria.
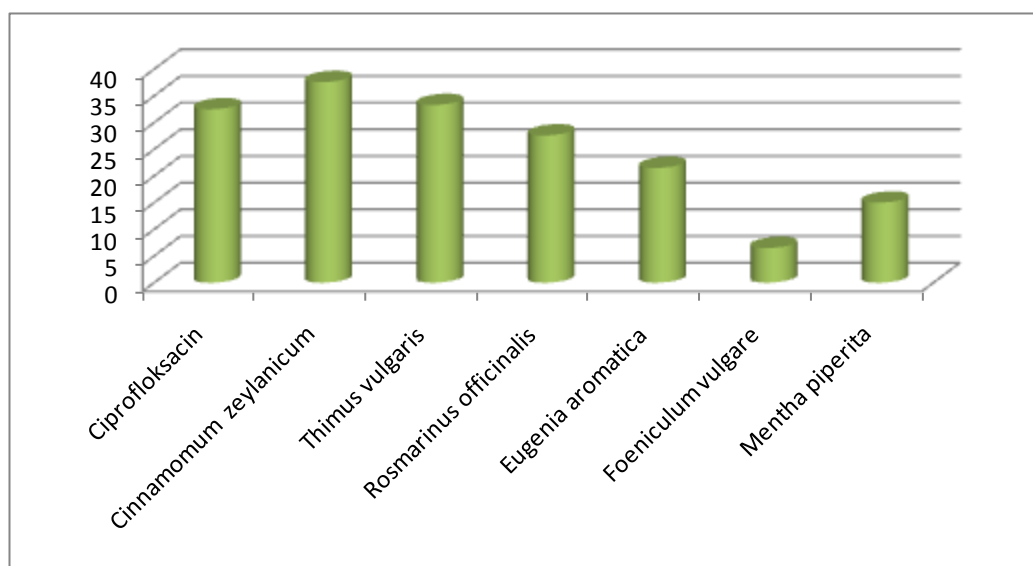
Figure 1. The comparation of maximal inhibition zones of etheric oils and antibiotic ciprofloxacin, mm

Results of our research are consistent with other researchers who are engaged in studies of influence of essential oils on the growth of pathogenic bacteria (Burt and Reinders, 2003; Dorman and Deans, 2000, Jovanović, Milosevic-Djurdjevic, 2008, Miletic *et al.,* 2009; Mitic *et al.*, 2005).

Based on the results obtained, the essential oils of cinnamon, thyme, rosemary and cloves can be recommended for use in pharmaceutical and food industries, in order to prevent the development of *Staphylococcus aureus* and diseases that these bacteria can cause.

CONCLUSION

Based on the performed tests it can be concluded that some antibiotic substances have not demonstrated effect on the growth of *Staphylococcus aureus*. Highest antimicrobial activity showed ciprofloxacin (32.5 mm).

Of all the tested essential oils, the strongest antimicrobial activity to inhibit the growth of *Staphylococcus aureus* has shown essential oil of cinnamon, as well as alcoholic solution in the proportion of 2:1 (37.6 mm) and pure essential oil of thyme (33.3 mm) and the size of the inhibition zones were most similar the effect of ciprofloxacin.

Essential oils of lemon, eucalyptus, orange and anise had no influence on the growth of *Staphylococcus aureus*.

On the basis of this investigation, it can be concluded that individually or combination of herbal essential oils can provide an effective mixture to inhibit the growth of *Staphylococcus aureus*, which achieves adequate guarding and preserving of food. Also, it may be recommend the use of cinnamon, thyme rising, rosemary and cloves in the pharmaceutical industry, and for the purpose of their use in the treatment of diseases caused by staphylococci.

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THE INFLUENCE OF FAT REPLACER ON INSTRUMENTAL PROPERTIES OF GLUTEN-FREE COOKIES

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ABSTRACT

A present study was conducted to investigate colour and texture of reduced-fat gluten-free cookies in which part of vegetable fat (30%, 40% and 50%) had been replaced by ground fibre-containing soybean bran in comparison to fullfat (no fat replacement) control cookies.

The colour of investigated reduced-fat gluten-free cookies was significantly darker compared to the fullfat control cookies indicating a positive effect of soybean bran on cookie colour making them more acceptable to the consumers.

The textural properties of the examined cookies indicate that increasing amounts of soybean bran in reduced-fat gluten-free cookies increased their fracturability and hardness compared to the full-fat cookie sample.

The obtained results indicated that the soybean bran could be successfully incorporated into the gluten-free cookie formulation as the fat replacer in terms of textural and colour properties.

Keywords: gluten-free cookies, fat replacer, soybean bran

INTRODUCTION

Coeliac disease is intolerance to the gliadin fraction of wheat and the prolamins of rye, barley and possibly oats (Marray, 1999). Therefore, celiac disease patients are recommended to be on a strict long-life diet, which usually lacks in certain essential nutrients (Thompson *et al.*, 2005). Insufficient amounts of nutrients in gluten-free products can be overcome by their fortification to achieve a balanced diet, i.e. to obtain added value products.

Cookies are very popular because they are ready-to-eat food which is characterized by long shelf-life and they are practical for storage and handling. They possess a high amount of vegetable fat (20-30% on flour weight basis) as one of the main ingredients besides flour, sugar, and water in biscuit or cookie formulation (Maache-Rezzoug *et al.*, 1998; Sudha *et al.*, 2007). Fat has a multiple function in cookies being responsible for tenderness, quality and texture of the final product (O'Brien, 2003). In addition, fat influences the rheological properties of cookie dough (Jissy, Leelavathi, 2007). Mechanical properties of biscuits depend on the fat component in cookie formulation (Baltsavias, Jurgens, van Vilet, 1999). Regarding the sensory properties fat contributes along with other ingredients to texture, mouth feel and overall sensation of lubricity of the product (Giese, 1996; Stauffer, 1998). Its presence is very important in the development of cookie dough for gluten-free products due to the specificity of gluten-free ingredients (Gallagher, Gormley, Arendt, 2004).

Despite the important role of fat in cookie formulations, high fat intake is usually followed by various health disorders such as obesity, high blood cholesterol and coronary heart diseases. High-fat foods are rich in calories which may be problematic for people struggling with energy balance (Giese, 1996). In addition, fat-containing products are not recommended to the patients who suffer from hyperlipidemia which leads to the development of blood vessel diseases and cardiovascular diseases, causing the highest morbidity and mortality worldwide (Kuulasmaa *et al.*, 2000; Menotti *et al.*, 2007). Due to this reason, many efforts have been made to reduce the fat content in foods regardless of the food category (gluten-containing or gluten-free foods) and replace it with various fat replacers (Shaltout, Youssef, 2007).

Fat could be partially substituted by ingredients that manifest functional properties similar to fat, while creation of a high-quality fat-free product seems impossible (Shukla, 1995). Therefore, fat replacers are recommended to replace up to 50% of fat in sweet bakery products (Zoulias, Oreopoulou, Tzia, 2002).

In creating of bakery products many fat replacers and mimetics can be used to replace fat or to imitate physical, textural and sensory properties (especially mouth feel) of real fats (Owisu-Apenten, 2005). Fibre based fat replacers are widely used in sweet bakery products regardless of the type and origin. Microcrystalline cellulose, methylcellulose gums (methylcellulose and hydroxypropylmethyl cellulose), pectins and hydrocolloid gums are some of commercially available fibre based fat mimatics (Shaltout, Youssef, 2007). On the other hand, there has been an increasing interest in recovering fibre-containing byproducts which may be used as an alternative source of fibre or as potential ingredients for fat replacement (Martínez-Cervera *et al.*, 2011). Peach fibre (Grigelmo-Miguel, Carreras-Boladeras, Martin-Belloso, 2001), potato pulp (Kaack, Pedersen, 2005), potato peels (Arora, Camire, 1994) and pectin-enriched materials from apple pomace (Min *et al.*, 2010) represent some of investigated fat replacers obtained by fruit and vegetable processing. Cereal milling fractions rich in fibre can also serve as the fat replacers. The studies with corn bran fibre (Jung, Kim, Chung, 2005), soluble fibre from corn and oats (Warner, Inglett, 1997) and fibre gel produced from rice bran (Inglett *et al.*, 2004) were conducted to investigate the possibilities of their utilization as the fat replacers.

The special benefit of fibre-containing byproducts used as the fat replacers is their fibre abundance which can significantly contribute to the functionality of the obtained low-fat products, especially in the category of gluten-free products known for their low nutritional and functional profile.

Soybean bran is obtained as a byproduct in soybean production. Having an insight into its proximate composition and dietary fibre content (Honig, Rackis, 1979), it seems that soybean bran could be used as a fibre-containing fat replacer in cookie formulation. No research has been conducted exploring the use of this type of bran as the fat replacer. In addition, the absence of gluten in soybean bran classifies it as a potential fat replacer in gluten-free food production.

The aim of the present study is to assess the effects of ground fibre-containing soybean bran used as a fat replacer at the level of 30%, 40% and 50% in the gluten-free cookie formulation on 1) the colour and 2) the textural properties of the produced gluten-free cookies.

MATERIAL AND METHODS

Materials
Soybeen bran is a byproduct from soybean production which was obtained from AD "Sojaprotein", Bečej, Serbia. Gluten-free mixture consisting of corn starch, corn flour, potato starch, potato flour, rice flour, guar gum, baking powder, and salt was obtained from "Nutri allergy center", Zemun, Serbia. Vegetable fat, glucose syrup, baking powder, salt, soy lecithin, corn grits and spices were commercially available.

Gluten-free cookie preparation
All ingredients were weighed together and mixed for 10 min in Farinograph mixing bowl (Brabender, Germany) tempered at 30 °C. The obtained dough was further processed. Pilot scale dough sheeter (Mignon, Italy) was used to sheet the dough to the desired thickness (4.5 mm). Cookies were shaped using a cutter (40×30 mm) and baked at 220 °C for 2 min and then at 160 °C for 14 min in a laboratory oven (MIWE gusto® CS, Germany).The obtained gluten-free cookies were first left to cool down at room temperature for 2 h and then they were packed and stored for 24 h in sealed polypropylene bags before the analysis. The control cookie formulation contained the following ingredients at the indicated level: gluten-free mixture, 100 g; vegetable fat, 30.18 g; glucose syrup, 10.71 g; spices, 4.64 g; corn grits, 3.57 g; salt, 1.79 g; soy lechitin, 0.71 g; baking powder, 0.54 g; and water, 43 g. In reduced-

fat gluten-free cookie formulations 30%, 40% and 50% of vegetable fat was replaced by an equal amount of the fat mimetic (soybean bran).

Colour evaluation of cookies

The colour of the cookie samples was measured using a Minolta Chromameter (Model CR-400, Minolta Co., Osaka, Japan) with granular attachment CR-A33b. All the samples were illuminated with D65-artificial daylight (2° standard angle). The colour values were expressed as L* (lightness/darkness), a* (redness/greenness) and b* (yellowness/blueness). The colorimeter was calibrated against a standard white plate (Y=84.8, x=0.3199, y=0.3377). The total colour difference (ΔE) between the reference (control) and cookie samples with soybean bran was calculated according to the following equation:

$$\Delta E = [(L_0^*-L^*)^2 + (a_0^*-a^*)^2 + (b_0^*-b^*)^2]^{1/2}$$

The parameters L_0^*, a_0^* and b_0^* refer to reference value, and L*, a* and b* refer to colour values of various cookies samples with added fat replacer.

The values used to determine whether the total colour difference was visually obvious were the following (Francis, Clydesdale, 1975):

ΔE < 1 – colour differences are not noticeable by the human eye

1 < *ΔE* < 3 – colour differences are not obvious to the human eye

ΔE > 3 – colour differences are obvious to the human eye.

Textural characteristics of cookies

Texture of cookie samples was measured with a texture analyzer (TA.XT Plus, Exponent Stable Micro System, UK) by using a compression test. For these measurements, texture analyser was equipped with a 30 kg load cell and 3-point bending rig (HDP/3PB). The settings used were: Option: Return to start; Test speed: 0.50 mm/s; Post-test speed: 5 mm/s; Distance: 5 mm; Trigger type: auto; Data acquisition rate: 500 pps. The maximum peak force from the force-distance graph was considered to be an indication of overall 'hardness' of the cookie samples and the linear distance was considered as an indication of 'fracturability'. Five repeat measures were taken for each type of cookie.

Hydroxymethylfurfural (HMF) analysis

Sample preparation

The extraction procedure was performed according to the method proposed by Rufián-Henares, Andrade and Morales (2006) with the modifications which were done by Petisca *et al.* (2014). Ten grams of sample was suspended in 5 mL water:methanol (70:30). The mixture was thoroughly stirred during 1 min and then 2.0 mL Carrez I and Carrez II solutions were added and centrifuged at 5000 rpm (4 °C) during 15 min, recovering the supernatant to a 15 mL flask. Two more consecutive extractions were made with 2 mL of water:methanol (70:30) until collecting 10 mL of supernatant. Two millilitres of this solution were centrifuged at 8000 rpm for 15 min before being analysed.

HPLC-DAD analysis

The chromatographic separation and quantification of HMF was performed using the HPLC method described by Ariffin, Ghazali and Kavousi (2014) and Tomasini *at al.* (2012) with some modifications.

Liquid chromatograph (Agilent 1200 series), equipped with a DAD detector and an Eclipse XDB-C18, 1.8 μm, 4.6 × 50 mm column (Agilent) was used for quantification of hydroxymethylfurfural in obtained extracts. Separation of the analyte was achieved with a column temperature of 30 °C and sample injection volume of 2 μL. The mobile phase consisted of two eluents, H_2O (0.1% HCOOH) (A) and methanol (B), delivered at a flow rate of 0.75 mL/min. The isocratic elution was employed with the ratio A:B (90:10, v/v). The DAD wavelength was set at 284 nm. The total run time of the analysis was 5 min.

RESULTS AND DISCUSSION

The soybean bran is fibre origin ingredient which was used at the level of 30%, 40% and 50% in the gluten-free cookie formulation to replace fat and imitate its functional and sensory properties with the aim to provide less calories compared to full-fat cookies.

Previous studies demonstrated that the textural properties of reduced-fat cookies varied depending on the type of fat replacers. Pectin-enriched materials from apple pomace used as a fat replacer up to 30% by the weight of shortening in cookie formulation contributed to a more tender texture and lighter surface colour (Min *et al.*, 2010). On the contrary, polydextrose and Dairytrim provided harder cookie texture (Zoulias, Oreopoulou, Tzia, 2002) and, therefore, these carbohydrate- and protein-based fat mimetics are not recommended to be used in high amounts as the fat replacers. Furthermore, the utilization of the jet-cooked oat bran did not result in significant difference in cookie hardness up to 20% shortening replacement but the cookies became lighter in colour as the replacer content was increased (Lee, Inglett, 2006).

The textural profiles of the examined cookies are shown in Figure 1. The values of hardness and fracturability increased with increasing levels of fat replacer in cookie formulation. Chevallier, Colonna and Lourdin (2000) considered that dough with higher content of fat has higher level of incorporated air and, consequently, lower density making cookies less hard. Pareyt *et al.* (2009) showed that fat reduction increased elastic properties of cookie dough and reduced cookie spread which would be responsible for more compact cookie structure. The force required to break biscuits containing 50% less fat than the control sample was almost three times higher than that required to break the control biscuits. This finding was in agreement with earlier results obtained by Sudha et al. (2007) and is also in line with our results.

In general, the textural measurement indicates that utilization of soybean bran as the fat replacer resulted in cookies with less tender texture suggesting its lower amounts in reduced-fat gluten-free cookies as acceptable.
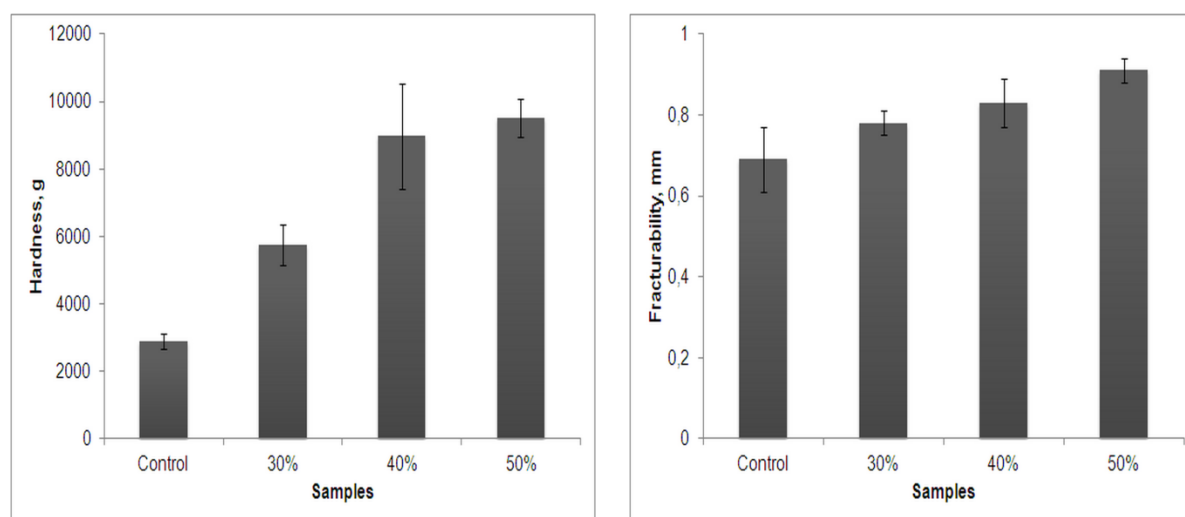
Figure 1. Textural properties (hardness and fracturability) of cookie samples
Vertical bars show ± SE of means.

Table 1 shows the effect of soybean bran on the surface colour of cookies. Fat replacement with soybean bran caused significant changes in L*, a* and b* values. The fat replacer provided significantly darker, less yellow and more red cookies compared to the control sample.

Table 1. Colour parameters of cookie samples

	L*(D65)	**a*(D65)**	**b*(D65)**	**ΔE**
Control cookie	83.12[c]	-0.47[a]	23.98[c]	
30%	74.67[b]	1.30[b]	22.70[b]	8.72
40%	71.41[a]	1.89[c]	22.30[ab]	12.06
50%	71.10[a]	2.05[d]	22.13[a]	12.42

Different letters in superscript within column indicate significant differences between cookie samples by LSD test (p < 0.05).

The colour changes in examined cookies resulted only from the increasing amounts of soybean bran (L*=73.32, a*=3.24, b*=24.00) in cookie formulation, i.e. they could not be addressed to the development of Maillard reaction products. Although thermal processing of foods, such as baking, results in the formation of Maillard reaction products, which are essential for final sensory attributes, such as surface colour, texture and flavour (Lindenmeier, Hofmann, 2004; Petisca *et al.*, 2014), it seems that gluten-free cookie formulation lacks compounds that are required as the reactants in Maillard reaction. This statement derives from the fact that neither HMF nor furfural was detected in examined cookie samples. These compounds are commonly studied as Maillard reaction and sugar pyrolysis intermediates formed during the thermal processing of foods (Capuano, Fogliano, 2011). The absence of Maillard reaction markers in all examined cookies and colour changes in reduced-fat cookies compared to full-fat ones indicate a positive effect of soybean bran on cookie colour making them more acceptable to the consumers.

CONCLUSIONS

The measurements performed in this study revealed that soybean bran could be successfully used as the fat replacer in the gluten-free cookie formulation, resulting in cookies of acceptable determined textural and colour characteristics.

ACKNOWLEDGEMENTS

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ANTHOCYANINS PROFILING BY HPLC-DAD-ESI/MS^n AFTER BIOTIC ELICITOR TREATMENTS IN *BRASSICACEAE* SPROUTS

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ABSTRACT

The anthocyanin profiles of three varieties of 8-day-old *Brassicaceae* sprouts, broccoli (*Brassica oleraceae*), "China rose" radish and "Rambo" red radish (*Raphanus sativus*) were studied using HPLC-DAD-ESI-MS^n and HPLC-DAD after biotic elicitor spray treatments with methyl jasmonate (25µM), jasmonic acid (150µM), salicylic acid (100µM), sucrose (176mM), glucose (277mM) and DL-methionine (5 mM). Ready-to-eat sprouts were treated during 5 days before harvest (day 3 to 7).

The most abundant type of anthocyanins was cyanidin and its derivatives, diglycosylated at C-3 and glycosylated at C-5, mainly acylated at 3 position (one or two cinnamoyl groups) and malonyl at hexose in 5 position, with qualitative and quantitative differences among varieties. All individual anthocyanins identified were increased by elicitor treatments, leading to the observed increase of total anthocyanin content (TAC). An increase of 40% of TAC was achieved after MeJA treatment in broccoli (17.51 mg · 100g F.W.), and by 50% in China rose radish (24.79 mg · 100g F.W.) and 30% Rambo red radish (241.77 mg · 100g F.W.) after glucose treatment.

The selection of ready-to-eat cruciferous sprouts rich in anthocyanins, as well as the appropriate elicitor treatment, is a candidate strategy to develop novel plant foods with beneficial nutritional and health properties.

Keywords: Brassica, Raphanus, anthocyanin, elicitors, sprouts.

INTRODUCTION

Even *Brassicaceae* vegetables are mainly recognized for their high content in glucosinolates, also these foods are rich in phenolic compounds and other nutrients and bioactive compounds (Manchali, *et al.*, 2012). Within phenolic compounds, anthocyanins are water-soluble flavonoids, that usually exist in plants in the form of glycosides and, not only are directly responsible of red and purple color of plants, but also protect against biotic and abiotic stresses (Harbone and Williams, 2000). Cyanidin and its derivatives, which possess two hydroxyl groups on the B-ring, are the most widely distributed among species. Anthocyanins content is affected by cultivars, environmental factors and the application of stresses, as elicitors. Exogenous application of elicitors has been considered as a suitable strategy for increase the content of secondary metabolites in the plants (Guo *et al.*, 2011a; Pérez-Balibrea, *et al.*, 2011). Broccoli, "Rambo" red radish and "China" rose radish sprouts, with different colour aspect, were selected as varieties with high amount of bioactive compounds, in order to study their anthocyanins pigments, and their increase after elicitation with phytohormones, sugars and the amino acid methionine. Considering the relatively low anthocyanin content in vegetables, creating new anthocyanin-rich varieties as a natural food, may contribute to stable intake of these compounds.

MATERIAL AND METHODS

Plant material

Seeds of broccoli (*Brassica oleracea* L. var. italica), red "Rambo" radish and "China rose" radish (*Raphanus sativus* L.) were provided by Intersemillas S.A. (Valencia) with commercial quality for sprouting lines. Hydrated seeds were spreaded on trays with cellulose and

irrigated with distilled water with sodium hypochlorite (5g·L^{-1}) for 8 days in a controlled environment chamber (16h light, 25ºC, 60%RH - 8h dark, 20ºC, 80%RH cycle; 400µmol·m^{-2} s^{-1} PAR) . Three replicates per treatment with elicitors were rapidly collected at day 8 stored at -80ºC prior analyses.

Elicitors
Jasmonic acid (JA, from SIGMA-ALDRICH) (150 µM), methyl jasmonte (MeJA, SAFC) (25 µM), salycilic acid (SA, Panreac) (100 µM), the oligosaccharides glucose (277 mM) and sucrose (146 mM) (SIGMA-ALDRICH), and the amino acid DL-methionine (Alfa Aesar GmbH) (Met, 5 mM) were selected as elicitors according to literature review. 30 mL of test solution per sample (10mL per tray), as exogenous spraying on the cotyledons, were applied from day 3 to 7 of sprouting, distilled water was used as control.

Sample extraction
Freeze-dried samples (100mg) were mixed with 1.5mL of methanol/water acidified with 1% formic acid (25:24:1, v/v/v). Samples were extracted in US bath for 1h, then, kept at 4ºC overnight, and sonicated again for 1h. After centrifugation (9500 xg, 5min), samples were filtered (0.45 µm PVDF) before HPLC analysis.

Anthocyanins analysis
According to Moreno, *et al.*, (2010), the identification of compounds was carried out in as Agilent HPLC-DAD 1100 with a mass detector in series. The mass spectrometry data were acquired in the positive ionization for anthocyanins (range from 100 to 1500 *m/z*). The quantification of these compounds was carried out in a HPLC-DAD system Waters, cyaniding 3-*O*-glucoside-β-glucopyranoside (Polyphenols, Norway) was used as external standard at 520 nm.

Statistical method
All analysis were conducted by triplicate, processed by SPSS 15.0. A multifactorial analysis of variance (ANOVA) and Ducan's Test were used to determine significant differences at *P* values < 0.05.

RESULTS AND DISCUSSION

The most abundant antchocyanins type identified was cyaniding derivatives, diglycosylated at C-3 and glycosylated at C-5 position, mainly with one or two acylated groups (sinapoyl, feruloyl, ρ-coumaroyl and caffeoyl), with quantitative differences among varieties. The qualitative (MS) study of [M]$^{+}$ *m/z* ions was carried out in HPLC-DAD-ESI-MS^{n}, allowing the detection of 14 anthocyanins in broccoli sprouts, 22 anthocyanins in "China" rose radish and 48 anthocyanins in red "Rambo" radish, however, only quantification results of the main anthocyanins are shown in this paper (Table 1).
All cyanidin compounds detected have been reported before in broccoli (Moreno *et al.*, 2014) while the composition of radish sprouts cultivars are reported here for the first time. Nonetheless, similar fragmentation patterns were found for cinnamoyl and malonyl derivatives anthocyanins (Matera, *et al.*, 2012; Park, *et al.*, 2014). A delphinidin-3,4-*O*-diglucoside was identified in broccoli sprouts for the first time. "China" rose radish not showed non-acylated anthocyanins. Red "Rambo" radish sprouts exhibited the widest range of anthocyanins, cyanidin was the predominant in this cultivar, also peonidin and delphinidin were found, according to Hanlon and Barnes (2011).

Table 1. Anthocyanins (mg·100g^{-1} F.W.) tentatively identified and quantified in broccoli, China rose radish and Rambo red radish sprouts by HPLC-DAD.

Compound	4.a. Broccoli							
	Control	MeJA	JA	SA	Glucose	Sucrose	Met	LSD$_{0.05}$
1. Cy 3-*O*-sophoroside-5-*O*-glucoside	0.38b	0.36b	0.27c	0.37b	0.58a	0.37b	0.36b	0.04***
2. Cy 3-*O*-(sinapoyl)soph-5-*O*-glc	0.72bcd	0.70cd	0.61d	0.68cd	0.98a	0.76bc	0.83b	0.04***
3. Cy 3-*O*-(*p*-coumaroyl)soph-5-*O*-glc	0.11bcd	0.12bc	0.13d	0.11cd	0.18a	0.12bc	0.13b	0.01*
4. Cy 3-*O*-(sinapoyl)soph-5-*O*-glc + Cy 3-*O*-(feruloyl)soph-5-*O*-glc	1.33c	1.63b	1.19c	1.26c	2.01a	1.35c	1.34c	0.05*
5. Cy 3-*O*-(*p*-coumaroyl, sinapoyl)soph-5-*O*-glc	2.00cd	2.14bc	1.76e	1.89de	3.05a	2.17bc	2.33b	0.07***
6. Cy 3-*O*-(feruloyl, sinapoyl)soph-5-*O*-glc	3.99bc	5.91a	3.53c	3.10c	5.76a	4.61b	5.63a	0.29***
7. Cy 3-*O*-(sinapoyl, sinapoyl)soph-5-*O*-glc	3.74c	6.65a	4.54b	3.02d	4.87b	4.61b	4.72b	0.23***
Total	12.27de	17.51a	12.03d	10.43d	17.43a	13.99bc	15.34b	0.59**
	4.b. China rose radish							
	Control	MeJA	JA	SA	Glucose	Sucrose	Met	LSD$_{0.05}$
1. Cy 3-*O*-soph-5-*O*-(malonyl)glc	0.03b	0.03ab	0.02b	0.03b	0.04a	0.03b	0.02b	0.004 n.d.
2. Cy 3-*O*-soph-5-*O*-(malonyl)soph	0.16cd	0.25a	0.17cd	0.21b	0.21ab	0.20bc	0.15d	0.12**
3. Cy 3-*O*-(caffeoyl)soph-5-*O*-(malonyl)glc	0.95de	1.08cd	0.60f	1.10c	1.29b	1.52a	0.84e	0.04**
4. Cy 3-*O*-(caffeoyl, feruloyl)sophe-5-*O*-(malonyl)glc	0.99cd	1.00d	0.88de	1.24b	1.52a	1.20c	0.85e	0.04***
5. Cy 3-*O*-(*p*-coumaroyl)soph-5-*O*-(malonyl)glc + Cy 3-*O*-(feruloyl)soph-5-*O*-(malonyl)glc + Cy 3-O-(sinapoyl)soph-5-O-(malonyl)glc	7.43c	10.7a	5.89d	8.48b	10.32a	9.72a	6.91c	0.33***
6. Cy 3-*O*-(disinapoyl)soph-5-O-(malonyl)glc + Cy 3-*O*-(caffeoyl, eruloyl)soph-5-*O*-(malonyl)glc	1.65c	1.84c	2.29b	2.48b	3.35a	1.74c	1.73c	0.13***
7. Cy 3-*O*-(feruoyl, sinapoyl)soph-5-*O*-(malonyl)glc + Pg-3-*O*-(feruloyl)soph-5-*O*-(malonyl)glc	2.49c	2.71b	2.58c	3.13c	3.77a	2.66c	2.17d	0.10***
8. Cy 3-*O*-(caffeoyl, sinapoyl)soph-5-O-(malonyl)glc + Cy 3-O-(caffeoyl, feruloyl)soph-5-O-(malonyl)glc	0.79e	0.9cd	1.11b	0.94c	1.22a	0.83de	0.65f	0.03***
9. Cy 3-O-(*p*-coumaroyl, sinapoyl)soph-5-O-(malonyl)glc or Cy 3-O-(diferuloyl)soph-5-O-(malonyl)glc	0.35cd	0.38bc	0.42ab	0.45a	0.44a	0.37c	0.3d	0.02**
Unidentified anthocyanins	0.99de	0.56e	1.64b	1.04c	2.63a	0.76cde	0.61de	0.12***
Total	15.83c	19.45b	15.65c	19.10b	24.79a	19.03b	14.23c	0.53***
	4.c. Rambo red radish							
	Control	MeJA	JA	SA	Glucose	Sucrose	Met	LSD$_{0.05}$
1. Cy 3-*O*-soph-5-*O*-glc	0.62b	0.52bc	0.6bc	0.79a	0.80a	0.56bc	0.49c	0.04***
2. Cy 3-*O*-(sinapoyl)soph-5-*O*-glc	1.1bc	1.03c	0.98c	1.40ab	1.57a	1.14bc	0.90c	0.09**
3. Cy 3-*O*-(sinapoyl)soph-5-*O*-(malonyl)soph	0.75d	0.79cd	0.95bc	1.37a	1.48a	0.99b	0.75d	0.06***
4. Cy 3-*O*-(sinapoyl)soph-5-*O*-glc	5.38cde	6.26b	5.11de	5.65bcd	8.35a	5.91bc	4.75e	0.21***
5. Cy 3-*O*-(feruloyl,sinapoyl)diglc-5-*O*-glc	5.3cd	5.53bc	4.75de	5.97b	7.61a	5.68bc	4.67e	0.18***

6. Cy 3-*O*-(feruoyl)soph-5-*O*-(malonyl)soph	11.27bc	11.29bc	10.77cd	12.5b	15.84a	11.61bc	9.63d	0.39***
7. Cy 3-*O*-(feruloyl, sinapoyl)soph-5-*O*-soph + Cy 3-*O*-(p-coumaryl)soph-5-*O*-(malonyl)glc	2.98c	2.98b	2.42d	3.28b	3.84a	3.15b	2.48c	0.09***
8. Cy 3-*O*-(p-coumaryl)soph-5-*O*-(malonyl)glc + Cy 3-*O*-(feruloyl, sinapoyl)soph-5-*O*-glc	23.5bc	24.04b	23.35bc	23.3bc	35.5a	24.82b	19.47c	1.37***
9. Cy 3-*O*-(sinapoyl)soph-5-*O*-(malonyl)glc + Cy 3-*O*-(feruoyl)soph-5-*O*-(malonyl)glc	50.37e	65.17b	53.49de	56.67cd	68.37a	57.53c	44.54f	1.05***
10. Cy 3-*O*-(p-coumaroyl, sinapoyl)soph-5-*O*-(malonyl)soph or Cy 3-*O*-(feruoyl, feruoyl)soph-5-*O*-(malonyl)soph	27.29c	28.82bc	22.93d	29.17bc	35.62a	30.50b	23.31d	0.59***
11. Cy 3-*O*-(feruoyl, sinapoyl)soph-5-*O*-(malonyl)glc	26.27c	28.96b	25.20c	25.19c	31.28a	26.4c	22.88d	0.66***
12. Cy 3-*O*-(p-coumaroyl, sinapoyl)soph-5-*O*-(malonyl)glc + Cy 3-*O*-(p-coumaroyl, sinapoyl)soph-5-*O*-(malonyl)soph or Cy 3-*O*-(p-coumaroyl,sinapoyl)soph-5-*O*-(malonyl)soph + Cy 3-*O*-(feruoyl, sinapoyl)soph-5-*O*-(malonyl)glc	15.19bc	15.87ab	13.22d	14.60bcd	17.34a	16.79a	14.16cd	0.47***
13. Cy 3-*O*-(p-coumaroyl, sinapoyl)soph-5-*O*-(malonyl)glc	3.41bc	3.35bc	2.78d	3.76b	4.39a	3.68b	2.98cd	0.18***
Unidentified anthocyanins	8.1c	6.79d	12.64a	7.96c	9.78a	8.10c	10.24b	0.34***
Total	181.5c	201.4b	179.2c	194.9b	241.8a	196.9b	161.3d	3.81***

[A]Mean values (n=3). a-d, Different lowercase-letters mean statistically significant differences between treatments.
[B], Least Dignificant Difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect.
Anova p value, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. $p > 0.05$
Abbreviations: Cyanidin (Cy), Pelargonidin (Pg), Sophoroside (Soph), Glucoside (Glc).

Anthocyanins were tentatively identified and quantified in HPLC-DAD by comparing retention times and spectra with the results of HPLC-MS^n identification. The most abundant anthocyanins in broccoli displayed M$^+$ at *m/z* 1125 (Cyanidin-3-*O*-(p-coumaroyl, sinapoyl)sophoroside-5-*O*-glucoside), 1155 (Cyanidin-3-*O*-(feruloyl,sinapoyl)sophoroside-5-*O*-glucoside) and 1185 (Cyanidin-3-*O*-(sinapoyl,sinapoyl)sophoroside-5-*O*-glucoside), representing about 10 mg·100g^{-1} F.W. of diacylated anthocyanins of the total (12.3 mg·100g^{-1} F.W.). "China" rose radish showed a most abundant anthocyanins at minute 30.5, comprising the elution of compounds with M$^+$ at *m/z* 1005 (Cyanidin-3-*O*-(p-coumaroyl)sophoroside-5-*O*-(malonyl)glucoside), 1035 (Cyanidin-3-*O*-(feruloyl)sophoroside-5-*O*-(malonyl)glucoside), 1065(Cyanidin-3-*O*-(sinapoyl)sophoroside-5-*O*-(malonyl)glucoside), representing 7.4 mg·100g^{-1} F.W., from the total (15.8 mg·100g^{-1} F.W.). The relevant anthocyanins in "Rambo" red radish sprouts showed M$^+$ also at *m/z* 1035 and 1065 (retention time 32'), representing almost 30% of the total (181.5 mg·100g^{-1} F.W.) (Table 1).

MeJA elicitor showed higher effects in *Brassicaceae* sprouts, increasing the total anthocyanins content (TAC) by 43%, 23% and 11% in broccoli, "China" rose radish and "Rambo" radish, respectively. JA and SA treatments not affected *Brassicaceae* sprouts, only SA increased TAC by 21% and 7% in "China" rose radish and "Rambo" red radish, respectively. The phytohormones, as signaling molecules, play an important role in plant defense signal transduction pathways, leading the biosynthesis of secondary metabolites from the stimulation of the PAL pathway (Beckers *et al.*, 2006).

Glucose and sucrose effectively enhance TAC in all varieties. Broccoli sprouts showed an increase of 40% and 14% of TAC, "China" rose radish an increase of 26% and 44%, and "Rambo" red radish an increase of 33% and 8%, after glucose and sucrose treatments, respectively. Guo *et al.*, (2011b), showed a higher transcription level of PAL in broccoli sprouts treated by sucrose compared to a control. Also osmotic stress could alter the

concentration of secondary metabolites in plant tissues, enhancing anthocyanin amounts (Guo *et al.*, 2011b), however, the induction of anthocyanin synthesis genes is likely to be sugar specific (Solfanelli, *et al.*, 2006).

The elicitor DL-methionine only showed an increase of 25% in TAC in broccoli sprouts and no effects were found in radish varieties. Danc, *et al.*,(2008) found reduced amounts of anthocyanin pigments in potato tubers after enhance methionine content due to the expression of specific genes.

CONCLUSIONS

The results supported the hypothesis that anthocyanin synthesis may allow the plant to develop resistance to some elicitor treatments by induction of anthocyanin biosynthesis gene expression and/or the stimulation of the PAL pathway. All individual identified anthocyanins were increased by elicitor treatment, leading to the observed increase of TAC. MeJA and sugars were considered the most effective elicitors in broccoli and radish sprouts, respectively. The selection of ready-to-eat cruciferous sprouts rich in anthocyanins, as well as the appropriate elicitor treatment, result novel strategies to develop plant food with beneficial nutritional and health properties.

ACKNOWLEDGEMENTS

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ANTIPROLIFERATIVE EFFECTS AND METABOLISM OF SULFORAPHANE AND GLUCORAPHANIN FROM BROCCOLI SPROUTS IN HUMAN COLON AND LIVER CANCER CELLS

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ABSTRACT

Broccoli sprouts (BS) provide a good source of glucosinolates, mainly glucoraphanin (GRA), which is hydrolyzed to the biologically active metabolite sulforaphane (4-methylsulfinylbutyl isothiocyanate, SFN). SFN is a naturally occurring cancer chemopreventive agent. The purpose of this study was to obtain the effective dose of a BS extract, the glucosinolate GRA and its metabolite SFN, which showed to be active in inhibiting the growth of three cancer cell lines, Caco-2 and HT-29 human colorectal carcinoma cells; and HEPG2 hepatocellular carcinoma cells by using the colorimetric assay technique MTT. The dose-dependent assay allowed establishing the half maximal inhibitory concentration (IC_{50}) for BS extract (1.67 µM, 1.62 µM and 3.2 µM, in Caco-2, HT-29, HEPG2 cell lines, respectively), and SFN (37.5 µM, 50.9 µM and 69.9 µM was observed in Caco-2, HT-29 and HEPG2 cells, respectively), however, glucoraphanin did not show an antiproliferative effect in the cells under study.

The absorption of sulforaphane metabolites from broccoli sprouts (SFN, SFN-glutathione and SFN-cysteine) by HEPG2 and Caco-2 cells during 3, 6, and 24h was assessed by a selective UHPLC/MS/MS procedure. Concentrations ranging 2 to 90 nmol/L were found within the cells, and concentrations ranging 1 to 60 nmol/L were found released in the medium, depending on the type of analyte under study.

The broccoli sprout extract and its bioactive isothiocyanate sulforaphane effectively inhibit proliferation of cancer cell lines: HT-29 and Caco-2 human colon cells; and HEPG2 human liver cells. Cells are capable of conjugative metabolism, since the SFN mercapturic derivatives could be identified in the incubation medium of HEPG2 and Caco-2 cell lines.

Keywords: *Brassica, Cytotoxicity, Absorption*

INTRODUCTION

There is consistent epidemiological evidence that a diet rich in vegetables, particularly cruciferous, is inversely related to the risk of suffer certain types of cancer. The chemopreventive benefit of these vegetables is attributed in part to the glucosinolates (GLS), however, the biological activity correspond to their hydrolysis metabolites isothiocyanates (ITC), which are known to stimulate Phase II carcinogen detoxifying enzymes, such as glutathione S-trasferase (GST) and quinone reductase. The most potent stimulator of such enzymes found in broccoli sprouts is sulforaphane (SFN) (1-isothiocyanato-4-(methylsulfinyl)-butane). Also the chemoprevention of SFN by blocking initiation via inhibiting phase I enzymes has been studied. Once cancer is initiated, SFN can act via several mechanisms that modulate cell growth and cell death signals to suppress cancer progression (Clarke, *et al.*, 2008). The inactive precursor glucoraphanin (GR) is hydrolyzed to sulforaphane (SFN) by the thiohydrolase myrosinase, found endogenously in broccoli, or by the microflora present in the colon.

The mechanisms by which isothiocyanates might exert their anticarcinogenic effects remain unclear and the evaluation of anticancer activity (antiproliferation) of SFN is very limited. Some recent results suggest that the chemopreventive activities of isothiocyanates may

involve other mechanisms in addition to the activation of detoxifying enzymes activities, as specific mechanisms that could also act at the DNA level or affect signal transduction pathways leading to growth arrest or cell death (Gamet-Payrastre *et al.*, 2000).

Caco-2 cells feature many characteristics of intestinal epithelial cells, representing a widely accepted in vitro system for the human metabolism. SFN and other ITC are known to be metabolized in the enterocytes and the liver through the mercapturic acid pathway (Angelino & Jeffery, 2014). An initial reaction between the –N=C=S group of ITC and the cysteine sulfhydryl group of glutathione (GSH) can take place spontaneously and enhanced by glutathione S-transferase (GST). SF-GSH metabolites are found in plasma and urine. The liver is able to carry out enzymatic modifications of the GSH moiety, forming cysteinylglycine-(cys-gly), cysteine-(cys); and the final N-acetyl-cysteine-(NAC-) conjugate is formed in the kidney.

The aim of this work was to examine the effect of lyophilized BS, as a food matrix, the glucosinolate GRA, and its metabolite SFN on the proliferation of three cancer cell lines, Caco-2 and HT-29 human colorectal carcinoma cells; and HEPG2 hepatocellular carcinoma cells using the colorimetric assay method MTT. Different concentrations of BS, GRA and SFN were tested in order to find their IC_{50}. The development of human preventive studies focusing on the components of cruciferous vegetables would be advisable if an inhibitory effect was detected *in vitro*. In this study also the absorption of SFN metabolites of BS extract was studied to identify the possible metabolism in Caco-2 and HEPG2 cells.

MATERIAL AND METHODS

Standards

Glucoraphanin (GR) and sulforaphane (SFN) were purchased from CRA-CIN (Rome, Italy) and Sigma (St. Louis, MO), respectively. The standards of SFN–gluthatione, SFN–cysteine, and SFN-N-acetylcysteine (SFN–GSH, SFN–Cys, and SFN–NAC, respectively) were from Santa Cruz Biotech (Santa Cruz, CA). All LC–MS grade solvents were obtained from J.T. Baker (Phillipsburg, NJ).

Plant material

Broccoli sprouts (BS) were germinated for 8 days, then were collected, rapidly frozen in liquid N_2 and lyophilized prior analysis. Then, dry samples were hydrolyzed following Cramer and Jeffery (2011) method (Cramer & Jeffery, 2011). All samples tested were filter by 0.22µm. The quantification of GRA and total GLS was carried out in a HPLC-DAD (Baenas, *et al.*, 2014; Mellon, *et al.*, 2002).

Citotoxicity assay

Caco2 and HT29 (human colorectal adenocarcinoma cell lines) and HepG2 (human hepatocellular carcinoma cell line) were maintained as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, and 1% penicillin/streptomycin (5,000 U/mL) (Lonza, Barcelona, Spain) in the presence of 5% CO_2 at 37°C and humidified atmosphereCells were plated in 96-well plates (1 x 10^4 cells/well) and grown for 24 h at 37°C in 5% CO_2. Cells were treated with different concentrations of sulforaphane (20, 10, 5, 1, 0.5, 0.1 and 0.05 µM in DMSO 0,1%), glucoraphanin (100, 85, 70, 55, 40, 25 and 10 µM in 0,1% DMSO) and extracted broccoli sprouts (containing 20, 10, 5, 1, 0.5, 0.1 and 0.05 µM of sulforaphane in DMSO 0,1%) dissolved in DMEM. After 24 h of incubation, cell viability was determined using the colorimetric MTT assay (Filipiak *et al.*, 2014). The assay was repeated with 3 independent experiment replications. The viability was calculated considering controls containing a solvent control (0.1% DMSO) as 100% viable. DMSO at experimental concentrations was nontoxic to cells.

Metabolism assay

Differentiated Caco-2 and HEPG2 cells (1.5 x 10^5 cells/well) were grown in a 6-well plates and incubated with extracted BS (containing 1 µM SF), during 3, 6 and 24 h. The analysis of SFN and its derivatives (SFN-GSH and SFN- Cys) in cell lines was carried out in a UHPLC/MS/MS method described by Dominguez-Perles, *et al.,* (2014) (Dominguez-Perles *et al.*, 2014).

Statistical analysis

All analysis were conducted by triplicate, processed by Graphpad Prism to determine IC_{50} and SPSS 15.0. to carried out a multifactorial analysis of variance (ANOVA) and Tukey's Test to determine significant differences at *P* values < 0.05.

RESULTS AND DISCUSSION

Analysis of broccoli sprouts

The concentration of GRA was analyzed by HPLC-DAD after the inactivation of the myrosinase activity to prevent the hydrosysis of GRA to SFN (Mellon *et al.*, 2002). Total GLS amount was 377.64 mg·100g fresh weight (F.W.), being GRA the major compound, 160 mg·100g F.W. of the total. In the hydrolyzed extracts of broccoli sprouts, only SFN was detected by UPLC/MS/MS and the concentration was 13 mg·100g F.W. This data is in line with previous reports (Baenas *et al.*, 2014; Dominguez-Perles *et al.*, 2014; Force, *et al.*, 2007).

Cell proliferation

To study the antiproliferative effect of BS and its metabolites on different cell lines, we examined their cytotoxicity on Caco-2, HT-29 and HEPG2 cells. The inhibition of cell growth by BS extract and its metabolites is shown in Figure 1. SFN has been shown to inhibit cell cycle progression, induce apoptotic cell death, and inhibit angiogenesis in a variety of cancer cell types.
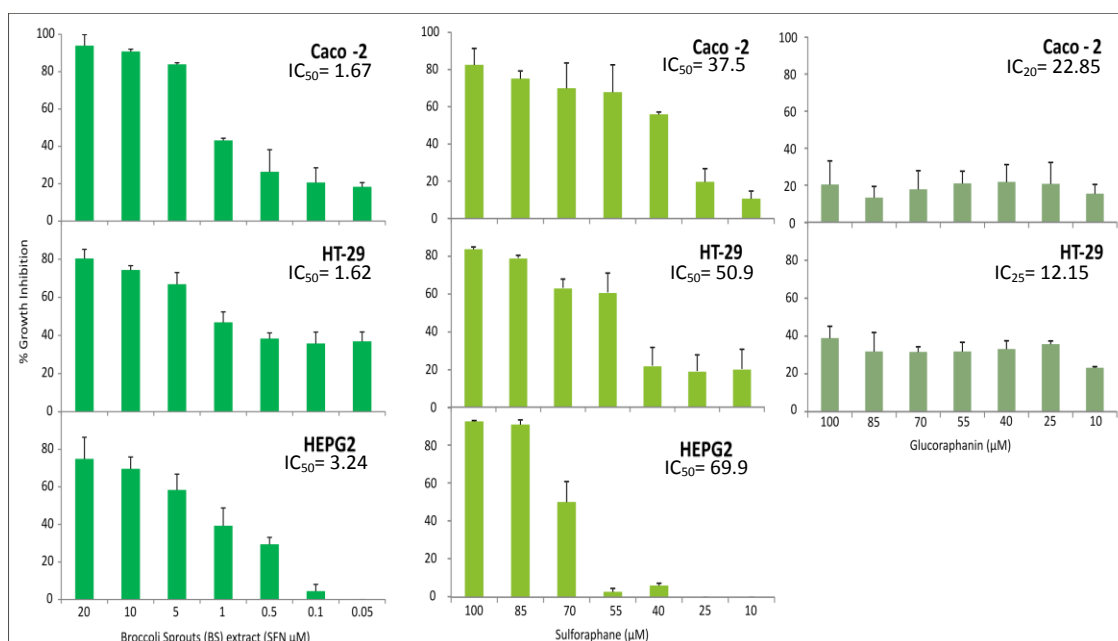
Figure 1. Growth Inhibition (%) of cell lines after application of BS extract, sulforaphane and glucoraphanin solutions. IC_{50} shows the half-maximal inhibitory concentration.

Data obtained was dose and time dependent for BS and SF, except for GRA, which did not achieve the half-maximal inhibitory concentration (IC_{50}) (Figure 1), consistent with observations made in other cancer cell types, including prostate cancer cells (PC3), colon cancer cells (HCT116) (Singh *et al.*, 2004), and Barrett esophageal adenocarcinoma cells (BEAC) (Qazi *et al.*, 2010).

The lowest IC_{50} was observed after BS application; the IC_{50} was 1.6 µM and 3.2 µM, in both Caco-2 and HT-29 cells lines, and in HEPG2, respectively. The BS extract showed the highest antiproliferative activity in all cells, even containing lower concentration of SFN. SFN molecule in low concentrations (10 – 0.05 µM) did not reduce the viability of cells (data not shown). The bioactivities of different samples varied with different cell lines. A IC_{50} of 37.5, 50.9 and 69.9 was observed in Caco-2, HT-29 and HEPG2 cells, respectively, after SF application, according to Lubeska *et al.*, (2012), who reported a IC50 of 33.4 µM. Gamet-Payrastre, *et al.*, (2000) showed that SFN induced a cell cycle arrest in a dose-dependent manner, followed by cell death via an apoptotic process. This cycle arrest was correlated with an increased expression of G_2/M phase cells and the level of cyclins A and B1.

Cell metabolism

The levels of SFN and its derivatives were measured in HEPG2 and Caco-2 cells. After treating the cells with BS extract containing 1µM SFN for 3, 6 and 24h, metabolites were analysed both in whole-cell lysates and in the culture medium (Figure 2). Results after 24h of exposing the HEPG2 cells to the BS extract could not be measured because of the high cytotoxicity of BS in this cell model.
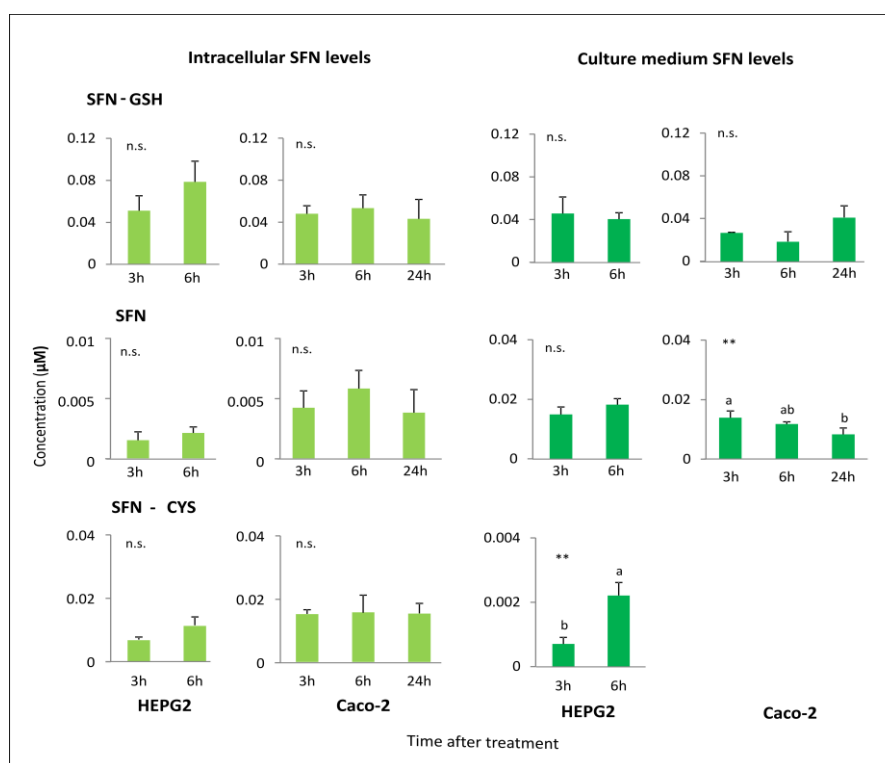
Figure 2. Concentration (µM) of sulforaphane (SFN) and its metabolites sulforaphane-glutathion (SFN-GSH) and sulforaphane-cysteine (SFN-CYS) in the interior of HEPG2 and Caco-2 cells and their release in the culture medium after the application of broccoli sprouts extract containing 1µM of sulforaphane. Lower case letters show statistically significant differences at **p<0.01 and n.s. not significant p>0.05.

SFN in cells, both intracellular and in the medium was principally accumulated as glutathione (GSH) conjugates. This process not only requires the action of the Phase II enzyme glutathione S-transferase (GST), it also depletes the cell of GSH, resulting in a rapid increase

in GSH production within the cell, therefore assisting carcinogen metabolism on two fronts. Phase II enzyme induction is considered the most likely contributing factor to the anticarcinogenicity of SFN, although the inhibitory effect on cell proliferation have been confirmed in other in vitro experiments (Frydoonfar, 2004). If the cells were treated with a GSH-depleting agent, the SFN could not be accumulated in the cells, and the subsequent induction of phase II enzymes was blocked (Kim, *et al.*, 2003).

Results showed also the presence of SFN and SFN-CYS, after the application of lyophilized BS extract to the HEPG2 and Caco-2 cells, in both media and cells (Figure 2). Concentration of SFN-CYS was similar in both cells lines After 6h, a higher content of SFN and its metabolites were found in both cell line lysates compared to 3h and 24h of incubation (Figure 2).

Cells are capable of conjugative metabolism, since, as we have shown in our experiments, SFN mercapturic derivatives could be identified in the incubation medium, as well as in the cell lysate. SFN effect as modulator of absorption and metabolism in enzymatic systems has been proved before (Lubelska *et al.*, 2012). Hence, these cell lines are a good model for the examination of metabolism regulation, even there are significant differences with human enterocytes (Petri *et al.*, 2003).

CONCLUSIONS

The results of this study indicate that broccoli sprouts and sulforaphane inhibit proliferation of cancer cell lines providing support to the role of *Brassica* foods in reducing the risk of certain cancers (Higdon, *et al.*, 2007), nonetheless, it is necessary to study the potential synergy of SFN combined with other food components, as in this work, broccoli sprouts extract, with lower sulforaphane concentration,had a greater antiproliferative effect than SFN its self, which may lose their bioactivity. On the other hand, concentrations of SFN-metabolites found in inside Caco-2 and HEPG2 cells showed absorption of SFN after the application of broccoli sprouts extract and its conjugation as mercapturic derivatives, assisting carcinogen metabolism in the cell. These results should encourage further *in vivo* assays to understand their metabolism and bioavailability, and preventive efficacy as therapeutic agents within the confines of a clinical trial for any form of cancer. The consumption of broccoli sprouts or their use as ingredient in food industry would enrich the composition in health-promoting bioactives of new foods.

ACKNOWLEDGEMENTS

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ANTIPROLIFERATIVE ACTIVITY OF LIPOPHILIC AND HYDROPHILIC TOMATO JUICE EXTRACTS IN HUMAN BREAST CANCER CELL LINE MCF-7

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ABSTRACT

High daily intake of tomato products is recommended, especially for the people with increased risk of cancer. Tomato juice contains different lipophilic (carotenoids, lycopene predominantly) and hydrophilic (phenolic compounds) phytochemicals that can contribute to its bioactivity. In this context, the aim of our research was to investigate the antiproliferative activity of both lipophilic and hydrophilic tomato juice extracts on the estrogen-dependent breast cancer cell line MCF-7.

Tomato juice, prepared in a kitchen juicer, was lyophilized, grinded and extracted with hexane to obtain lipophilic extract. After extraction, the part of the residue was re-extracted with ethanol to obtain hydrophilic extract. Dried extracts were dissolved in DMSO and used for the determination of the antiproliferative activity against breast cancer cell line (MCF-7) after 24 and 72 hours using MTT assay. Different concentrations of extracts were used to determine the concentration dependence and calculate the IC_{50} values.

Antiproliferative activity of both extracts showed concentration dependant behaviour. Hexane tomato juice extract expressed much higher antiproliferative activity after 24 hours of incubation (IC50=770.7 μg/mL) in comparison to ethanolic extract, which induced maximal cytotoxicity of only 24,38%. However, after 72 hours of incubation, the difference between IC_{50} values of hexane and ethanolic extracts was less prominent (798.00 μg/mL and 866.93 μg/mL, respectively). Higher antiproliferative activity of hexane extract in the first 24 hours (acute phase) could be explained by the higher bioavailability of carotenoids compared to the phenolic compounds, presumably because of their lipophilicity.

Keywords: tomato, extracts, antiproliferative activity, phenolic compounds, lycopene

INTRODUCTION

High daily intake of fruits and vegetables, including juices is recommended, especially for the people with health issues, such as cardiovascular diseases, cancer, neurological conditions and type 2 diabetes (Wootton-Beard and Ryan, 2011). Numerous epidemiological studies have suggested that a high consumption of tomatoes and tomato products can reduce risk of several types of cancer, most notably those of prostate, breast, and digestive tract (Giovannucci *et al.*, 2002; Omoni and Aluko, 2005). This beneficial effect of tomato consumption is linked to lycopene, major carotenoid in tomato fruits which after ingestion of fresh tomato or tomato products becomes predominant carotenoid in human plasma (Agarwal and Rao, 2000; Hadley *et al.*, 2003). Beside epidemiological evidence of a possible protective effect of lycopene against cancer, several cell culture studies have demonstrated the anticarcinogenic potential of lycopene (Omoni and Aluko, 2005). Lycopene has been found to inhibit proliferation of several types of cancer cells, including those of breast, lung, and endometrium (Heber and Lu, 2002). The results of research conducted by Levy *et al.* (1995) showed that lycopene is far more effective cancer cell growth inhibitor than α- and β-carotene.

Tomato contains other phytochemicals, predominantly phenolic compounds, which may also contribute to its anti-carcinogenic potential (Canene-Adams *et al.*, 2005). Phenolic compounds are supposed to have protective role against several chronic diseases, including cancer, but there are still too few intervention studies to make any final statement about their influence on health (Soto-Vaca *et al.*, 2012). Flavonoids present a large group of phenolic compounds which are known for their bioactivity (Erlund, 2004). Phytochemical extracts from fruits and vegetables exert strong antioxidant and antiproliferative activities, and the major part of total antioxidant activity comes from the combination of phytochemicals, such as phenolics and carotenoids (Liu, 2004).

The incidence of breast and endometrial cancers has been rising in the last decades in the Western countries, which has been attributed to increased exposure to estrogens (Hirsch *et al.*, 2007). There are several studies conducted on human estrogen-dependent breast cancer cell line MCF-7 which confirmed antiproliferative effect of lycopene. It has been shown that lycopene selectively inhibit the growth of MCF-7 human breast cancer cells but not MCF-10 mammary epithelial cells (Uppala *et al.*, 2013). Several mechanisms have been proposed for the explanation of lycopene action on MCF-7 cell line, including inhibition of estrogen signalling of both 17β-estradiol and genistein (Hirsch *et al.*, 2007) and modulation of the gap junction intercellular communication in this cell line, as observed for other cancer cell lines (Fornelli *et al.*, 2007). However, in other study (Prakash *et al.*, 2001) it was demonstrated that carotenoids inhibit the growth of both estrogen-dependent and estrogen-independent human breast cancer cell lines, indicating that estrogen receptor status is important, but not essential factor for the responsiveness of breast cancer cells to carotenoid treatment.

In this context, the aim of our research was to investigate the antiproliferative activity of both lipophilic and hydrophilic tomato juice extracts on the estrogen-dependent breast cancer cell line MCF-7. Additionally, as a part of these experiments, content of lycopene and phenolic compounds was determined in both extracts.

MATERIAL AND METHODS

Tomato juice was prepared using kitchen juicer (Gorenje, Velenje, Slovenia), and fresh juice was lyophilized for 72 h (Martin Christ GmbH, Osterode am Harz, Germany). 4 g of lyophilized sample (obtained from 55.6 g of tomato juice) was extracted with n-hexane (8 x 20 mL) in ultrasound bath at ambient temperature, to obtain lipophilic extract. The extract was filtered through filter paper and one part of the dried residue was re-extracted with ethanol (40 mL) for 24 hours on shaker at room temperature, to obtain hydrophilic extract. The solvents from both extracts were removed by evaporation in vacuum at 37 °C, using rotary evaporator. The samples were then dissolved in dimethyl sulfoxide (DMSO), a widely accepted solvent for bioassays.

Lycopene content in extracts was estimated spectrophotometrically (Specord M40, Carl Zeiss Jena, Germany) using method described by Rao *et al.* (1998). Absorbance of the extracts dissolved in hexane was measured at 502 nm against a hexane blank. Concentration of lycopene was calculated using the extinction coefficient (E) in % of 3150.

Total phenolic content in extracts of tomato juice was determined according to method of Singleton *et al.* (1999), adapted for detection on plate reader (Multiskan Ascent, Thermo Electron Corporation, USA). 125 µl of 0,1M Folin-Ciocalteu reagent was added to 25 µl of sample. After 10 minutes, 100 µl of 7.5% w/v sodium carbonate was added and reaction mixture was incubated for 2 hours. Absorbance was read at 690 nm after incubation period. In order to eliminate the interferences, correction was prepared by replacing the volume of reagents with the same volume of distilled water. Standard curve was prepared for gallic acid, and total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of extract. Experiments were performed in triplicate.

The flavonoid content of hydrophilic extract was measured spectrofotometrically in a 96-well plate reader using modified method of Chang *et al.* (2002). 30 µl of sample was mixed with 90 µl of methanol, 6 µl of 0.75 M aluminium trichloride hexahydrate, 6 µl of 1 M sodium acetate trihydrate and 170 µl of distilled water. Absorbance was measured at 414 nm after 30 minutes of incubation. Correction was prepared in the way indicated above, with aluminium trichloride hexahydrate solution replaced with the equivalent volume of distilled water. Standard curve was prepared using quercetin. Experiments were repeated three times, and results were expressed as mg quercetin eq/g of extract.

MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium with 4.5% of glucose (DMEM, PAA Laboratories) supplemented with 10% fetal calf serum (FCS). For the experiment, the cells were seeded in a 96-well microplate (5000 cells per well). After 24 h incubation, the growth medium was replaced with 100 µl of medium containing hexane and ethanolic extracts at four different concentrations (33.3, 100, 300 and 900 µg/mL). Untreated cells served as the control, and ellagic acid was used as a positive control. After 24 and 72 h, the cell viability was determined by the proliferation test MTT assay (Mosmann 1983), which is based on the colour reaction of mitochondrial dehydrogenase in living cells with MTT reagent. At the end of the treatment period, MTT was added to each well (50 µg/100 µl /well), which was then incubated at 37 °C in 5% CO_2 for 3 h. The coloured crystals of produced formazan were dissolved in 100 µl acid-isopropanol (0,04 M HCl in isopropanol). The absorbance was measured at 540 nm and 690 nm on plate reader (Multiskan Ascent, Thermo Electron Corporation, USA). The absorbance was calculated from the difference of two absorbances: $A=A_{540}-A_{690}$. Percentage of cytotoxicity was calculated as the ratio of treated group absorbance and the control group absorbance, multiplied by 100. Experiments were performed twice in triplicate, and the obtained results were expressed as IC_{50} values (sample concentration that inhibited 50% of the net cell growth). IC_{50} values were calculated from the cytotoxicity (%) - extract concentration (µg/mL) plot using the Origin v. 6.0 graphing and data analysis software (1999).

RESULTS AND DISCUSSION

Bioactive compounds present in tomato (lycopene, total phenolics, and flavonoids) were determined in both lipophilic and hydrophilic extracts (Table 1.). Flavonoids were not determined in lipophilic extracts due to the strong interference with matrix at given wavelength. Lycopene content of extracts was much lower than their total phenolic content, even in lipophilic extract. Nevertheless, this extract contained about 100 times higher amount of lycopene than hydrophilic. The total phenolic content in both extracts was around 5.5 % of the dry extract. Phenolic compounds in lipophilic tomato extract were previously reported by Toor and Savage (2005). However, when calculated on the 100 g of tomato juice fresh weight, amount of phenolics comprised within lipophilic fraction was almost negligible (1.4 mg GAE in comparison with 48.6 mg GAE). It can be calculated from the results presented in Table 2. that flavonoids comprise 13.5% of total phenolics in tomato juice ethanolic extract. This value, as well as total flavonoid content calculated per 100 g tomato juice fresh weight (6.57 mg quercetin equivalents), is similar to those obtained for whole tomatoes in previous researches (Lenucci *et al.*, 2006).

Table 1. Lycopene content, total phenolic content, and total flavonoid content of hexane and ethanolic extracts of tomato juice

Tomato juice extract	Lycopene content (mg/g extract)	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg quercetin equivalents/g extract)
Lipophilic	1.460±0.098	65.54±2.62	n.d.
Hydrophilic	0.020±0.003	48.13±9.28	6.50±0.38

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

Antiproliferative activity of fresh (without thermal treatment) tomato juice extracts was investigated on MCF-7 breast cancer cell line. IC_{50} values were calculated after different incubation periods from the percentages of cytotoxicity obtained using different extract concentrations (Table 2.). The antiproliferative activity of tomato juice lipophilic extracts could be discussed in comparison with lycopene, since similar growth inhibition of LNCaP human prostate cancer cells was obtained by hexane extract of tomato paste and water soluble lycopene (Hwang and Bowen, 2005). Lipophilic (hexane) extract used in this study gave similar IC_{50} values after 24 and 72 hours of incubation, indicating rapid cell growth inhibition. Fornelli *et al.* (2007) demonstrated that minimal inhibitory concentration of lycopene on MCF-7 cell proliferation obtained using MTT assay did not change after 72 hours of incubation. On the other hand, results obtained for hydrophilic (ethanolic) extract differed markedly after 24 and 72 hours of incubation. In the first 24 hours (acute phase), this extract did not inhibit cell growth enough to enable the calculation of IC_{50} value, since maximal cytotoxicity was only 24.38%. However, after 72 hours of incubation, the difference between IC_{50} values of hexane and ethanolic extracts was less prominent. It was presumed that hydrophilic extract needed more time to express its antiproliferative effect.

Table 2. IC_{50} values of the net cell growth for the tested extracts

Sample	IC_{50} (µg/mL)	
	24 h	**72 h**
Lipophilic tomato juice extract	770.72	797.87
Hydrophilic tomato juice extract	24,38*	866.93
Ellagic acid (positive control)	59.27	59.13

*Results are presented as mean (n=6). *Maximal cytotoxicity (%) with 900 µg/mL of hydrophilic extract.*

Ćetković *et al.* (2012) investigated the cell growth effects of tomato waste hydrophilic extract (80% ethanol) in MCF-7 cell line by SRB test, and antiproliferative effect was observed at higher concentrations (≥12.5 mg/mL) after 48 hours of incubation. Similarly, Choi *et al.* (2001) analysed extracts of different tomato varieties in 80% methanol for cancer cell-inhibiting effects by the MTT assay. After 48 hours of incubation, the examined tomato extracts promoted growth in normal liver cells, showed little effect in proliferation of normal lung cells, and mildly inhibited growth of lung cancer cell. However, the growth of lymphoma cells was promoted at lower concentrations (10 µg/mL) and inhibited at higher concentrations (100 µg/mL). Moreover, cell growth inhibitory activity of certain fruit and vegetable juices was less expressed when water-insoluble components were excluded during extraction (Boivin *et al.*, 2009). Higher antiproliferative activity of hexane extracts in the first 24 hours could be explained by the higher bioavailability of carotenoids from the medium compared to the phenolic compounds, presumably because of their lipophilicity. In general, phenolics are known to have poor bioavailability (Soto-Vaca *et al.*, 2012).

CONCLUSIONS

Lipophilic (hexane) extract of tomato juice had higher lycopene content, while content of phenolic compounds in both extracts was similar. Flavonoids comprised 13.5% of total phenolics in tomato juice ethanolic extract. Lipophilic extract had higher antiproliferative activity against estrogen-dependent breast cancer cell line MCF-7 after 24 h (acute phase), which could be explained by the higher bioavailability of carotenoids compared to the phenolic compounds. After 72 hours of incubation, the difference in IC_{50} values between lipophilic and hydrophilic extracts was less prominent.

ACKNOWLEDGEMENTS

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THE INFLUENCE OF DIFFERENT POSTHARVEST TREATMENTS ON THE SENSORY QUALITY OF TWO TOMATO VARIETIES AFTER STORAGE

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ABSTRACT

The aim of this research was to compare the influence of different postharvest treatments used for disinfection (H_2O_2), prevention of tissue softening ($CaCl_2$), and delay of the ripening process through inhibition of respiration (CO_2) applied solely and in combination to the tomatoes harvested at different ripening stages on the sensory quality after storage.

Two tomato varieties ("Camry" and "Zouk") were harvested in mature green and turning ripening stage. Fruits harvested at turning stage were treated with one of the following treatments: washing with H_2O_2, dipping in 1% $CaCl_2$ solution, and keeping under CO_2 for 24 hours; in addition, one batch was treated with all three treatments. Tomatoes harvested at mature green stage were treated with both H_2O_2 and $CaCl_2$ or stored without treatment. All fruits were stored for 14 days in semi-controlled conditions while non-treated turning tomatoes, stored at room temperature, were used as a control. Descriptive sensory evaluation was performed by a trained panel after the storage period. Textural characteristics and colour were determined instrumentally before and after the storage.

All colour and texture parameters changed significantly compared to the results obtained before storage for both mature green and turning tomato samples. After 14 days of storage, the control samples had significantly lower lightness (L*) values, while "Zouk" had significantly higher red colour intensity (+a*) for all treatments. Regarding sensory evaluation, there were no significant differences between treatments in terms of taste after storage. The results of both sensory and instrumental analysis showed that the treatment with CO_2 was the most suitable for the preservation of tomato freshness.

Keywords: *tomato, postharvest treatment, sensory evaluation, colour, texture*

INTRODUCTION

Different chemical treatments are used for disinfection of fresh produce, such as chlorine based disinfectants (Kitinoja and Kader, 2002; Tzortzakis, 2010). However, in the last decades there has been growing demand for the application of non toxic chemicals in agriculture, because of environmental and health concerns as well as ineffectiveness of these treatments on a wide range of microorganisms (Lurie, 1998; Tzortzakis, 2010). Application of hydrogen peroxide for disinfection of both whole and fresh cut fruits instead of routine process of wash water chlorination up to 200 ppm was explored (Sapers and Sites, 2003; Ukuku, 2004). Treatment of fresh tomatoes with 5% H_2O_2 at 60°C showed greater reduction of *Escherichia coli* NRRL B 766 and *Salmonella* cocktail than treatment with 200 ppm free Cl_2 (Sapers and Jones, 2006). Moreover, dipping of eggplants and sweet red peppers in 0.5% Sanosil-25 (compound that contained 48% H_2O_2 and 0.05% Ag^+ as a stabilizing agent) reduced development of *Botrytis cinerea* and *Alternaria alternata* after storage period in comparison with untreated control (Fallik et al., 1994).

Calcium (Ca^{2+}) is an essential plant nutrient which plays important role in cell wall structure, plant growth and development, as well as ripening and senescence of horticultural crops (Aghdam et al., 2012). Calcium ions create bridge between pectin chains with negatively charged carboxyl groups creating structures called "egg-boxes" (Prasanna et al., 2007; Aghdam et al., 2012). Calcium, usually either as a solution of calcium chloride or of calcium lactate, is commonly used for the preservation of firmness in the fresh-cut fruits and vegetables production, as well as part of commercial anti-browning formulations with

ascorbic acid (Toivonen and Brummell, 2009). Pinheiro and Almeida (2008) investigated the effect of pH and calcium on pericarp firmness and pectin solubility in tomato fresh cut fruit, and treatment with calcium chloride had a significant effect on firmness retention in disks from turning and ripe tomato fruits.

Storage of fruits and vegetables in a very low O_2 atmosphere can have several beneficial effects, such as reducing respiration rate, inhibiting ethylene production, and ripening delay (Fallik et al., 2003). The use of increased levels of carbon-dioxide in modified atmosphere packaging of fresh cut tomato is well known (Artes et al., 1999; Gil et al., 2002). On the other hand, 24 h anoxia treatment with N_2 was used successfully by Fallik et al. (2003) to reduce development of *Botrytis cinerea* rot on tomato fruits, simultaneously preserving their quality.

Postharvest treatments can cause changes in the sensory properties of tomato, leading to consumer preferences (Barrett et al., 2010). Therefore, sensory analysis must be performed to determine whether treated tomatoes can meet consumers' expectations. In addition, most of the previous research focused primarily on studying the sensory properties of fresh tomatoes only (Hongsoongnern and Chambers, 2008).

Considering all mentioned above, the aim of our research was to compare the influence of postharvest treatment with H_2O_2, 1% $CaCl_2$ solution, and 24 h anoxia under CO_2 atmosphere applied solely and in combination to the tomatoes harvested at different ripening stages on the sensory quality of two tomato varieties after storage.

MATERIAL AND METHODS

Two tomato varieties ("Camry" and "Zouk"), grown in the commercial greenhouse (Gložan, Serbia), were harvested at the mature green and turning ripening stage. The samples of both varieties harvested at turning stage were treated with one of the following treatments: washing with 3% hydrogen peroxide, dipping in 1% calcium chloride solution for 5 minutes, and keeping under carbon dioxide for 24 hours; in addition, one batch was treated with all three treatments. Tomatoes harvested at mature green stage were treated with both H_2O_2 and $CaCl_2$ or stored without treatment. Afterwards, all fruits were placed in plastic trays and stored in semi-controlled conditions for 14 days. Storage temperature and relative humidity were measured twice a day, at 8:00 am and 2:00 pm. Their values ranged from 14.4 to 19.9°C and from 35 % to 55 %, respectively. One tray of untreated tomatoes for each variety was stored at room conditions for 14 days and used as a control sample.

Sensory evaluation of tomatoes was performed in two replications by seven trained sensory panellists after storage period. Technique for intensity scaling, which includes labelling a mark on a line to indicate the intensity of appearance, colour, hardness, odour and taste, was applied. The panellists made a mark on a numbered line (1-9) to indicate the intensity of the appropriate properties. Central reference point (5) was used to represent the value of a standard or baseline product on the scale. All samples were coded with three random numbers and presented simultaneously to the assessors. Water was used as a palate cleanser after each sample.

Colour values of tomatoes were determined by a Chroma Meter CR-400 (Konica Minolta Co., Ltd.,Osaka, Japan), using a D_{65} light source and the observer angle at 2°. Five tomatoes per batch were chosen randomly for colour measurement at six points (two locations between the equator and the stem; two on the equatorial region; and two between the equator and the blossom end). The results were expressed as CIE L* (lightness), CIE a* (+a*= redness, -a* = greenness), and CIE b* (+b* = yellowness, - b* = blueness).

Penetration and TPA (Texture Profile Analysis) tests were performed on the three randomly chosen tomatoes from each sample using a TA.XT Plus Texture Analyser (Stable Micro Systems, England, UK). Fruit skin strength was determined by penetration (GRP1_P2) of each fruit at the blossom end with a 2 mm diameter stainless steel flat cylinder probe (P/2) and a load cell of 5 kg. For the measurement of texture profile (TPA.PRJ), the fruit samples were compressed twice with a 100 mm diameter stainless steel cylinder (P/100), while a 30 kg load cell was equipped. Both instrumental settings (GRP1_P2; TPA.PRJ) were taken from

the sample projects of the software package (Texture Exponent Software TEE32, version 6,0,6,0, Stable Micro Systems, England, UK).

Results were expressed as mean±standard deviation for all replications. Analysis of variance (ANOVA) and Duncan's multiple range test were used to compare means at 5% significance level by the data analysis software system STATISTICA (2013).

RESULTS AND DISCUSSION

The sensory quality of tomato samples was defined by evaluation of colour, appearance, hardness, odour and taste of fruits. Sensory evaluation was performed after the storage period when all samples became suitable for consumption and the obtained results are presented in Table 1. However, tomatoes that were ripened from green maturity stage did not ripen uniformly, with decolourized spots on the surface. There were no significant differences between the tomato samples in terms of taste. Similarly to our findings, in experiment conducted by Fallik et al. (2003), anoxia treatment did not change tomato flavour after 8 days of storage. Regarding odour, both variety "Camry" used as a control and treated with $CaCl_2$ differed significantly, with control sample assessed with the highest score, and treated sample assessed with the lowest.

Green non treated fruits of variety "Zouk" received lowest scores for colour because they failed to ripen uniformly, while turning samples of the same variety treated with all three treatments received highest scores, implying that they could be overripe. All tomato samples received lower appearance scores, partly as the consequence of skin wilting caused by the low relative humidity in the storage. This property expressed the greatest variability, with "Zouk" kept under CO_2 atmosphere evaluated with scores closest to the standard values. This sample was also assessed as the hardest. Generally, tomatoes that were treated with CO_2 alone or in combination received higher scores for hardness, which could be the consequence of delayed ripening.

Table 1: Sensory scores of differently treated turning tomatoes after 14 days of storage

Cultivar	Ripening stage	Treatment	Colour	Appearance	Hardness	Odour	Taste
Camry	turning	non treated*	5.0±0.0[ab]	3.3±0.6b[cd]	3.7±0.6[b]	5.3±1.53[a]	5.0±0.0[a]
	turning	$CaCl_2$	5.0±0.0[ab]	2.3±0.6[e]	4.0±1.0[b]	2.7±0.58[b]	4.7±1.5[a]
	turning	H_2O_2	5.3±0.6[ab]	3.0±0.0[cde]	4.0±1.0[b]	3.3±1.15[ab]	5.0±1.0[a]
	turning	CO_2	5.0±0.0[ab]	3.0±0.0[cde]	4.7±0.6[ab]	3.3±1.15[ab]	5.0±0.0[a]
	turning	CO_2 + H_2O_2 + $CaCl_2$	4.7±0.6[ab]	3.7±0.6[abcd]	4.7±1.5[ab]	5.0±2.00[ab]	3.7±0.6[a]
	green	non treated	5.0±0.0[ab]	3.7±0.6[abc]	4.0±1.0[b]	3.0±1.00[ab]	3.7±1.2[a]
	green	H_2O_2 + $CaCl_2$	4.7±0.6[ab]	3.3±0.6[bcd]	4.0±0.0[b]	3.7±1.15[ab]	4.3±1.0[a]
Zouk	turning	non treated*	5.3±0.6[ab]	3.0±1.0[cde]	4.3±0.6[ab]	4.7±1.15[ab]	5.0±2.7[a]
	turning	$CaCl_2$	5.0±0.0[ab]	3.3±0.6[bcd]	3.7±0.6[b]	3.3±0.58[ab]	4.0±1.0[a]
	turning	H_2O_2	5.0±0.0[ab]	4.0±0.0[ab]	4.3±1.2[ab]	3.0±1.73[ab]	3.7±0.6[a]
	turning	CO_2	5.0±0.0[ab]	4.3±0.6[a]	5.7±1.2[a]	4.0±1.00[ab]	4.3±1.2[a]
	turning	CO_2 + H_2O_2 + $CaCl_2$	5.7±1.2[a]	2.7±0.6[de]	5.0±0.0[ab]	3.3±0.58[ab]	4.0±0.0[a]
	green	non treated	4.3±0.6[b]	3.0±0.0[cde]	5.0±0.0[ab]	3.3±1.15[ab]	5.0±0.0[a]
	green	H_2O_2 + $CaCl_2$	5.3±1.2[ab]	3.0±0.0[cde]	4.7±0.6[ab]	4.7±1.53[ab]	5.3±1.2[a]

Samples were stored at room temperature.
Values with the different superscript within a column are statistically different ($P < 0.05$).

Results of instrumental measurements of colour and texture performed on examined samples are summarized in Table 2. Unripe tomatoes differed significantly both among themselves and from ripe tomatoes in terms of L* and a* colour values. L* values decreased, a* values increased from minus (green colour) to plus (red colour), while b* values decreased slightly during fruit ripening. Regarding varieties examined, "Camry" generally had lower a* and b* colour values than "Zouk".

Control samples (non treated fruits stored at room temperature) of both varieties had the lowest values of L* parameter, as well as one of the lowest values of skin strength and hardness, indicating they were overripe. The highest a* values were obtained for "Zouk" treated with CO_2. This is in accordance with the results obtained by the sensory panel, which scored this sample closest to the standard values (5) of colour and appearance. Fallik et al. (2003) found that tomatoes that were submitted to the anoxia treatment with N_2 did not differ significantly by hue values from control samples.

Table 2: Color values and texture parameters of differently treated green and turning tomatoes before and after 14 days of storage

Cultivar	Storage period (days)	Ripening stage	Treatment	L*	a*	b*	Skin Strenght (g)	Hardness (g)
Camry	0	green	non treated	55,1±2,7[a]	-0,6±6,2[g]	25,4±2,1[cde]	881±91,2[a]	23053±3143[a]
	0	turning	non treated	46,9±2,2[d]	18,3±7,9[e]	26,1±1,9[bcd]	820±23,7[ab]	22882±1844[a]
	14	turning	non treated*	39,9±2,1[i]	24,0±3,2[cd]	24,1±3,0[efgh]	357±100[j]	7027±351[d]
	14	turning	$CaCl_2$	41,6±2,2[fg]	22,9±3,3[d]	25,4±2,8[de]	386±68,9[ij]	6614±809[d]
	14	turning	H_2O_2	41,4±1,9[fgh]	23,1±3,6[d]	23,9±2,3[fgh]	424±95,7[hij]	7152±937[cd]
	14	turning	CO_2	41,1±1,4[fgh]	22,3±3,8[d]	23,8±2,5[gh]	564±54,7[efg]	10129±753[b]
	14	turning	$CO_2 + H_2O_2 + CaCl_2$	42,3±2,7[ef]	21,9±3,3[d]	25,3±2,0[def]	615±108[def]	7762±823[bcd]
	14	green	non treated	41,7±1,4[fg]	22,8±3,9[d]	23,6±2,8[h]	687±78,1[cde]	9084±2209[bcd]
	14	green	$H_2O_2 + CaCl_2$	40,6±2,4[ghi]	21,2±3,1[d]	22,8±2,4[h]	545±86,7[fgh]	6882±1175[d]
Zouk	0	green	non treated	51,2±2,1[b]	-5,9±7,1[h]	29,4±3,1[a]	794±105[abc]	22116±2505[a]
	0	turning	non treated	48,0±3,1[c]	15,1±10,5[f]	27,2±2,2[b]	715±22,9[bcd]	22769±1582[a]
	14	turning	non treated*	40,2±1,8[hi]	27,5±3,5[ab]	25,1±2,5d[efg]	518±27,7[fghi]	7103±1562[cd]
	14	turning	$CaCl_2$	41,3±2,6[fgh]	26,6±4,4[abc]	27,1±2,5[b]	406±55,3[ij]	9277±1629[bcd]
	14	turning	H_2O_2	41,4±2,0f[gh]	26,5±3,9[abc]	25,1±2,8[defg]	455±75,9[ghij]	8824±1196[bcd]
	14	turning	CO_2	42,1±1,6[ef]	28,7±3,1[a]	26,7±2,2b[c]	505±79,0[fghi]	10059±248[b]
	14	turning	$CO_2 + H_2O_2 + CaCl_2$	41,5±2,1[fg]	27,4±3,6[ab]	27,1±2,2[b]	548±25,9[fgh]	8353±79[bc]
	14	green	non treated	42,9±1,9[e]	26,1±3,8[abc]	26,8±2,4[b]	350±60,6[j]	9960±821[bcd]
	14	green	$H_2O_2 + CaCl_2$	41,2±2,0[fgh]	25,7±4,0[bc]	23,2±2,9[h]	418±54,2h[ij]	10662±1763[b]

Samples were stored at room temperature.
Values with the different superscript within a column are statistically different (P < 0.05).

Different texture measurement tests were carried out to explore the possible changes in tomato texture caused by treatments applied in this study. The obtained results are presented in Table 2. The peak force of penetration test presents the mechanical strength of the tomato skin. Examined tomato samples were grouped in even 10 homogenous groups by skin strength, but no general trend was observed except significantly higher values obtained for green and turning tomato samples.

Texture Profile Analysis (TPA) was performed to determine further textural characteristics of tomato. The hardness was recorded as the peak force of the first compression. Other parameters measured and calculated by TPA test could not be used for the comparison with the unripe tomatoes, since they were cracked during the test.

However, unripe (green and turning) tomatoes differed significantly from ripe tomatoes in terms of hardness. Previous studies have shown that TPA test compresses a piece of food two times in a motion that imitates the action of the jaw, and textural parameters calculated from the resulting force-time correlate well with sensory evaluation of those parameters (Bourne, 2002). Variety "Zouk" generally had harder fruits than "Camry", which is in accordance with the results obtained by the sensory panel. Moreover, fruits of both varieties kept under CO_2 atmosphere had significantly higher hardness values than most of examined samples, which was also observed by the panellists. It should be noted that samples dipped in 1 % calcium chloride solution did not have significantly higher skin strength or hardness as expected. This could be due to the fact that tomato skin contains liposoluble substances which make it almost impermeable for calcium ions, especially when infiltration is not applied. On the contrary, mature green, turning and ripe tomato disks vacuum-infiltrated with 50 mM $CaCl_2$ were firmer from control sample both after 4 h and 5 days of storage (Pinheiro and Almeida, 2008).

CONCLUSIONS

The results of sensory evaluation showed that there were no differences between the samples in terms of odour and taste. Colour score of all samples was close to the standard value, while the appearance of the samples, on the contrary, received low scores with the exception of "Zouk" treated with CO_2. This sample was also assessed as the hardest by the sensory panel. Regarding instrumental properties, all samples changed significantly during 2 week storage, indicating that they ripened under these conditions. Variety "Zouk" had generally higher a* and b* values, as well as hardness determined by TPA test. These instrumental measurements were in accordance with the results obtained by the sensory panel. Keeping turning tomato fruits for 24 h under CO_2 atmosphere before storage exhibited the most favorable effect on the sensory quality and instrumental characteristics.

ACKNOWLEDGEMENTS

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CHEMOMETRIC ANALYSIS OF ANTIOXIDANT ACTIVITY OF LETTUCE

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ABSTRACT

Half maximum inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound to competitively inhibit a specific biological function. As a quantitative measure, it indicates how much of a particular substance is needed to inhibit a given biological process by half. The aim of this study was to establish models for half maximal inhibitory concentration of lettuce, using their biological properties (phenols, chlorophylls, total carotenoids, vitamin C). Antioxidant activity, expressed as IC_{50}, was determined in methanolic extracts of samples. Samples were extracted 24h in dark using methanol. In order to select biological properties that best describe the IC_{50} of the investigated lettuce, principal component analysis (PCA) was carried out by Statistica version 10.0 software. Hierarchical cluster analysis (HCA) was conducted in order to confirm the grouping already obtained by the PCA and multiple linear regression (MLR) was used for establishing the mathematical models. The complete regression and cluster analysis were carried out by NCSS 2007 and GESS 2006 software. The predictive ability of the established models was evaluated by standard statistical measures and *cross*-validation parameters. Given results should be treated as preliminary for the prediction of half maximum inhibitory concentration.

Keywords: *lettuce, multiple linear regression, hierarchical cluster analysis, principal component analysis*

INTRODUCTION

The highest content of antioxidants such as vitamin A and C, carotenoids, flavonoids and polyphenolic compounds can be found in vegetables and fruits. Antioxidants prevent the attack of free radicals, reducing the risk of carcinogenic diseases. Lettuce (*Lactuca sativa L.*) belongs to the family *Asteraceae* and it is an important leaf vegetable because of the high content of biologically active compounds such as vitamins, mineral and organic substances. Additionally, owing to its high content of micronutrients (copper, zinc, iron and magnesium), lettuce is important in the human diet.

Chemometric analysis involves performing calculations on measurements of chemical data and it has a great importance in modern sciences. If any chemical problem occurs, a large number of solutions, e.g. chemometric analysis, can be applied. As predictive technique, modelling of the properties of chemical systems is done in order to predict new properties or behavior (Podunavac-Kuzmanović *et al.*, 2012; Jevrić *et al.*, 2013; Kovačević *et al.*, 2013a; Kovačević *et al.*, 2013b).

Principal component analysis (PCA) is a useful statistical technique for reducing the amount of data when there is correlation present, retaining as much information as possible. As a result of this analysis, similarities or dissimilarities among the analysed data can be revealed, using given score and loading plots.

A method for dividing a group of objects into classes so that similar objects are in the same cluster, is known as hierarchical cluster analysis (HCA). Cluster hierarchy is displayed as a tree diagram called dendrogram, where horizontal axis represents the distance or dissimilarity between the clusters.

Multiple linear regression (MLR) has to quantitate the relationship between more than one independent variables and a dependent variable. In MLR analysis it is very important to define the optimal number of independent variables included in the MLR model, to avoid

over-parametrization of the mathematical model and the chance of correlation between the variables (Minovski *et al.*, 2011).

The aim of this paper was to test the antioxidant activity of lettuce extract, expressed as IC_{50}, and to make the correlation between the tested samples.

MATERIAL AND METHODS

Investigated samples of lettuce were grown at fields of Faculty of Agriculture, University of Novi Sad. The research was conducted on lettuce samples of the type leaf - *Levistro* and oak leaf lettuce - *Murai*. Samples were stored under following conditions: greenhouse with a cube (day 15 - 18 °C, night 10 - 12 °C), refrigerator with a cube (temperature 1.8 - 1.9 °C, relative humidity of air 92 - 94 %) and refrigerator without a cube (temperature 1.8 - 1.9 °C, relative humidity of air 92 - 94 %). A cube represents organic substrate, with dimensions 5 × 5 × 5 cm.

Antioxidant compounds were determined using HPLC and spectrophotometric method. For determining total phenolic content, samples were extracted in methanol solution. The samples were shaken (GFL, Schuttelapparate Shaker, Germany, Model 3015) in the dark, at room temperature for 24h and filtered. Total phenolic content was determined by the Folin-Ciocalteu procedure (Kähkönen *et al.*, 1999) and absorbance was measured at 765 nm (Šumić *et al.*, 2013). For chlorophyll and total carotenoids determination, spectrophotometric method was used. Samples were extracted in acetone and filtered with vacuum pump. Absorbance was measured in acetone solution at 662, 644 and 440 nm. For determining vitamin C (L-ascorbic acid) content, samples were extracted in 3 % m-phosphoric added in 8 % acetic acid. Activated carbon was added to remove the color. Solution was filtered through filter paper (blue label) and a membrane syringe filter with diameter pore of 0.45 μm. Vitamin C was determined using HPLC system (Agilent 1100, USA), with C-8 column and DAD detector (Šumić *et al.*, 2013).

For determining the free radical-scavenging activity DPPH assay was used. The free radical-scavenging activity was determined using spectrophotometric method (Espin *et al.*, 2000). Final concentrations were obtained, 2.0, 4.0 and 6.0 mg of lettuce/ml and the absorbance was measured at 517 nm. Antioxidant activity was expressed as IC_{50} which represents the concentration of extract solution required for obtaining 50 % of the radical scavenging capacity (Šumić *et al.*, 2013).

The conduced PCA, HCA and MLR analysis were carried out by NCSS&GESS (NCSS 2007 and GESS 2006, 2010) and Statistica v 10.0 software (STATISTICA, 2011). Clustering is based on Ward's linkage method (Ward, 1963) and Euclidean distance. In established MLR models multicollinearity should be avoided. The impact of multicollinearity is checked by Variance Inflation Factor (VIF) and values greater than 10 indicate multicollinearity (Marquardt and Snee, 1970; O'Brien, 2007; Young *et al.*, 2008). Established MLR models have to be statistically valid.

RESULTS AND DISCUSSION

The content of phytochemicals in the analysed lettuce samples is presented in Table 1.

PCA resulted in a model that explains 86.02% of total variance, with the two significant PCs. Score and loading values for the first two PCs are presented in Fig. 1. The first principal component explains up to 64.32% of the variability and the second 21.70%. Loading graph indicates that all phytochemicals have a negative influence on PC1, while vitamin C content has a positive influence. Along the PC2 axis, phenolic content has the most negative and vitamin C content the most positive influence. PC1 separates lettuce samples according to their chlorophyll a and chlorophyll a + b content. It can be concluded that sample 5 has the smallest value of chlorophyll a and chlorophyll a + b content and it is positioned at the positive end of PC1 axis. Analogously, sample 22 is positioned at the negative end of PC1 axis, as the sample with the largest value of chlorophyll a and chlorophyll a + b content.

All other samples are set according to the increase in chlorophyll a and chlorophyll a + b content, from the positive toward negative end of PC1 axis. Additionally, sample 13 is different from all other on the basis of very high values of vitamin C content. Samples 18, 22 and 29 have the highest values of chlorophyll a, total carotenoids and chlorophyll a + b content.

Table 1. *Content of phytochemicals in the analysed lettuce samples*

Sample	Cultivars	Postharvest conditions	IC$_{50}$	total phenolic content mg/100gDM	chlorophyll a mg/100gDM	chlorophyll b mg/100gDM	total carotenoids mg/100gDM	chlorophyll a + b mg/100gDM	vitamin C mg/100gDM
1	*Levistro*	Greenhouse	2.846	416.672	4.139	1.987	1.490	6.126	10.929
2	*Levistro*	Greenhouse with a cube	6.420	206.016	6.452	1.971	2.151	8.423	20.077
3	*Levistro*	Greenhouse with a cube	5.437	167.367	5.536	1.964	1.786	7.500	14.968
4	*Levistro*	Greenhouse with a cube	6.185	259.584	4.015	1.095	1.642	5.109	16.859
5	*Levistro*	Greenhouse with a cube	6.026	165.149	3.302	1.101	1.258	4.403	11.653
6	*Levistro*	Refrigerator with a cube	3.963	376.191	4.887	1.396	1.920	6.283	14.100
7	*Levistro*	Refrigerator with a cube	5.746	226.018	4.745	1.230	1.757	5.975	16.168
8	*Levistro*	Refrigerator with a cube	2.474	544.202	5.056	1.498	2.060	6.554	14.059
9	*Levistro*	Refrigerator with a cube	4.441	368.458	5.124	1.590	1.767	6.714	16.485
10	*Levistro*	Refrigerator with a cube	7.869	169.457	4.562	1.095	1.642	5.657	12.096
11	*Levistro*	Refrigerator *	3.340	484.480	6.030	1.508	2.345	7.538	14.983
12	*Levistro*	Refrigerator *	4.486	331.046	6.030	1.508	2.178	7.538	14.756
13	*Levistro*	Refrigerator *	6.843	200.944	5.565	1.739	1.913	7.304	26.242
14	*Levistro*	Refrigerator *	4.196	419.563	5.155	1.718	1.718	6.873	13.639
15	*Levistro*	Refrigerator *	5.292	340.717	4.036	1.345	1.345	5.232	14.915
16	*Murai*	Greenhouse	4.774	371.844	6.581	2.011	2.377	8.592	12.302
17	*Murai*	Greenhouse	1.533	769.394	7.729	2.254	2.899	9.984	11.680
18	*Murai*	Refrigerator with a cube	2.389	377.409	9.340	2.576	3.382	11.755	15.910
19	*Murai*	Refrigerator with a cube	1.746	481.851	6.969	1.621	2.431	8.590	9.944
20	*Murai*	Refrigerator with a cube	4.946	292.638	6.697	2.131	2.283	8.828	13.689
21	*Murai*	Refrigerator with a cube	0.824	820.281	6.775	2.062	2.504	8.837	10.489
22	*Murai*	Refrigerator with a cube	3.865	361.541	9.404	2.978	3.135	12.382	14.741
23	*Murai*	Refrigerator with a cube	3.087	349.717	8.040	2.345	2.848	10.385	11.896
24	*Murai*	Refrigerator with a cube	1.460	736.789	7.216	2.062	2.577	9.278	15.390
25	*Murai*	Refrigerator with a cube	1.545	713.934	6.046	1.634	2.288	7.680	13.594
26	*Murai*	Refrigerator with a cube	3.949	347.533	7.362	2.454	2.607	9.816	13.654
27	*Murai*	Refrigerator *	2.690	420.007	6.989	3.047	2.330	10.036	13.302
28	*Murai*	Refrigerator *	3.588	373.638	7.589	2.381	2.679	9.970	13.852
29	*Murai*	Refrigerator *	2.810	371.120	8.373	2.528	3.002	11.058	22.850
30	*Murai*	Refrigerator *	1.496	611.031	6.327	2.623	2.160	8.951	11.959
31	*Murai*	Refrigerator *	3.565	466.038	8.012	2.773	2.928	10.632	11.681

* without a cube

HCA was performed in order to confirm the grouping of the samples already obtained by the PCA. Dendrogram in Fig. 2 shows two well-separated clusters. First cluster contains samples with higher values of chlorophyll a and chlorophyll a + b content and second one samples with lower values of chlorophyll a and chlorophyll a + b content. Clustering is based on the chlorophyll a and chlorophyll a + b content and it is the same as on the PC1-PC2 score plot (Fig. 1).
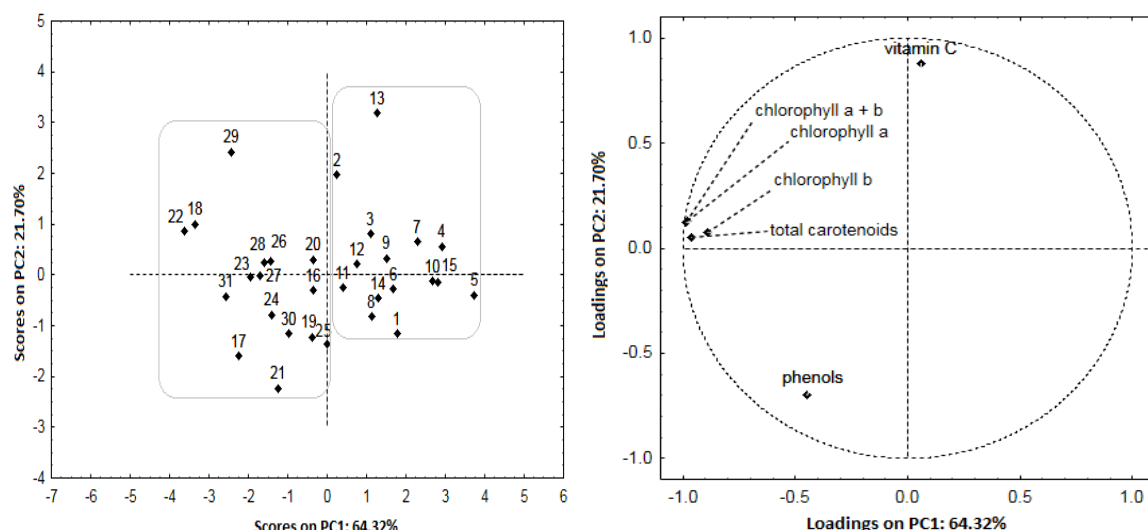
Figure 1. Score values and factor loadings of phytochemicals for 31 lettuce samples.
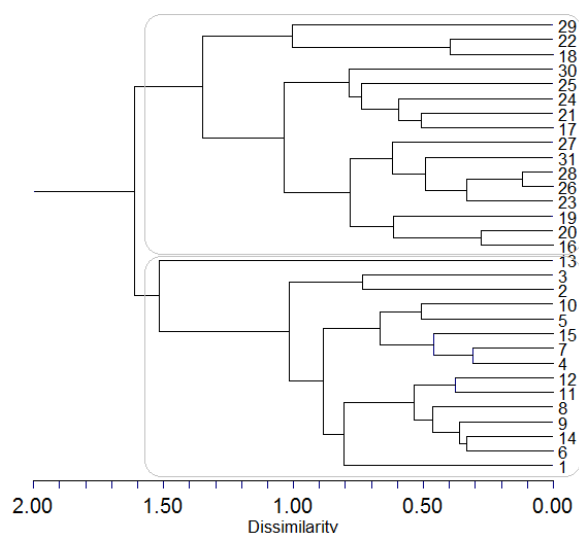
Figure 2. Dendrogram of 31 examined lettuce samples.

As a result of MLR analysis, four statistically significant models, free of multicollinearity, were obtained and they are given in the Table 2. It can be observed that all phytochemicals (total phenols, chlorophylls and total carotenoids) have negative influence on the IC_{50}, except vitamin C. For testing the quality of the predictive power of established models the leave-one-out (LOO) method was used (Table 3). High values of r^2_{cv} and r^2_{adj} (higher than 0.5) and *PRESS* values significantly less than *TSS* for all five models indicate the high predictive ability of the MLR model. In equations 1-4 all phytochemicals have the negative influence on the IC_{50}, except vitamin C content.

The plots of residual *versus* the experimentally observed IC_{50} values shows that the residuals are randomly distributed around y = 0 axis (Fig. 3). On the result of *cross*-validation parameters and plots given in Fig. 3, it can be concluded that the best models are 1 and 2. Based on the same criteria, models 3 and 4 are satisfactory.

Table 2. Statistical parameters of MLR models established

Variables			Multiple Linear Regression: $y = a + b \cdot x_1 + c \cdot x_2$							Eq.
y	*x₁*	*x₂*	*a*	*b*	*c*	*r*	*F*	*s*	*VIF*	
IC₅₀	total phen.	chlorophyll b	8.8579	-0.0083	-0.8467	0.8405	73.77	0.7434	1.1	1
IC₅₀	total phen.	chlorophyll a + b	9.0352	-0.0082	-0.2278	0.8374	72.12	0.7505	1.1	2
IC₅₀	total phen.	chlorophyll a	8.9352	-0.0082	-0.2788	0.8298	68.24	0.7680	1.1	3
IC₅₀	total phen.	total carotenoids	8.9115	-0.0080	-0.8054	0.8265	66.69	0.7753	1.2	4

Table 3. Cross-validation parameters of MLR models established.

Equation	1	2	3	4
r^2_{cv}	0.8059	0.7985	0.7873	0.7837
r^2_{adj}	0.8291	0.8258	0.8176	0.8141
PRESS	18.8266	19.5484	20.6350	20.9812
TSS	97.0128	97.0128	97.0128	97.0128
PRESS/TSS	0.1941	0.2015	0.2127	0.2163
S_{PRESS}	0.7793	0.7941	0.8159	0.8227
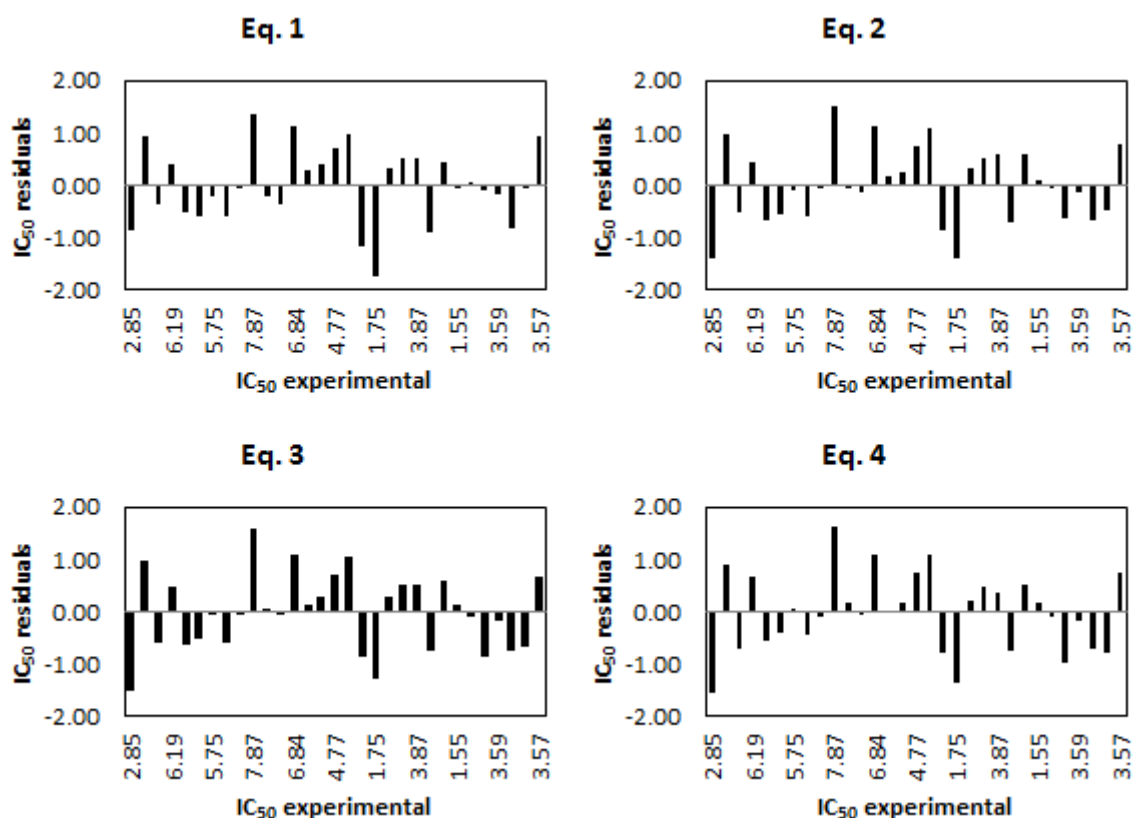
Figure 3. Plots of residual values versus the experimentally obtained IC₅₀ values.

The main aim of the conducted correlation analysis was to determine the ability to predict antioxidant activity, expressed as IC₅₀, of investigated lettuce samples using phytochemicals content. Established MLR models could be used in applied conditions in order to avoid conducting of the DPPH assay.

CONCLUSIONS

The aim of this study was to find the most important phytochemicals affecting antioxidant activity (expressed as IC_{50}) and to determine the ability to predict antioxidant activity of investigated lettuce samples. PCA showed that lettuce samples were separated according to their chlorophyll a and chlorophyll a + b content. The results of HCA confirmed the grouping of the samples obtained by PCA. The usefulness of the established models was confirmed by standard and *cross*-validation statistical parameters. Comparison of the experimental and residual values confirmed that established MLR models can be successfully used in prediction of antioxidant activity in lettuce samples. Predictive ability of presented models allows us to estimate the IC_{50} for similar lettuce samples and reduces the analysis time of antioxidant activity.

ACKNOWLEDGEMENTS

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CHEMOMETRIC ANALYSIS OF THE INFLUENCE OF PHENOLS, VULGAXANTHIN AND BETANIN CONTENTS ON ANTIOXIDATIVE ACTIVITY OF BEETROOT EXTRACT

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ABSTRACT

The present study is based on chemometric analysis of the correlations between antioxidative activity of beetroot extracts and phenols, vulgaxanthin and betanin contents in the extracts. Preceding crops, such as cabbage, cauliflower, kohlrabi and broccoli, were grown on the same soil before beetroot. Regression analysis was carried out applying linear (LR) and multiple linear regression (MLR) approaches. The best LR and MLR correlations were established for extracts obtained from beetroot with cauliflower, kohlrabi and broccoli as preceding crops. The basic statistical measures and cross-validation parameters confirmed the prediction ability of the established models, which can be used for prediction of antioxidant activity of the obtained extracts. LR models indicate the increase of antioxidant activity with increase of phenols, vulgaxanthin and betanin contents in the beetroot extracts.

Keywords: beetroot, antioxidant activity, chemometrics, regression analysis

INTRODUCTION

Beetroot (*Beta vulgaris*) belongs to *Chenopodiaceae* family. It is classified as root vegetable. In Western Europe 90% of produced beetroot is used in preserved vegetables and 10% is processed into juice or food colour. It has a characteristic red colour. It is a good source of natural pigments betalains (redish-purple betacyanins and yellowish-orange betaxanthins). Beetroot is a rich source of antioxidants, phenolic compounds being the most important. In polar solvents, beetroot extracts and beetroot peel extracts show relatively high antioxidant activity.

In the present study, the analysis of antioxidant activity of 64 beetroot extracts was carried out. The beetroot was cultivated in soil after different preceding crops (cabbage, cauliflower, kohlrabi and broccoli). The concentrations of phenols, vulgaxanthin and betanin in obtained extracts were determined.

Chemometric approach was applied in order to establish correlations between the concentrations of phenols, vulgaxanthin and betanin and antioxidant activity of beetroot extracts. Chemometrics, as a relatively young scientific discipline, has been applied in many studies (Kovačević *et al.*, 2014; Podunavac-Kuzmanović *et al.*, 2013; Gadzuric *et al.*, 2014; Jevrić *et al.*, 2013). It is applied to solve both descriptive and predictive problems in experimental life sciences, especially in chemistry. It is a highly interfacial discipline which use methods frequently employed in core data-analytic disciplines such as applied mathematics, multivariate statistics and computer science, in order to address problems in chemistry, biology, biochemistry, medicine and chemical engineering. Chemometric analysis can be carried out in such way that the results appear much better than they really are. Because of that, the most commonly used technique for model validation is cross-validation technique, which indicates real predictive ability of the model.

MATERIAL AND METHODS

The contents of betanin and vulgaxanthin in beetroot extracts were determined applying spectrophotometric method (von Elbe *et al.*, 2001). The absorbances of extracts were measured at 538, 476 and 600 nm. The corrected absorbances were calculated on the basis of the following formulas: $A_1 = 1.095 \times (a − c)$; $Z = a − A_1$; $A_2 = b − Z − (A_1 / 3.1)$, where: a – absorbance of the sample at 538 nm, b – absorbance of the sample at 476 nm, c – absorbance of the sample at 600 nm, Z – absorbance of impurities, A_1 – absorbance of betanin (corrected for the contribution of colored impurities), A_2 – absorbance of vulgaxanthin I (corrected for the contribution of betanin and colored impurities). The following formulas were used for calculation of betanin (**B**) and vulgaxanthin I (**V**) contents:

B = $(V_m \times A_1 \times DF) / (m \times 1120)$ [mg/100g]   **V** = $(V_m \times A_2 \times DF) / (m \times 750)$ [mg/100g]

V_m – extract volume (ml), m – mass of the sample taken for the analysis (g), DF – dilution factor.

The extraction of phenolic compounds was carried out according to the method introduced by Gonzales-Gomez *et al.*, 2010 with certain modifications. Folin-Ciocalteu method was applied for determination of total content of phenolic compounds using chlorogenic acid as a standard (Kähkönen *et al.*, 1999). The absorbance was measured at 765 nm. The results were expressed in mg equivalent of chlorogenic acid per 100 g of dry matter.

Antioxidant activity of the obtained beetroot extracts was expressed as IC_{50} value which is the concentration of the extract solution needed for 50% of antiradical activity. Radical scavenging capacity (RSC) was determined by DPPH test. IC_{50} values were determined on the basis of linear dependence between RSC and beetroot extract concentrations.

MLR is a widely applied regression method (Podunavac-Kuzmanović *et al.*, 2013; Jevrić *et al.*, 2013). The general purpose of MLR analysis is to quantitate the relationship between several independent or predictor variables and a dependent variable. MLR model is built with descriptive variables using the least squares methods to minimize the residuals. General MLR model is: $y = a + b_1 \cdot x_1 + b_2 \cdot x_2 + \cdots + b_n \cdot x_n$, where y is the quantitative property to predict (dependent variable), x_n an independent (descriptive) variable, a the intercept, and b_n the regression coefficient for x_n. The main restriction of MLR analysis is the case of large descriptors-to-compounds ratio or multicollinear descriptors in general. For construction of MLR models it is very important to avoid multicollinearity. Variance inflation factor (VIF) is a diagnostic tool used to check the impact of multicollinearity in MLR models. VIF greater than 5 indicates multicollinearity.

The statistical quality of the generated MLR equations was measured by using the standard statistical parameters (Pearson's correlation coefficient (R), F-test (Fisher's value), root mean square error (RMSE) and coefficient of variation (CV%)), and cross-validation parameters (cross-validated coefficient of determination (R^2_{cv}), adjusted coefficient of determination (R^2_{adj}), predicted residual sum of squares (PRESS) and total sum of squares (TSS)). The correlation coefficient values closer to 1 represent the better fit of the regression, and high values of the F-test indicate that the model is statistically significant. Standard deviation expresses the variation of the residuals or the variation about the regression line, and should have a low value for the regression to be significant. Lower PRESS value is, the better the predictability of the model. If PRESS value is less than TSS value, the model predicts better and can be considered statistically significant. TSS values are in terms of the dependent variable y. In many cases, R^2_{cv} and R^2_{adj} are taken as a proof of the high predictive ability of estimated mathematical models. High values of these statistical characteristics (R^2_{cv}, $R^2_{adj} > 0.5$) indicate high predictivity of the equations.

Statistical analysis (LR and MLR analysis and cross-validation procedure) was carried out by Statistica v.10, NCSS 2007 and Microsoft Excel 2010 software.

RESULTS AND DISCUSSION

In the first step of the chemometric analysis, simple LR was carried out on the set of 64 samples of beetroot extracts, including all samples regardless the preceding crops, based on IC_{50} values and contents of phenols, vulgaxanthin and betanin in each analyzed sample. IC_{50} values were used as dependent variable, while contents of phenols, vulgaxanthin and betanin were used as independent variables. The results of LR analysis are presented in Fig. 1.
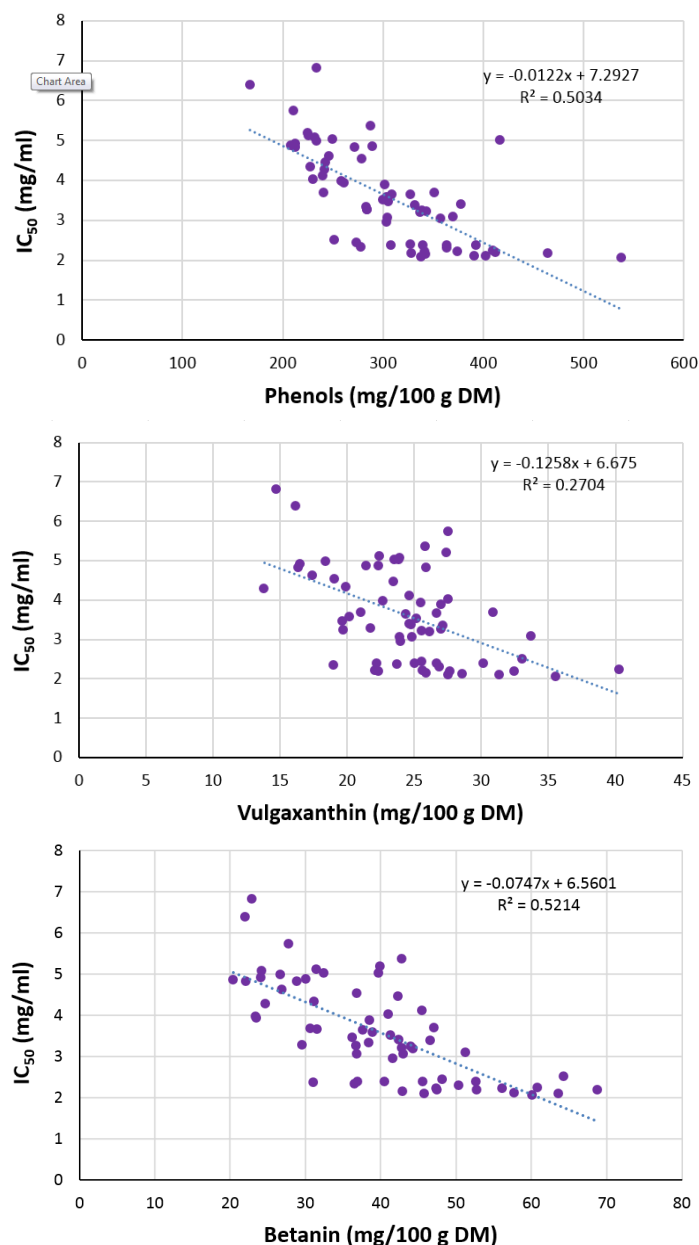
Figure 1. Linear relationships between phenols, vulgaxanthin and betanin contents and antioxidant activity of the analyzed beetroot extracts.

The obtained LR models were subjected to leave-one-out (LOO) cross-validation. The standard statistical and validation parameters of these LR models are shown in Table 1. As it can be seen from these results, the best statistical performance has the model which correlate betanin content and IC_{50} values. This model has highest correlation/determination coefficients (R, R^2_{adj}, R^2_{cv}) and F value and lowest error (RMSE), PRESS value, PRESS/TSS

ratio and CV%. However, the predictive power of this model is not quite satisfactory, since it is slightly under the limit ($R^2_{cv} > 0.5$) defined by Golbraikh-Tropsha criteria (Golbraikh and Tropsha, 2002).

Table 1. The statistical measures and cross-validation parameters of LR models.

Statistical parameters	Independent variable		
	Phenols content (mg/100 g DM)	Vulgaxanthin content (mg/100 g DM)	Betanin content (mg/100 g DM)
R	0.7095	0.5200	0.7221
R^2_{adj}	0.4954	0.2586	0.5137
R^2_{cv}	0.4611	0.2259	0.4930
RMSE	0.8462	1.0258	0.8307
PRESS	48.18	69.21	45.33
TSS	89.41	89.41	89.41
PRESS/TSS	0.54	0.77	0.51
CV%	23.55%	28.55%	23.12%
F	62.9	23.00	67.6
p (α = 0.05)	0.000000	0.000011	0.000000

Therefore, the LR modelling was carried out on the data set which contained the extracts obtained from different beetroot samples which had been cultivated on the soil with different preceding crops (cabbage, cauliflower, kohlrabi and broccoli). The best linear model was obtained for cauliflower preceding crop (Fig. 2), whose statistical characteristics are the following: R = 0.8408, R^2_{adj} = 0.6860, R^2_{cv} = 0.6384, RMSE = 0.5665, PRESS = 5.54, TSS = 15.33, PRESS/TSS = 0.36, CV% = 14.87%, F = 33.78 and *p* = 0.00005.

Preceding crop: cauliflower

$y = -0.018x + 9.1741$
$R^2 = 0.707$
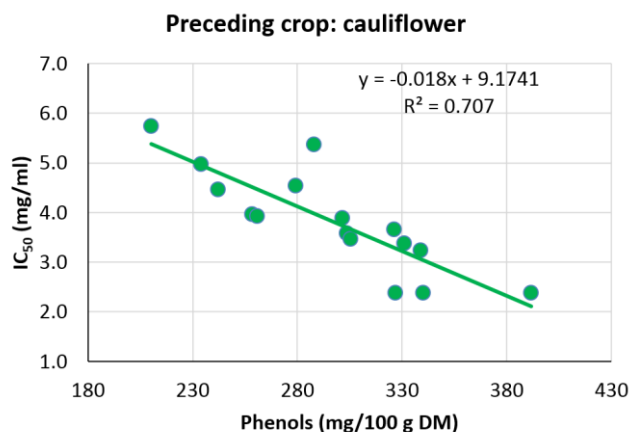
Figure 2. Linear relationship between phenols content and antioxidant activity of the analyzed beetroot extracts when cauliflower was used as preceding crop.

In order to obtain mathematical models with much better predictive power than the already established ones, MLR method was applied on the whole data set of 64 samples. MLR resulted in two acceptable models which have better statistical performance than LR models. First model (Eq. 1) is a typical MLR model, however the second model (Eq. 2) is a polynomial. Although polynomial regression fits a non-linear model to the data, as a statistical estimation problem it is linear, in the sense that the regression function is linear in the unknown parameters that are estimated from the data. For this reason, polynomial regression is considered to be a special case of MLR.

$IC_{50} = 7.5584 - 0.04624\ \mathbf{B} - 0.00699\ \mathbf{P}$ Eq. 1
$IC_{50} = 12.2110 - 0.0377\ \mathbf{B} - 0.03796\ \mathbf{P} - 0.00009\ \mathbf{B^2} + 0.00005\ \mathbf{P^2} + 0.0000003\ \mathbf{B\ P}$ Eq. 2

B – Betanin content (mg/100 g DM), **P** – Phenols content (mg/100 g DM).

Graphical representations of Eqs. 1 and 2 are presented in Fig. 3, and their statistical parameters are presented in Table 2. These two models are quite similar according to their statistical performance, but much better than LR equations. Also, multicollinearity was not detected according to variance inflation factor (VIF < 5).

a) Eq. 1
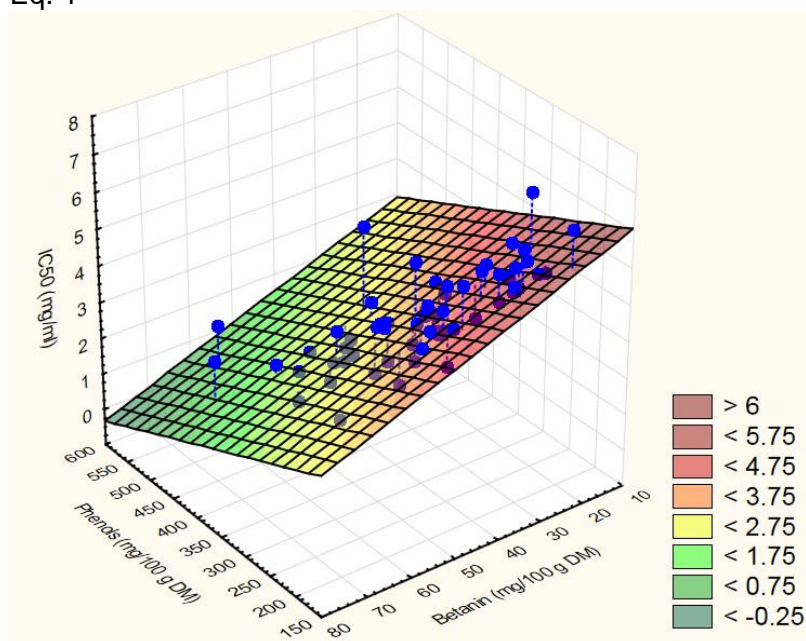
b) Eq. 2
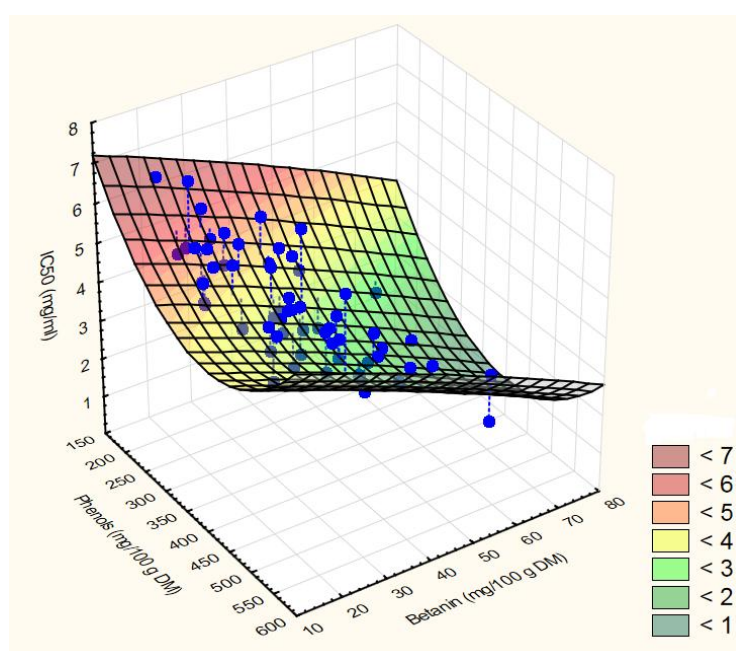
Figure 3. Multiple linear regression model (a) and polynomial regression model (b) of phenols and betanin contents and antioxidant activity (IC_{50}) of the analyzed beetroot extracts.

It is obvious from Fig. 3 that higher concentrations of phenols and betanin in the analyzed beetroot extracts have significant influence on increase of antioxidant activity (lower IC_{50} value).

Table 2. The statistical measures and cross-validation parameters of MLR models.

Statistical parameters	MLR models	
	Eq 1.	Eq. 2
R	0.7826	0.8301
R^2_{adj}	0.5997	0.6622
R^2_{cv}	0.5659	0.5345
RMSE	0.7537	0.6924
PRESS	38.81	41.62
TSS	89.41	89.41
PRESS/TSS	0.43	0.47
CV%	20.97%	19.27%
F	48.2	25.7
p (α = 0.05)	0.000000	0.000000
VIF	1.83	-

CONCLUSIONS

The established LR models showed already expected trend in dependence between antioxidant activity and contents of phenols, betanin and vulgaxanthin in beetroot extracts. However, MLR analysis included two independent variables (phenols and betanin contents) in prediction of antioxidant activity. MLR resulted in two reliable models which have better statistical performance than LR equations, hence they should preferably be used in approximate prediction of antioxidant activity, expressed as IC_{50}, of beetroot extracts.

ACKNOWLEDGEMENTS

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ORGANIC PRODUCTION AS NATIONAL BRAND

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ABSTRACT

World market of organic products, food and beverage is of very fast growing kind. However, demand for these products in the market is much higher, than it is the offer. This trend creates significant opportunities for producers and exporters of less developed countries. Serbia has sufficient quality of agricultural land as well as and rich agro industrial tradition, so it can be expected, that these benefits will be converted into export opportunities of well organized organic production. The concept of organic production combines economic and social aspects of agricultural production. Its goal is to enhance biological processes within production and to emphasize the preventive approach of controlling process and products. Since offer of organically produced food in the European market is less than demand, it would be significantly for Serbia to develop organic production and to forward derived organic products toward foreign markets. Also, this trend encourages new promotional campaigns with aim of greater sale of such products which are being implemented by producers, as well as and by vendors. Techniques, which can be used, are unlimited application and are excellent way for producers and traders to display their creativity. It is very important that many countries, among them and our country, adopted policy of support of organic production, processing and placement products, which represents an additional motivation.
Keywords: organic products, standards of quality, production management, ecological aspects, branding

INTRODUCTION

Consumption of organically produced food in developed countries is on the rise, while offer still can not satisfy such demand. This situation opens up possibility for countries with lower level of development to increase and forward production of organic alimentary products to the international market.

According to statistics, in the world organic production is increasing at the rate of 20% per year, due to the growing consumers' interest.

Various factors that motivate consumers to buy and consume organic products are primarily health, and then style of life and environmental protection.

Reduction in organic food purchasing, could be result of higher prices and inadequate distribution channels. In the following period, it should be expected growth of organic food markets. Leading countries in this chain are USA, Germany, France, Great Britain, Austria, and other countries.

Sell of organic products tripled in the countries of European Union. Consumers primarily buy this kind of food because of healthy reasons (46%) or better taste (40%).

In some developed countries, the organic agriculture represents a significant part of the total agricultural production. In Denmark includes 13%, in Austria 10%, or in Switzerland 8%. The biggest organic products market is Germany, with an annual growth rate of 10% and, as such, more than twice is bigger then the French market.

Certain states are predominantly importers of these products, and among them the biggest is Great Britain. Opposite example is Austria, a country that fully and independently satisfies its needs. A large market in Europe is Germany, which imports 50%-60% of organic products. French and Italian markets are also of very fast growing kind markets (Wier et. al, 2002).

Significant organic products in Western Europe are fruits and vegetables, in the Central Europe are cereals and bread, and in the Scandinavian and Alpine states are dairy products.

Research indicates the fact that the organic food market will grow as long as buyers believe in its' safety (Rimal et. al, 2002). Fear of genetically modified products is the most frequently reason for purchasing. It was found out that higher price does not have a significant impact on purchase decision, but fear from genetic engineering and genetically modified organisms.

THE ORGANIC FOOD PRODUCTION CONCEPT

In order to understand organic food production advantage, it should be analyzed production in conventional terms. Differences among these food production procedures, exist primarily in the concept of quality.

Conventional production means, that on basis of samples are determined biological and physiological chemical characteristics. Use of chemical means makes the essence of the conventional mass production.

In addition, costs of inputs are significant and very high. This indicates that conventional agriculture is a technological production, with an intensive use of mineral fertilizers, pesticides, veterinary preparations and hormones. Fact is that this way of production causes whole range of negative, and environmental and social problems (Williams et. al, 2001.).

It is estimated that in the EU, about 20% of all groundwater contains larger amounts of agrochemicals' debris, than it is foreseen by regulations. As a result, there are pollution of drinking water and expressed negative impact on health of people and animals.

That is why developed countries choose for the introduction of restrictions on use of mineral fertilizers. By defining maximum quantities, they control presence of specific chemical elements in the ground and water (above all, quantities of nitrogen and phosphorus).

Statistic data indicate the fact, that today in the countries of the EU, about 25% more agricultural products are produced, but, and about 20% are discarded, which negatively affects to the products prices.

Until recently, the EU member states were stimulated producers for greater production, through subsidies for mineral fertilizers, pesticides, seeds and the rest. Today, producers are constantly monitored with the whole set of regulations, in order to stop excessive production.

Regardless of this information, reality is that consumers of conventional produced food have worries about the fact that in food can be identified residues of pesticides, nitrite, heavy metals and veterinary preparations. And that is directly affecting the product quality.

Organic agriculture is developing as a response to obvious deterioration of alimentary products quality and reportedly environmental degradation. Its' basic motive is harmonization of development with market needs and preservation of the living environment.

In organic food production is present preventive concept of quality, which provides production control in all phases (from soil preparation up to the receipt of finished products and its' market realization).

According to this methodology, special importance have determination of biological and physical-chemical properties of products, then control over production process and to date documentation and product traceability.

The organic food production of high quality food is part of sustainable development concept, which strives to ecologically clean, economically payable and ethics reasonable production.

Numerous researches are conducted in part of comparisons of nutritive values of organic and conventional food (Fillion, L, et. Al, 2002).

Results showed that organic food contains 27% more vitamins C, 22% more iron and around 15% less nitrate. There are differences in the quality of presence proteins, all in favor of organic food.

Also, the presence of larger amounts of omega 3 fatty acids and antioxidants, contribute to the increased biological values (Pejanović, R, et. Al, 2009.). Facts suggest the full justification of accelerated development of organic agriculture.

ORGANIC FOOD PRODUCTION DEVELOPMENT

Organic production in Serbia started with developing recently and is of newer date. According to the Ministry of Agriculture, about 900 organic food producers were registered. In addition, area under organic plant production in the conversion period was 5,000 hectares, while in organic production status were around 3,000 hectares.

In contrast with this data, expert analysts say all the time that our export material is large and that demand for organic products is on the rise. It emphasizes need for development acceleration of this production sector.

There is no doubt that organic food is more health safer than standard, which confirm following parameters:

- Researches show that food manufactured in this way possess larger quantities of nutritive elements, minerals, vitamins and compounds with antioxidant capacities, as well as
- The fact that the organic food world market was worth 18 billion$ ten years ago, and approximately 60 billion$ is worth at present day, indicates significant advantages.

Among major countries that have recognized essence and advantage of this food production way is India, with its 500 thousand producers. To large group of producers belong Australia, Spain and Italy, first in part of fruits and vegetables production. While Germany is one of the largest organic products market in Europe.

These benefits are identified and at us, as data of the Serbian Chamber of Commerce confirmed:

- Serbia had about 9,000 hectares under organically produced crops in 2013, and this is increase of 32% compared to the previous year.

It is undisputed that organic food production is our great business opportunity. For implementation is necessary to implement organic manner of production and to adopt its principles.

Organic agriculture essence is composed of:

*The health principle
- Production of food with high nutritive values,
- Usage of agricultural production systems, in which basic factors and economic principles are in balance with the environmental parameters.

*The ecology principle
- Production is based on risk assessment, precautionary measures and preventive measures,
- It is not allowed to use genetically modified organisms.

*The responsibility principle
- Production should be managed responsibly in order to protect the health and the environment.

We can say that future of Serbia lays in organic food production, which should be the main pillar of country's economic recovery.

In accordance with this was conducted review of National Action Plan for development of organic production in Serbia in November 2011. Addition was made to set out goals and implementation measures in 2009. (Marz et al. 2013.).

Essential elements of National development plan of organic production may be:

- Support for organic production as integral part of national agricultural policy and rural development policy and
- The organic production regulated by law, i.e. that is in line with EU standards.

In Serbia there are legal adjustment rules for production of organic food market for the EU market. These are the Laws on food safety and the law on organic farming, which are basis for alimentary products export (Đokovic, G. et. Al 2013.). Undoubtedly, organic food is Serbian big chance.

CONCLUSION

Serbian potentials in organic products production are certainly great and it should be of optimal usage. First of all, with adoption of Eco-regulation 2092/91, was appointed minimum of requirements that have to be met in order to register product as organic and as such offer it on the market.

Also, with Environment regulation 2078/02 is defined form of support for producers who are involved in this production. In that way defined support, means financial assistance, continued education of producers and encouraging scientific research in the production field, according to concepts of organic agriculture.

Serbia has shown its readiness to support and new trends, by adopting National rural development program from 2011 to 2013. By this program is presented in detail status of agricultural sector. On the basis of that, preventive measures have been adopted through whole evaluating system, in order to prepare farmers and processors for EU market.

Results of such method of work have shown that our chance is in special manufacturing sectors. According to produced quantities, these are sectors for production of fruits, vegetables and crops. In addition to fresh and processed fruits and vegetables in different forms, great potential for substantial investments may be found in soybean. Genetically unmodified soybean, can be a big market potential and can become another Serbian brand.

It is undisputed, that after presented data, we can determine that the organic food production is our greatest economic opportunity. Why is it not happening yet? It is sufficient to say, that struggle for alimentary products market control is very harsh.

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BROCCOLI INGREDIENTS: INNOVATION FROM THE AGRIFOOD SCIENCE TO THE SPIN-OFF EXPERIENCE

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ABSTRACT

Broccoli (*Brassica oleracea* var. *Italica*) appears to be one of the best inducers of mammalian detoxification enzymes (also known as phase II enzymes) associated with a reduced risk of suffering different chronic conditions and diseases including carcinogenic processes and cardiovascular pathologies. Additionally, it has been suggested as a health-promoting food for the digestive tract. Broccoli byproducts (harvest remains) are responsible of important environmental problems, since ¾ of the plant is discarded after harvest. This bioburden has only been used as animal feedstuff or as a source of glucosinolate standards. However, the boosted broccoli crop productions for the growing EU markets in the last few years makes unbearable to manage such amount of byproducts generated by this agri-food activity with a marked seasonality and strong economic relevance in the area. The integrated study "from farm to health" at CEBAS-CSIC (the Spanish Research Council Institute in Murcia), developing healthier foods enriched in bioactive phytochemicals, is uncovering new possibilities for the use of broccoli: edible fresh foods with added value of high-density in chemopreventive glucosinolates, biotechnological ingredients for cosmetics, patented ingredients for culinary and industrial dietetic food products, agricultural applications in green chemistry, etc. Therefore novel opportunities and strategies for economic activities in the EU arena were envisaged: The development of a spin-off company to offer a pipeline of scientifically based and certified products for commercialization – Aquaporins & Ingredients SL. Current developments on bioactive compounds from new sources in agricultural commodities open new opportunities in the convergence of food/pharma, with the transfer of technology from public R&D teams that could be incorporated in technologically based companies with a broad spectrum of possibilities in the global era of the food science for health.
Keywords: Brassica, Bioactive, Glucosinolates, Start-ups, Ingredients

INTRODUCTION

The regular intake of broccoli has been widely related to health promotion which boosted the broccoli production and exports worldwide in the last years. Currently, this commodity is mainly consumed as florets (head inflorescences) and less distributed as sprouts, and the main inflorescence of a broccoli plant represents only one-fourth of the aboveground biomass of this vegetable. There is a great potential for using broccoli by-products as source of health-promoting bioactives and nutrients for feed, food, dietary supplements and drugs. Recent studies showed that broccoli by-products ranged in the health-promoting composition values of previously reported data for inflorescences, the edible part (Dominguez-Perles et al., 2010, 2011). Therefore, there is room for innovative opportunities in transforming agrowaste in sources of bioactive ingredients for the benefit of the food and drug industry.

Broccoli consumption and health

The dietary intake of broccoli (*Brassica oleracea* var. *Italica*), contributes to the prevention of distinct chronic diseases, carcinogenic processes, and cardiovascular pathologies, as well as to improve gut health. Several epidemiological studies that evaluated the physiological effects of the dietary consumption of cruciferous vegetables demonstrated an effective roll in

health promotion. However, the efficacy of each cruciferous food is closely related to the portion and number of servings consumed on a weekly basis. Current animal feeding studies provided accurate information on the specific activity of broccoli in health promotion since epidemiological studies on human populations do not differentiate between the effects of the ingested separate cruciferous vegetables as single factor affecting the condition or disease under study.

The 'healthy food' broccoli attributes are well documented as based on its nitrogen-sulphur compounds (glucosinolates and isothiocyanates), phenolic compounds (hydroxycinnamic acid derivatives and flavonoids), carotenoids, and essential nutrients (vitamins A, B, C, K, E, etc., and mineral K, Na, S, P, Fe, Se, Zn, etc.), which are present in broccoli edible parts and byproducts has been widely characterized and recent data on their level in broccoli by-products (Moreno et al., 2006; Domínguez-Perles et al., 2009).

The glucosinolates in general, and glucoraphanin in particular, which is hydrolyzed by myrosinase to sulforaphane (SFN), are widely studied as responsible of the broccoli anticancer properties. The activity of glucosinolates in chemoprevention of diseases is due to their capacity to induce detoxification of enzymes (particularly phase II enzymes) (Moreno et al., 2006; 2007; Dominguez-Perles et al., 2012). Additionally, the broccoli intake is also important for health and wellbeing for its high content in natural antioxidants such as, vitamin C, carotenoids, and phenolic compounds (especially flavonoids), that reduces the reactive oxygen species (ROS) generation under stressful conditions (Moreno et al., 2006, 2007).

The broccoli consumption has been also related to the prevention of heart disease since takes part in the regulation of the homocysteine serum level, which constitutes a risk factor in the development of coronary diseases. Moreover, the relative positive effect of the broccoli bioactive compounds has been shown as more intense in the smokers than in non-smokers. An anti-inflammatory effect of the broccoli consumption was also described due to the SFN that reduced the secretion of pro-inflammatory molecules by macrophages *in vitro* and inhibited the central transcription factor activation in inflammatory processes and cancer (NF-*κβ*). The dietary fiber of broccoli has also been reported as beneficial for the digestive transit (Moreno et al., 2006; Dominguez-Perles et al., 2012; Martínez-Ballesta et al., 2013).

Broccoli by-products as source of bioactive ingredients

The main source of scientific information on the phytochemical load of broccoli nowadays is available from the edible and marketable inflorescences. However, less than 33% of the total aboveground biomass of broccoli crops corresponds to florets. The aboveground by-products (leaves, stalks, secondary heads when present, non-commercial heads) may represent from > 65% to > 95% of the total production (Figure 1). This is making broccoli an environmentally-costly agrifood activity with tons of agrowaste without any envisaged use and generating environmental problems in the production areas of the world. The adverse agronomic and environmental growth conditions in many of the production areas (i.e., SE Spain) may induce premature or non-blooming and the total lost of the marketable production, converting all the biomass of a season in unprofitable bioburden.

Within the wealth of bioactive compounds present in broccoli, the differential class of compounds in the cruciferous foods is the nitrogen-sulphur compounds: glucosinolates and their cognate bioactive isothiocyanates. The content of the glucosinolates in the broccoli by-products has been shown to be in the healthy range of the inflorescences, with higher amounts in stalks than in leaves. The same is true for the phenolic compounds with leaves presenting between 2 and 4-times higher amounts of phenolic compounds than inflorescences. Both leaves and stalks present the phenolics in the range of health-promoting foods. The minerals and vitamins in leaves and stalks are also in the range described for broccoli inflorescences (Moreno et al., 2007; Rodríguez-Hernández et al., 2012).
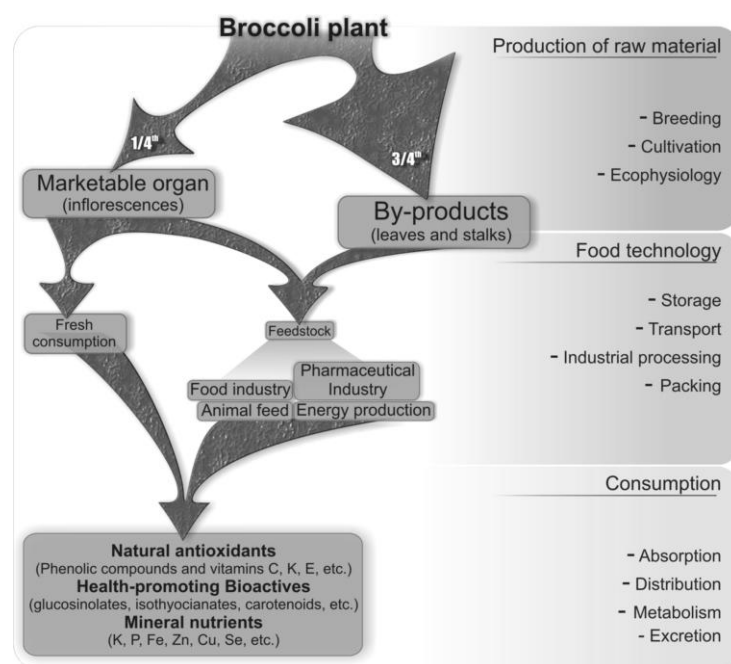
Figure 1. Processing broccoli and the potential for innovative practices using byproducts.

The potential of broccoli by-products for the industry

The use of broccoli by-products has been restricted to the production of some fiber for foodstuff and feed and the glucosinolate-standard and isothiocyanate extractions. Nowadays, the potential use as cost-effective source of ingredients for the food and drug industry is getting much more attention from the scientific community (Dominguez-Perles et al., 2010, 2011, 2012). The bioconversion into high-added-value products from broccoli by-products has focused the attemption of the scientific community to develop methods for the determination and isolation of sulforaphane (SFN) as single ingredient. This method requires the conversion of glucoraphanin to SFN to be performed on both fresh and lyophilized broccoli by-products with a high efficiency. On the other hand, the use of the agro-wastes (harvest remains) as animal feed (for goats and sheep) reduced somehow the environmental effects of this annual accumulations in large production sites as found in SE Spain (Murcia region), but this activity is not enough to convey all the by-products generated from this economically relevant activity (main producer for the EU). Additionally, the use of the exceeding biomass from broccoli crops among a large variety of agro-resources for energy production or as feedstock may increase in the next decades in competition with food production to feed future generations. One of the most relevant handicaps of the agrifood activity is the environmental impact of the by-products generated. This condition is not only related to the broccoli crop but also with other agricultural productions. It is becoming more and more important to transform bioburden into bioactives and broccoli by-products as source of glucosinolates, phenolics, vitamins and minerals are a good example.

The spin-off company Aquaporins & Ingredients developed and patented ingredients from broccoli by-products (PCT ES2011/070760) and plant membranes enriched in transporter proteins (PCT ES2012/070366) to attend the current demand of natural ingredients without chemicals or artificial additives to be incorporated in foods, plant protection, pharmaceuticals and cosmetics, more appealing and attracting to the consumer and able to help in the use of co-products or by-products in the agri-food and industrial activities (zero-waste policies).

CONCLUSIONS

Novel opportunities and strategies for economic activities in the EU arena were envisaged: The development of a spin-off company to offer a pipeline of scientifically based and certified products for commercialization – Aquaporins & Ingredients SL, and current developments on bioactive compounds from new sources in agricultural commodities open new opportunities in the convergence of food/pharma, with the transfer of technology from public R&D teams that could be incorporated in technologically based companies with a broad spectrum of possibilities in the global era of the food science for health

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EFFECT OF KCl-PRIMING AND METHYL JASMONATE ON SECONDARY METABOLITES PRODUCTION IN TWO VARIETIES (WHITE AND RED) OF *BRASSICA OLERACEA* L. UNDER NaCl STRESS

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ABSTRACT

In this work, glucosinolates, anthocyanins and phenolic compounds were determined in two different (red) and (white) *Brassica oleracea* L. sprouts, under non-saline (control) and saline (150 mM NaCl) conditions and after 50 mM KCl-priming or methyl jasmonate (25 μM) elicitation. Differences in the genotype response were observed. The antioxidant capacity of the red cabbage, determined by the amount of phenolic compounds and anthocyanins, was higher in the red cabbage in relation to the white genotype. Also, KCl-priming and MeJa elicitation reduced in the red cabbage both, phenolic compounds and anthocyanins content, whereas they remained unaltered in the white cabbage. However, indole glucosinolates were increased in both cultivars by MeJa and although in the white cabbage aliphatic glucosinolates were reduced by MeJa, in the red cabbage, salinity alleviated this reduction maintaining similar levels to control sprouts pointing out the importance of the combined NaCl and MeJa elicitation to increase indole glucosinolates without a reduction in the aliphatic content.
Keywords: anthocyanins, Brassica oleracea, glucosinolates, methyl jasmonate, phenolic compounds

INTRODUCTION

Cruciferous plants represent an important source of health-promoting phytochemicals such as phenolic compounds and glucosinolates. These secondary metabolites are involved in plant defense and were accumulated in response to environmental stress (Kliebenstein, 2004). Genetic studies have indicated that changes in gene expression can lead to important variations in secondary metabolite profiles under stress conditions. These changes can include glucosinolate biosynthesis genes as well as a set of genes involving in plant defense signaling pathways, e.g., jasmonic acid signaling pathway (Sasaki *et al.,* 2001, Sasaki-Sekimoto *et al.,* 2005, Perotto *et al.,* 2013). In fact, jasmonic acid and related compounds have been widely used as elicitors to mimic insect feeding or wounding (Mikkelsen *et al.,* 2003, Mewis *et al.,* 2005, 2006).

In addition, it has been reported that priming may improve germination and emergence of several seed species proving its effectiveness to improve crop establishment on saline environments (Ashraf *et al.,* 2001, Basra *et al.,* 2005). However, the effect of seed-priming on the secondary metabolite content or the effect of the elicitors on the content of different bioactive compounds has been understudied.

In this work, the content of glucosinolates, anthocyanins and phenolic compounds were determined in two different (red) and (white) *Brassica oleracea* L. sprouts, under non-saline (control) and saline (150 mM NaCl) conditions and after 50 mM KCl-priming or methyl jasmonate (25 μM MeJa) elicitation. Different genotype responses relative to these secondary metabolites were evaluated.

MATERIAL AND METHODS

Plant Material and Germination Conditions

Seeds were rinsed in distilled water and immersed in 5 g·L^{-1} sodium hypochlorite under aeration for 24 h. After pouring off the soaking water, the seeds were weighed (day 0) and spread evenly on trays (5 g per tray) lined with cellulose growth pad (CN Seeds, U.K.) and irrigated everyday with the different solutions tested. Three replicates (trays) were transferred into a controlled environment chamber with a 16 h light/8 h dark cycle and air temperatures of 25 and 20 °C, respectively. The relative humidity (RH) was 60% (day) and 80% (night). Photosynthetically active radiation (PAR) of 400 µmol m^{-2} s^{-1} was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Hamburg, Germany; Sylvania F36W/GRO, Danvers, MA, USA) and metal halide lamps (Osram HQI.T 400 W, Munich, Germany). Aliquots of 5 g of seeds were frozen in liquid nitrogen and stored at −80 °C pending phytochemical analysis.

Seed-priming

Seeds were placed for 10 hours in Petri dishes lined with filter paper, soaked with KCl (50 mM), (PKCl), and air dried for 24 h. Then, the seeds were weighed lined with cellulose growth pad and irrigated with 10ml of Milli-Q water or 150 mM NaCl applied from day 3 to day 7.

Treatment with the elicitor

The phytohormone methyl jasmonate (MeJA) (25 µM) was dissolved in 0.2% ethanol in Milli-Q water (MeJa). MeJa was applied as exogenous spraying on the cotyledons (not as soaking or irrigation solution) with 30 mL oftest solution per sample (10 mL per tray) from day 3 to day 7 of sprouting (5 days of treatment) using Milli-Q water as control (C). A control with 0.2% ethanol and Milli-Q water was also used (Ce) and the half of the seeds were irrigated with Milli-Q water and the other half with150 mM NaCl.

Extraction and determination of anthocyanins

To analyse anthocyanins, each sample (100 mg) was extracted in polypropylene-capped tubes using 2 mL of MeOH:formic acid:H$_2$O (25:1:24, v/v/v), for 12 h at 4 ºC, followed by centrifugation (10000 × g at 4 ◦C) and recovery of the supernatant, which was filtered through a 0.2-μm inorganic-membrane filter (ANOTOP 10 plus, Whatman, Maidstone, U.K.). The extraction procedure was repeated 3 times. The aqueous solutions were quantified by HPLC–DAD using cyanidin-3-O-β-glucopyranoside asexternal standard (Polyphenols, Norway), following the identification of peaks in samples tested in HPLC-DAD-ESI-MSn (Moreno *et al.,* 2010). The total ion chromatograms were recorded as2 alternating, automatic-scan events: full-scan mass spectra (MS)and MS/MS for fragmentation of the most-abundant molecular ions.

Extraction and determination of glucosinolates and phenolic compounds

Freeze-dried samples (100 mg) were extracted with 1.5 mL of methanol 70% V/V in a US bath for 10 min, then heated at 70 °C for 30 min in a heating bath, with shaking every 5 min using a vortex stirrer, and centrifuged (17500 × g, 15 min, 4 °C).Supernatants were collected, and methanol was completely removed using a rotary evaporator. The dry material obtained was redissolved in 1 mL of ultrapure water and filtered through a 0.45 µm Millex-HV13 filter (Millipore, Billerica, MA, USA). Glucosinolates and phenolic compounds were determined by HPLC according to Domínguez-Perles *et al.,* 2010.

RESULTS AND DISCUSSION

In white cabbage, no significant changes in the total phenolic compounds after the different treatments were observed (Figure 1A). However, in red cabbage the total phenolic compounds were decreased by KCl-priming and MeJa in a similar way in both, non-saline

and saline plants (Figure 1B) comparing to the control, being the reductions higher for the KCl-priming treatment. Similar results were found in broccoli sprouts, where MeJa diminished the antioxidant capacity decreasing phenolic compounds (Barrientos-Carvacho *et al.,* 2014). However, in radish sprouts (7 days from germination) irrigation with 1 mM MeJa showed a 41% increase in total phenolic compounds content with respect to non-treated plants (Young *et al.,* 2005). Thus, the Brassicaceae response to MeJa relating to the phenolic concentration depends on the genotype and the experimental conditions such as elicitor amount and the method of application. In any case, the phenolic compounds levels in the red cabbage were higher than in the white cabbage, even after reductions by the MeJa elicitation.

Also, in red cabbage, the effect of salinity was only relevant in the control plants when ethanol was added to the irrigation water.
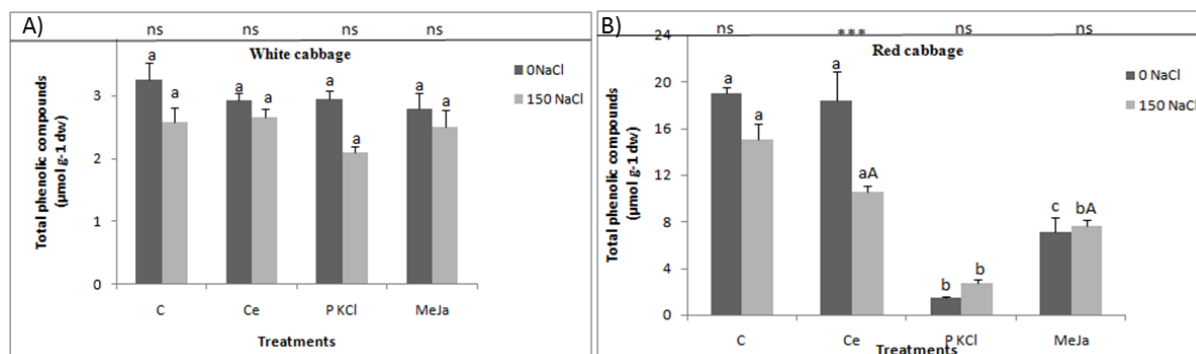
Figure 1. Total phenolic compounds in white (A) and red (B) cabbage control plants (0mM NaCl) and salt-treated plants (150 mM NaCl); when they were irrigated with Milli-Q water (C), sprayed with Milli-Q water plus 0.2% ethanol (Ce), previously primed with KCl (50 mM) (PKCl) and plants sprayed with MeJa (25 µM) (MeJa).

In white cabbage, no effect of KCl, and MeJa on anthocyanins levels was observed under non-saline and saline conditions (Figure 2A). However, in red cabbage, KCl and MeJa reduced the anthocyanins content regarding control plants (Figure 2B) probably due to the fact that anthocyanins levels were 10 folds higher in this genotype comparing to the white cabbage. Also, salinity had a significant effect in the ethanol-control plants of the red cabbage.
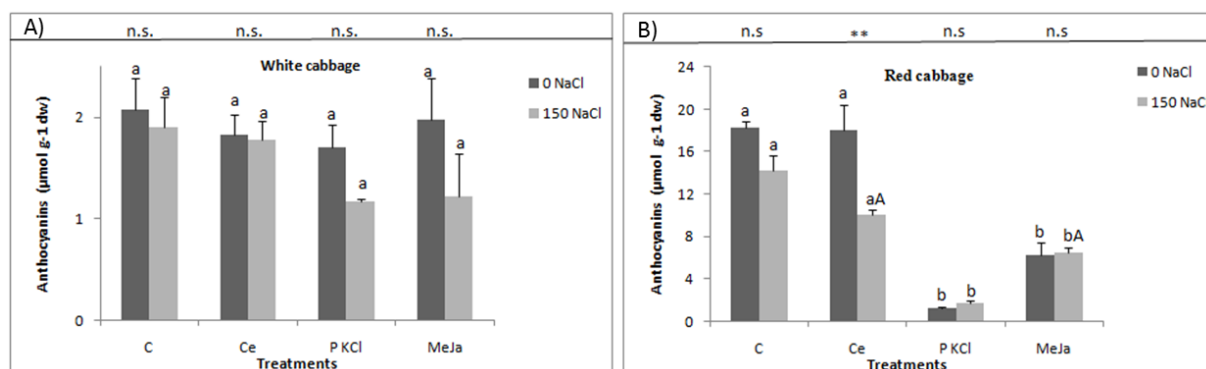
Figure 2. Anthocyanins in white (A) and red (B) cabbage control plants (0mM NaCl) and salt-treated plants (150 mM NaCl); when they were irrigated with Milli-Q water (C), sprayed with Milli-Q water plus 0.2% ethanol (Ce), previously primed with KCl (50 mM) (PKCl) and plants sprayed with MeJa (25 µM) (MeJa).

Total aliphatic and indole glucosinolates were determined (Figure 3). Aliphatic glucosinolates were decreased by MeJa in the white cabbage genotype producing similar reductions in non-

saline and saline plants (Figure 3A). In red cabbage, total aliphatic glucosinolates were reduced by KCl-priming and MeJa under non-saline conditions but not under salinity (Figure 3B).

However, total indole glucosinolates were increased by MeJa in the two genotypes, under both, non-saline and saline treatments, being the increment higher in white cabbage plants comparing to red cabbage plants (Figure 3C, 3D). Similarly, the effect of MeJa on the glucosinolates profile was determined in broccoli sprouts and indole glucosinolates responded to the induction with MeJa (Pérez-Balibrea *et al.*, 2011b).

Additionally, NaCl has been used as an elicitor and its application at a concentration of 100 mM during 3 and 5 days to radish sprouts increased total polyphenols content (Yuan *et al.*, 2010). However, in this work a 150 mM NaCl application elicited only aliphatic glucosinolates in the red cabbage genotype.
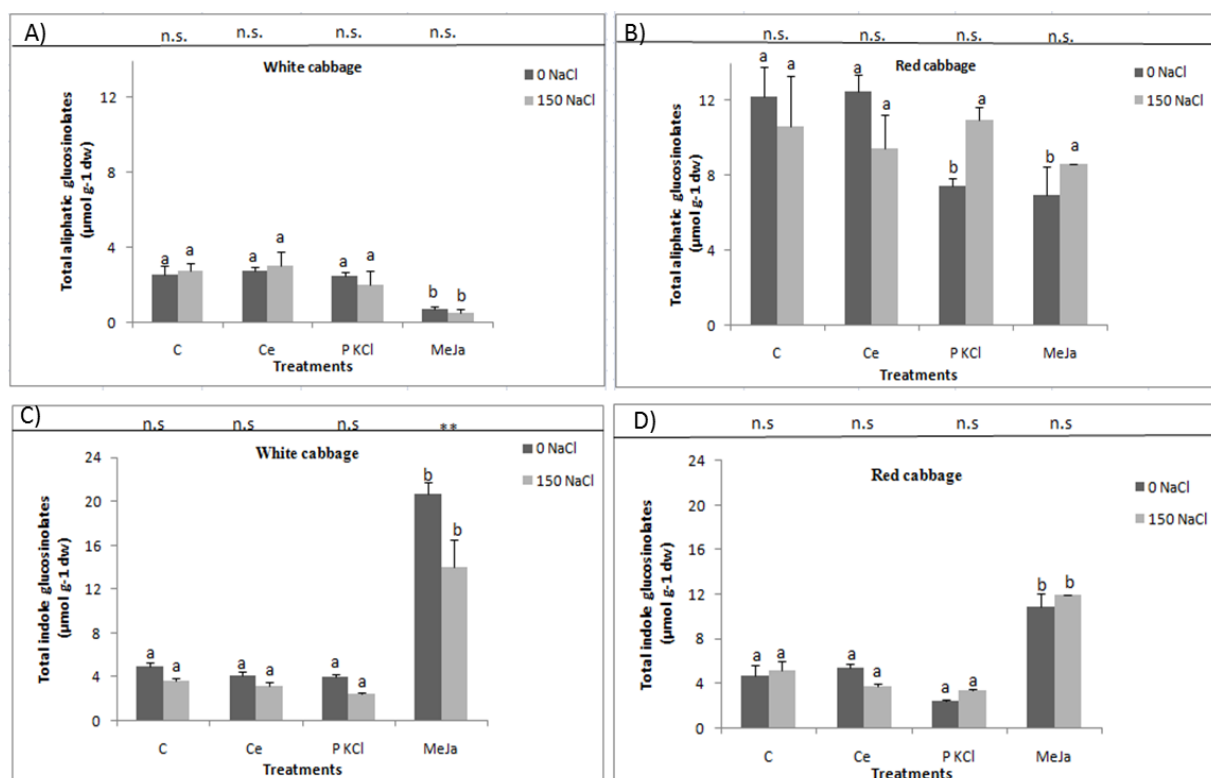
Figure 3. Total aliphatic (A,B) and indole (C,D) glucosinolates in white and red cabbage control plants (0mM NaCl) and salt-treated plants (150 mM NaCl); when they were irrigated with Milli-Q water (C), sprayed with Milli-Q water plus 0.2% ethanol (Ce), previously primed with KCl (50 mM) (PKCl) and plants sprayed with MeJa (25 μM) (MeJa).

CONCLUSIONS

Brassicaceae response to elicitation, relative to the amount of secondary metabolites, may depend on the genotype as well as the experimental conditions. Thus, whereas in white cabbage, elicitation had no effect on phenolic compounds content in red cabbage, the antioxidant capacity of the red cultivar was reduced. Similar reductions were found for anthocyanins, however, the increased amount of these antioxidants (10 folds higher) in the red genotype with regard to the white one, must be considered. In both cultivars, MeJa significantly increased indole glucosinolates and while in white cabbage aliphatic glucosinolates were reduced by MeJa, in the red cabbage, salinity alleviated this reduction maintaining similar levels to control sprouts. Therefore, the combined NaCl and MeJa elicitation may result efficient to increase indole glucosinolates without a reduction in the aliphatic content.

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STUDY ON THE APPLICATION OF THE CHITOSAN-ALGINATE CAPSULES AND ALGINATE-CHITOSAN-ALGINATE CAPSULES IN ETHANOL FERMENTATION

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ABSTRACT

In this study the possibilities of the yeast immobilization in chitosan - alginate and alginate - chitosan – alginate capsules and their application in ethanol fermentation were investigated. All the capsules were with liquid core and they were prepared from 3 % Ca- alginate beads. It was found that the increase in the coating time had a negative effect on the ethanol yield. Simultaneously, the additional coating with alginate with low concentrations led to the formation of a membrane which was the main reason for the increase in the mechanical stability of the capsules. The results were used for the determination of a proper combination of the coating time and the alginate and chitosan concentrations for the formation of mechanically stable capsules.

Keywords: encapsulation, ethanol fermentation, mechanical stability

INTRODUCTION

In recent years the use of ethanol as motor fuel increased because of the increasing environmental concerns regarding greenhouse gas emission. The implementation of immobilized cell systems nowadays provide the ethanol industry with a method not only for maintaining high cell concentrations in the bioreactors, but also reducing processing time without sacrificing product quality (*Margaritis and Kilonzo, 2005*).

Microencapsulation is one of the promising methods for microorganism immobilization. In contrast to entrapment where the cells are trapped in a polymer matrix, the encapsulation process enclose the cells within a thin semi-permeable membrane (*Park and Chang, 2000; Ylitervo et al., 2011*). This membrane allows the bidirectional diffusion of nutrients, oxygen and waste and provides the protection of the inner cells from both mechanical stress and toxic compounds, which might do harm to the entrapped cells (*Gorka et al., 2003; Qi et al., 2006*).

A variety of polymers, such as chitosan, polyacrylates, alginate, polyamino acids, and polyamides, have been used to make microcapsules (*Qi et al., 2005*). With the advance in the study of chitosan as biomaterial, alginate-chitosan (AC) and alginate–chitosan–alginate (ACA) microcapsule has been developed where the positively charged chitosan has been used to make polyelectrolyte complex with negatively charged alginate.

The aim of this study was to investigate the possibilities of ethanol production with yeast cells immobilized in alginate-chitosan and alginate-chitosan-alginate microcapsules.

MATERIALS AND METHODS

Microorganisms and medium

The experiments were carried out with dry yeast strain *Saccharomyces cerevisiae Actiflore BO213*, purchased from Laffort, France.

The culture medium has the following composition (g/dm^3): glucose – 118.40; $(NH_4)_2SO_4$ – 2; KH_2PO_4 – 2.72, $MgSO_4·7H_2O$ –0.5; yeast extract – 1. All the culture media were sterilized in autoclave at 121 °C for 20 min.

Cell Immobilization

The yeast suspension (yeasts were rehydrated according to the manufacturer instructions) was added to a 3% w/v sodium alginate solution and subsequently dropped into a 2% w/v $CaCl_2$ solution. The cell concentration in the beads was 10^7 CFU/cm^3 of gel. The beads were left for 30 min in $CaCl_2$ and were then placed into a 0.2 % w/v chitosan solution in 1% acetic acid. Equal parts of alginate beads stayed in the chitosan solution for 30, 60, 90 and 120 min, respectively. Afterwards, all of the chitosan-alginate beads were washed with sterile water. All the beads stayed in a 0.05 M sodium citrate solution for 30 min to obtain microcapsules with liquid core (AC 30, AC 60, AC 90 and AC 120). Part of these alginate-chitosan microcapsules were used for fermentations and the others were placed in 0.2 % w/v alginate solution for 60 min (ACA 30, ACA 60, ACA 90 and ACA 120). All microcapsules were stored in saline before fermentation.

Fermentations

For each variant 14 g microcapsules were introduced in 200 cm^3 sterile medium. The fermentations were carried out at 30°C in fermentation bottles equipped with airlocks. The free cell fermentation was used as a control sample. The free cells suspension, containing 10^7 CFU/cm^3 was added to 200 cm^3 sterile culture media.

Analytical Methods

The mechanical stability of particles was determined visually by monitoring the moment when the capsules destruction started.

The fermentation rate was determined by measuring CO_2 evolution indirectly. Therefore, the bottle weight loss was recorded at every 12 hours until the 84th hour for each of the investigated variants. The fermentation rate of immobilized cells was compared to the same of free cells.

The fermentation parameters were determined according to (*Kopanarova et al., 2014*). The ethanol concentration was measured according to:

$$E = \frac{92\Delta m}{88.V_{fer}} \tag{1}$$

where: Δm – the bottles weight loss, g; V_{fer} – volume of the fermenting media, dm^3; 92– molecular weight of 2 moles ethanol; 88 – the molecular weight of 2 moles CO_2;

The ethanol yield was determined according to:

$$\alpha = \frac{E}{k.S_0} \tag{2}$$

where: E – ethanol concentration, g/dm^3; к – theoretical yield – (for glucose k=0.51); S_0 – initial substrate concentration, g/dm^3;

The substrate concentration during the fermentation was calculated according to:

$$S = S_0 - \frac{E}{0.464} \tag{3}$$

where: 0.464 – summarized ethanol yield per substrate unit (the theoretical ethanol yield was reduced with the yield of biomass and secondary metabolite during fermentation);

Fermentation kinetics

Kinetic characteristics were determined according to *Kostov et al., 2012* and *Kostov et al., 2010*. For this purpose, the Monod model is integrated into the following differential equations system:

$$\left|\begin{array}{l} \dfrac{dX}{d\tau} = \left(\mu_{\max} \dfrac{S}{K_{sx}+S} \right) X \\[3mm] \dfrac{dE}{d\tau} = \left(q_{p\max} \dfrac{S}{K_{sp}+S} \right) X \\[3mm] \dfrac{dS}{d\tau} = -\dfrac{1}{Y_{x/s}} \dfrac{dX}{dt} - \dfrac{1}{Y_{p/s}} \dfrac{dE}{dt} \end{array}\right. \tag{4}$$

where: the first equation in the system represents the change of biomass concentration, the second one - the ethanol production rate, and the third one - the substrate consumption rate.

RESULTS AND DISCUSSION

The results for fermentation dynamics are shown on Figure 1. The fermentation dynamics were similar for the immobilized and free cells.

a) Chitosan coating for 30 min

b) Chitosan coating for 60 min

c) Chitosan coating for 90 min

d) Chitosan coating for 120 min
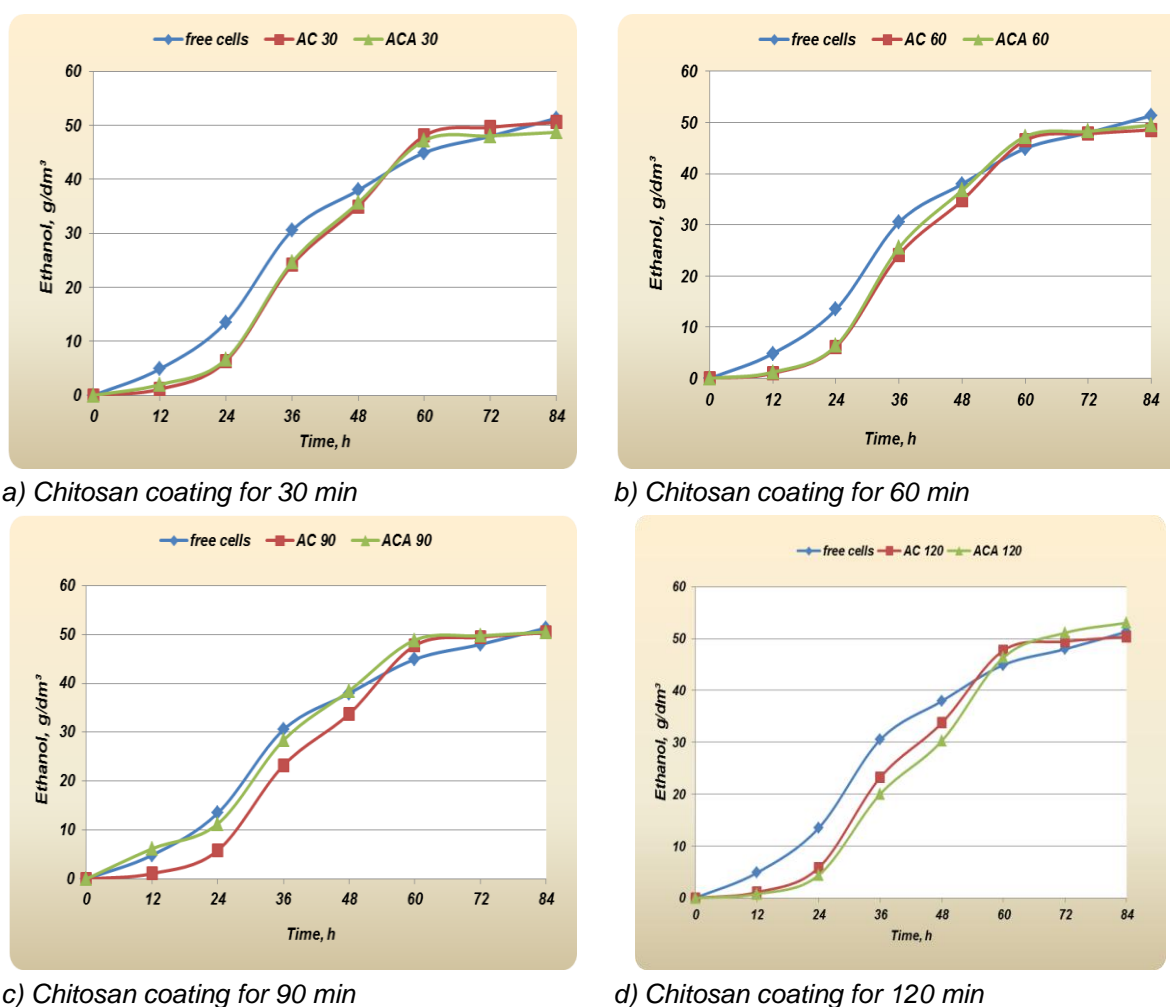
Figure 1. Fermentation dynamics of free cells and microcapsules of alginate-chitosan (AC) and alginate-chitosan-alginate (ACA) prepared by chitosan coating for 30, 60, 90 and 120 min, respectively

Free cells started fermentation faster than the immobilized ones and within 48 hours the ethanol yield was approximately 62% of the theoretical ethanol yield. The slow start of the immobilized cells was explained with the existed diffusion barriers. Although the fermentation

rate was similar for the free and immobilized cells, the presence of the barrier did not allow the ethanol to leave the capsules which was the main reason for the lower ethanol concentration within the first 48 hours. More interesting results on the fermentation dynamics were recorded between the 48th and 72nd hour. It can be assumed that during this period the ethanol concentration in the capsules reached a certain value, which led to the quickly overcome in the concentration differences and the accumulation of comparable ethanol volumes between the immobilized and free cells in fermenting media. At the end of the fermentation the ethanol concentration is slightly higher for the immobilized cells. Therefore, it can be hypothesized that the membrane protects cells by product inhibition.

It is interesting to note that the additional alginate layer had no effect on the fermentation dynamics when the time for chitosan coating was 30 and 60 min, respectively (Figure 1a and 1b). The increase in the time for chitosan coating led to discrepant results. Figure 1c shows that the ethanol concentration of the ACA microcapsules has higher at the beginning of the fermentation. At the end of the fermentation the results were similar to those for the ethanol produced by AC microcapsules. Figure 1d presents slightly higher ethanol concentration for AC microcapsules compared to the ACA microcapsules at the beginning of the fermentation. At the end of fermentation the AC microcapsules produced slightly lower ethanol.

Figure 2 shows the results on the ethanol yield at the 24th, 48th and 72nd hour. The data for the ethanol yield confirmed the observed fermentation dynamics. The ethanol yield was higher for free cells until the 48th hour and then the immobilized cells showed increase in the ethanol yield.
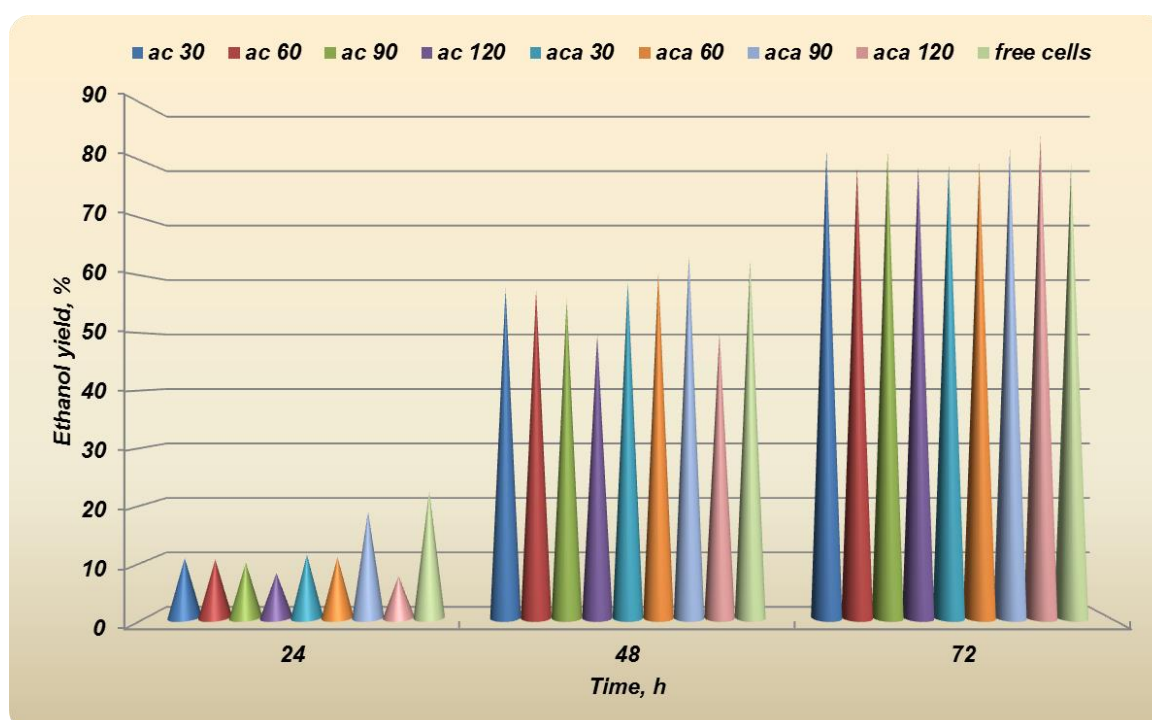
Figure 2. Ethanol yield at fermentation with free and immobilized cells

The general trend observed in the fermentation dynamics and ethanol yield was the fermentation rate decrease with the increase in the time for capsules preparation. It could be estimated by the use of kinetic parameters of the capsules. The kinetic characteristics (specific growth rate μ_{max} and ethanol production rate q_{pmax}) are defined according to the Monod model and they are presented in Table 1.

Table 1 show that the immobilization did not affect remarkably the biomass growth, but it had a significant impact on the cells primary metabolism. The data indicated that the immobilized cells showed a little higher specific growth rate than the free cells. Therefore, it can be

suggested that the AC and ACA membranes protect the cells from substrate and product inhibition, which is a prerequisite for rapid yeast growth.

Table 1. Kinetic parameters of free and immobilized cells

Variant	μ_{max}, h^{-1}	q_{pmax}, g/(g.h)
Free cells	0.347	2.070
AC30	0.492	0.472
AC60	0.497	0.785
AC90	0.519	0.785
AC120	0.451	0.871
ACA30	0.448	0.412
ACA60	0.401	0.612
ACA90	0.491	0.651
ACA120	0.442	0.721

Unlike the specific growth rate, the ethanol production rate of immobilized cells was between 2.6 and 5 times lower than the same of free cells. However, the concentration of ethanol produced by immobilized cells was similar to that of free cells, particularly because of the greater total amount of capsules in the fermentation bottles. The data indicate that there was a reduction of approximately 20% in the specific ethanol production rate for ACA microcapsules compared to the AC microcapsules. Therefore, it can be concluded that the process of microcapsules formation affected the ethanol production. It may be considered to be a disadvantage of the immobilized cells system. Nevertheless, the said drawback is compensated by the use of immobilized cells mainly in continuous fermentation systems. According to Naydenova et al., 2014 the system productivity is a parameter, which can be used for a transfer of batch fermentation system to a continuous mode. The system productivity is defined as the maximum concentration of the produced ethanol for a certain time in a specified volume. The immobilized cells showed relatively high system productivity (Figure 3). It can be expected that in a continuous fermentation system it will be well above average for the experiments – 2.96 g/(dm^3.h). Although the system productivity with free cells was slightly higher than the same with immobilized cells in batch fermentation, the results will be contrary in a continuous mode.
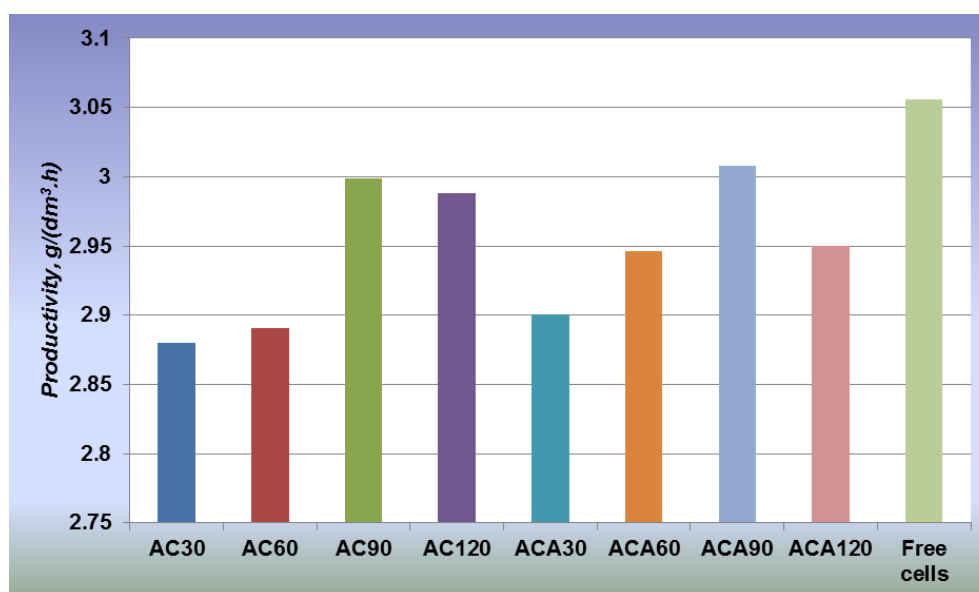
Figure 3. System productivity with free and immobilized cells

The process of crosslinking enhances the mechanical stability of the capsules. AC capsules with a liquid core were with good elasticity which facilitated the CO_2 removal. The addition of an outer layer of alginate did not affect the capsules elasticity. The visual observations showed that the ACA capsules were destroyed more difficultly than the AC ones. Thus, the additional layer improved the mechanical stability of the capsules. This fact should be taken into account in subsequent research, particularly in the development of continuous fermentation system.

CONCLUSION

The possibilities for ethanol production with AC and ACA microcapsules with a liquid core were investigated. The results showed that the additional alginate layer led to the increase in the mechanical stability of all the capsules and it affected the fermentation in different ways depending on the time for chitosan coating. The additional alginate layer had no influence on the fermentation dynamics when the time for chitosan coating was 30 and 60 min. However, it led to the decrease in kinetic parameters of the capsules compared to AC microcapsules. The additional layer led to faster fermentation and slight differences in the kinetic parameters values between ACA 90 and AC 90 microcapsules. The ACA 120 started fermentation more slowly than AC120 but at the end they produced more ethanol. Therefore, it can be summarized that the optimal kinetic and mechanical characteristics will have double-layered capsules when time for chitosan coating is between 90 and 120 min.

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ANTIBIOTIC RESISTANCE OF AUTOCHTHONOUS POTENTIAL PROBIOTIC BACTERIA

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ABSTRACT

Traditional cheeses represent great source for isolation and selection of lactic acid bacteria and potential application as starter cultures or potential probiotic bacteria. Nowadays, probiotic criteria (ability to survive gastrointestinal tract, antimicrobial activity, antibiotic resistance) and clinical trials, became very important for selection of autochthonous potential probiotic bacteria. Some potential probiotic strains may carry transmissible plasmid-encoded antibiotic resistance genes. The aim of this work was to evaluate the antibiotic resistance of 85 autochthonous potential probiotic strains isolated from traditional Serbian cheeses. Antibiotic resistance was determined for ampicillin, vankomycin, oxacillin, neomycin, chloramphenicol, gentamycin, tetracycline, erythromycin, kanamycin, penicillin, streptomycin using disk diffusion test and E-test. All the strains showed resistance to kanamycin, while most of the LAB strains showed resistance to streptomycin (94%) and vancomycin (82%). Slightly less number of strains showed resistance to gentamicin (65%), neomycin (56%) and oxacillin (53%). All strains showed sensitivity to penicillin, while several strains showed resistance to ampicillin, erythromycin, tetracycline and chloramphenicol. Strains showed minimal inhibitory concentracion (MIC) for streptomycin ≥ 24 µg mL^{-1}, gentamicin ≥ 48 µg mL^{-1}, oxacillin ≥ 1.5 µg mL^{-1}, tetracycline ≥ 3 µg mL^{-1} and erythromycin ≥ 1 µg mL^{-1}. The results obtained in this study indicate that resistance of autochthonous potential probiotic lactic acid bacteria isolated from traditional Serbian cheeses to antibiotics, is very important feature in characterisation and selection of strains for application as potential probiotic bacteria in production of functional food.

INTRODUCTION

Lactic acid bacteria (LAB) represent heterogeneous group of bacteria which are widely spread in the nature: gastrointestinal and urogenital tract of human and animals, dairy products, numerous fermented food, etc. These bacteria traditionally present natural microflora of different fermented dairy product, such as cheeses, as well as different cereals, fermented sausages, vegetables, etc.

According to the definition given by FAO/WHO (2002) probiotics are „live microorganisms which when administrated in adequate amounts, confer a health benefits on the host". Many LAB are used as probiotic bacteria due to their potential health benefits to the consumer health. Regarding this, LAB represent important part of food industry.

Lactic acid bacteria strains isolated from traditional made cheeses constitute a reservoir of unexplored potential in biotechnology. Considering the fast growing interest for application of probiotic strains in food production, it could be presumed that it is possible to isolate some strains, with potential probiotic ability, among the autochthonous strains (Radulovic *et al.*, 2010). In order to be used as probiotics LAB should fulfill essential characteristics which include the following: (1) recognition as safe (GRAS; generally recognized as safe); (2) viability during processing and storage; (3) antagonistic effect against pathogens; (4) capability to survive in the intestinal ecosystem and (5) adherence to the intestinal epithelium

of the host among others (McFarland *et al.*, 1997; Begley *et al.*, 2005; Vesterlund *et al.*, 2005; Lin *et al.*, 2006), as well as sensitivity to the antibiotic. Considering the importance of antibiotic resistance of LAB in food chain, antibiotic susceptibility of autochthonous potential probiotic bacteria is a very important criteria for their selection (Radulovic *et al.*, 2012).

The extensive use of antibiotic in animal husbandry for therapeutic and prophylactic use as well as growth promoters (Barton, 2000, Singer *et al.*, 2003), lead to the use of antibiotic as integral part of feed. Autochthonous LAB isolated from dairy products obtained from animals that have been treated with antibiotics, could carry antibiotic resistant determinates (Teuber *et al.*, 1999). This resistance could be transmitted to the human population through food chain as one of the main pathways for the transmission of antibiotic resistant bacteria from animals to humans (Singer *et al.*, 2003). Although many strains are not pathogenic, autochthonous LAB could constitute a reservoir of genes conferring resistance to antibiotics which might be transferred to pathogenic strains (Lukasova and Sustackova, 2003). The circulation of genes coding for antibiotic resistance from beneficial LAB in the food chain via animals to humans is a complex problem (Radulovic *et al.*, 2012). Therefore, there is a need to evaluate the safety of autochtonous potential probitic lactic acid bacteria regarding their ability to acquire and disseminate antibiotic resistance determinants in selection of LAB.

The aim of this work was to examine resistance of 85 autochthonous lactic acid bacteria to different antibiotic and to determine minimal inhibitory concentration of antibiotics to the LAB.

MATERIAL AND METHODS

Traditional cheeses
Autochthonous lactic acid bacteria were isolated from traditional made white brined Sjenicki cheese. Samples of cheeses were collected from different household in region of Sjenica in Serbia. The collected samples of cheeses were kept in sterile bags at 4°C and analyzed within the following 24 h.

Isolation and selection of lactic acid bacteria
From each cheese samples, 10 g were weighed aseptically and transfered into a sterile Stomacher bag under unseptic conditions and homogenized in 90 mL of sterile sodium citrate 20% (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. Lactobacilli were isolated from plates which were incubated in anaerobic conditions (Gas Pak, BBL, Germany) on MRS agar (Oxoid, CM 361) for 48 h on 30°C and 45°C. Lactococci were accumulated on M17 agar (Oxoid, CM 785) under aerobic conditions on 30°C and 45°C for 48 h.

After incubation, few single colonies were randomly picked from MRS and M17 agar plates and streaked on new agar plates for purification. Pure Gram-positive and catalase-negative isolates were cultivated in MRS and M17 broth overnight and stored under -80°C in MRS and M17 broth supplemented with 20% glycerol. Isolates were revitalized by two consecutive transfers on MRS broth for lactobacilli on 37°C and M17 broth for lactococci on 30°C.

Overall, 161 pure cultures were isolated form Sjenicki cheese and 85 strains were selected based on their technological characterisation and identification with API system (BioMerieux, France). The following 85 strains of LAB were examined for antibiotic resistance: *Lactococcus lactis* (twenty-one strains), *Lactococcus cremoris* (seven strains), *Leuconostoc* (five strains), *Lactobacillus plantarum* (twenty-eight strains), *Lactobacillus paracasei* (nineteen strains), *Lactobacillus brevis* (three strains) and *Lactobacillus pentosus* (two strains).

Testing for antibiotic resistance
Selected autochthonous LAB were tested for their resistance to 11 antibiotic: penicillin (10U), ampicillin (10 µg), vancomycin (30 µg), streptomycin (10 µg), kanamycin (30 µg), erythromycin 15 µg), tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), neomycin (30 µg) and oxacillin (1 µg). Antibiotics were produced by Becton, Dickinson and

Company, USA. Testing was performed using the standard disc diffusion test (National Committee for Clinical Laboratory Standards, 1993).

Determination of antibiotic minimal inhibitory concentracion
E-test strips (BioMerieux, France) for determination of minimal inhibitory concentration of antibiotic susceptibility for gentamycin, oxacillin, tetracycline, streptomycin and erythromycin were used according to the manufacturer's instruction. Briefly, a bacterial suspension was made by picking a few colonies of LAB from MRS or M17 agar plates using sterile loop and transferred to sterile sodium chloride solution (0.9%) to reach density corresponding to the McFarland 0.5 value. Using sterile swab, the suspension of isolates was swabbed on appropriate agar plates in three directions, rotating the plates for 60 degrees each time to evenly distribute the suspension of isolate. The E-test strips were placed on air dried surface of plates. For lactobacilli and lactococci isolates were used MRS and M17 agar plates which were incubated anaerobically at 37°C for 48 h and aerobically at 30°C for 48 h, respectively. The minimal inhibitory concentration (MIC) for each antibiotic was read as the lowest antibiotic concentration in which the growth was inhibited.

RESULTS AND DISCUSSION

The results obtained for antibiotic resistance of selected autochthonous strains using the disk diffusion method are shown in table 1.
Among 85 strains of autochthonous potential probiotic lactic acid bacteria, none of the strains was totally susceptible to the tested antibiotics and some of the strains showed multiple resistances. Results showed that all strains were susceptible to penicillin, while one strain of *Lb. plantarum* and *Lb. brevis* showed resistance to amplicilin. Also, resistance to erythromycin, tetracycline and chloramphenicol, regardless the species strains, was present in only several strains which is in accordance to the results of Herros et al., 2005. Autochthonous potential probiotic lactic acid bacteria showed resistance to kanamycin, while almost all strains were resistant to streptomycin and vancomycin. These results are in accordance to the several papers (Herros *et al.*, 2005, Zhou *et al.*, 2005, Kastner *et al.*, 2006) which indicate that LAB are normally resistant to the principal types of antibiotics.

Table 1. Resistance of 85 strains of autochthonous potential probiotic lactic acid bacteria determined by disk diffusion test

	Lc. lactis	*Lc. cremoris*	*Leuconostoc*	*Lb. plantarum*	*Lb. paracasei*	*Lb. brevis*	*Lb. pentosus*
Number of strains	21	7	5	28	19	3	2
Ampicillin (10 µg)	0	0	0	1 RS	0	1 RS	0
Chloramphen. (30 µg)	1 RS	0	0	0	1 RS	0	0
Erythromycin (15 µg)	0	0	0	1 RS	0	0	0
Gentamicin (10 µg)	15 RS	4 RS	4 RS	20 RS	11 RS	1 RS	0
Kanamycin (30 µg)	21 RS	7 RS	5 RS	28 RS	19 RS	3 RS	2 RS
Neomycin (30 µg)	12 RS	4 RS	2 RS	15 RS	11 RS	2 RS	1 RS
Oxacillin (1 µg)	11 RS	2 RS	1 RS	17 RS	10 RS	2 RS	2 RS
Penicillin (10U)	0	0	0	0	0	0	0
Streptomycin (10 µg)	21 RS	6 RS	5 RS	26 RS	18 RS	2 RS	2 RS
Tetracycline (30 µg)	1 RS	1 RS	0	3 RS	0	0	0
Vancomycin (30 µg)	18 RS	6 RS	3 RS	24 RS	15 RS	2 RS	2 RS

RS-resistant strains

The incidence of antibiotic resistance of potential probiotic bacteria varied notably depending on the minimal inhibitory concentration (MIC). Among the strains which were tested for streptomycin, one *Lb. plantarum* strain showed ≥24 µg mL^{-1} for MIC, while the rest of the tested strains showed significant higher concentration (192-1024 µg mL^{-1}). A broad range of

MIC distributions of potential probiotic bacteria was detected for erythromycin, 0.19 µg mL^{-1} for one *Lb. paracasei* strain, 0.50 µg mL^{-1} for *Lb. plantarum* and *Leuconostoc*, 1 µg mL^{-1} for *Lb. plantarum, 4* µg mL^{-1} for two *Lc. lactis* strains and 6 µg mL^{-1} for one *Lb. plantarum* strain. Tetracycline MIC distributions was detected for *Lc. lactis* 0.064 µg mL^{-1}, 0.094 µg mL^{-1} and 6 µg mL^{-1}, 3 µg mL^{-1} for *Lb. planatrum* strain and 4 µg mL^{-1} for *Lc. cremoris* strain. A wide range of gentamycin MIC distributions was determined, 48 µg mL^{-1} for *Lb. plantarum* and *Lb. paracasei*, 64 µg mL^{-1} for *Lb. paracasei* strain, 96 µg mL^{-1} for three *Lb. plantarum* strains and one *Lb. paracasei* strain, 128 µg mL^{-1} for *Lc. cremoris* and *Lb. paracasei* and 256 µg mL^{-1} for one *Lc. lactis* and *Lb. paracasei* strain. For oxacillin, MIC range of antibiotic susceptibility was clearly narrow, 1.5 µg mL^{-1} for *Lb. paracasei*, 2 µg mL^{-1} was detected for six *Lb. paracasei* strains and one *Lc. lactis* strain, and 3 µg mL^{-1} for one *Lb. plantarum* strain.

These results agree with other studied on probiotic bacteria (Hummel *et al.*, 2007, Matto et al., 2007, Korhonen, 2010).

Knowledge on the antibiotic resistance of autochthonous potential probiotic bacteria is still limited. However, resistance to certain antibiotic is not unusual for autochthonous LAB which includes potential probiotic and starter cultures (Florez *et al.*, 2005, Katla et al., 2001).

Results of many studies, including this one, indicate that genera *Lactobacillus, Leuconostoc* and *Lactococcus* are generally quite sensitive to clinically relevant antibiotic such as penicillin, ampicillin, erythromycin and tetracycline. In contrast, some resistance appear to be intrinsic for LAB, like vancomycin, streptomycin, kanamycin and gentamycin (Kastner et al., 2006, Korhonen, 2011).

Determination of MIC breakpoint values for LAB is very important, considering the fact that it may affect on the decision on whether resistance could be considered as intrinsic. Furthermore, there is a not standard and the National Committee for Clinical Laboratory Standards does not establish MIC breakpoints for LAB, except the *Enterococcus* spp.

CONCLUSIONS

The food chain was considered as the main route of transmission of antibiotic resistant lactic acid bacteria between the animals and human population. Fermented dairy products and fermented meats, which are not heat-treated before consumption, provide a vehicle for antibiotic resistant LAB with a direct link between the animal indigenous microflora and the human gastrointestinal tract. Results from the present study suggest that autochthonous lactic acid bacteria used as potential probiotic bacteria isolated from traditional cheeses might carry and possibly spread antibiotic resistance determinants. Since there has been a significant rise in the consumption of probiotic products, it is important that probiotics should be tested for the presence of transferable resistance genes before being used as probiotic bacteria. In the future research, continuous attention should be paid to the selection of probiotic strains free of transferable antibiotic-resistance determinants.

ACKNOWLEDGEMENTS

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INFLUENCE OF FREEZE-DRYING ON VIABILITY AND BIOCHEMICAL PROPERTIES OF AUTOCHTONOUS LACTIC ACID BACTERIA

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ABSTRACT

The aim of this study was to examine the influence of freeze-drying on autochthonous lactic acid bacteria *Lactobacillus plantarum* 564, *Lactobacillus paracasei* 08, *Lactococcus lactis* 563, *Lactococcus lactis* 565 (Department for Food Microbiology, Faculty of Agriculture, University of Belgrade). Their ability to tolerate freeze-drying process was examined with determination of viability and acidification activity. Four different medium were used for freeze-drying, 10% reconstituted skim milk (RSM), 10% RSM+1% glycerol, 10% RSM+1.5% gelatin and 10% RSM+1% glycerol+1.5% gelatin. Strains were freeze-dried in ALPHA 1-4 freeze-dryer (Martin Christ, Osterad am Harz, Germany). Before freeze-drying, count of examined strains was around 10^9 cfug^{-1}. Cell number of all autochthonous strains after freeze-drying remained at the same level. The acidification activity of freeze-dried *Lc. lactic* 563 and *Lc. lactis* 565 strains were changed, but still at satisfactory level. Nevertheless, *Lb. plantarum* 564 and *Lb. paracasei* 08 showed reduced acidification activity after freeze-drying in all mediums. The results indicate that freezing mediums and freeze-drying conditions are suitable for *Lc. lactis* 563 and *Lc. lactis* 565 strains. However, freeze-dried *Lb. plantarum* 564 and *Lb. paracasei* 08 showed unsatisfactory biochemical properties. Therefore, alternative freezing medium and methods are essential to afford protection and unchanged biochemical properties for these autochthonous strains.

Keywords: autochthonous lactic acid bacteria, freeze-drying, acidification activity, viability

INTRODUCTION

In dairy-fermentation industry, which encompasses production of e. g. cheese, yoghurt, sour cream etc., lactic acid bacteria (LAB) play central role in process of production. The most important LAB, in production of dairy fermented products, belong to the genera *Lactococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* (Daly *et al*., 1998). In food industry LAB may be used as a starter cultures and/or probiotic bacteria. Use of LAB in food industry largely depends on their preservation, which guarantees long-term stability and viability of LAB (Carvalho *et al.,* 2003). In industrial conditions, LAB may be distributed in liquid, spray dried or freeze-dried form. Freezing and freeze-drying has been extensively used for the preservation and distribution of LAB as a starter cultures or probiotics. However, during process of freeze-drying, bacterial cells are exposed to low temperature, to the formation of ice-crystals and to the remove of water within the cells. Freeze-drying might cause denaturation of DNA and sensitive proteins, cell membrane damage and decreased viability of cells (Leslie *et al*.,1995). In order to use LAB as a starter cultures or probiotic in food industry, it is necessary to optimize freeze-drying process. Viability of cells during process of freeze-drying depends of many factors: initial concentration of microorganisms, growth conditions, drying medium and protective agents, freezing rate, storage conditions (temperature, atmosphere, relative humidity) and rehydratation conditions. (Andersen *et al*., 1999; Carvalho *et al.,* 2002; Desmons *et al.,* 1998; Morgan *et al,.* 2006). In freeze-drying process, protectants have an important role in conservation of viability. Protectants should provide protection to the cells at low temperature during freeze-drying process and provide a good matrix to allow stability and easy rehydratation. Many different substances have been used for testing their protective action, polyols, polysaccharides, disaccharides, amino acids,

proteins, minerals, salts of organic acids and vitamins-complex media in combination with skim-milk like as drying medium. (Champagne *et al.* 1991; Huba´lek 2003). One more critical factor in freeze-drying process is cooling rate. In case when cooling is slow enough, water will have time to flow out of the cell by osmosis, but in case when cooling is too fast, the cell will not lose water quick enough to maintain equilibrium and ice crystal form intracellularly which will cause cell damage (Zhao *et al.* 2005). However, protection afforded given by a given additive and optimum rate of freezing during process of freeze-drying will varies from species to species (Font de Valdez *et al.* 1983; Champagne *et al.* 1991; Sanders *et al.* 1999).

So, the aim of this study was to evaluate the effect of protective agents and freezing temperature on the viabilities and biochemical properties of four autochthonous LAB, when subjected to a freeze-drying process.

MATERIAL AND METHODS

Bacterial strains
Autochthonous LAB *Lactobacillus plantarum* 564, *Lactobacillus paracasei* 08, *Lactococcus lactis* 563, *Lactococcus lactis* 565 belong to Department for Food Microbiology, Faculty of Agriculture, University of Belgrade. These LAB strains were isolated from Serbian traditional cheeses and selected based on their biochemical characteristics. *Lb. plantarum* 564 and *Lb. paracasei* 08 were grown anaerobically at 30°C for 48 h on MRS agar (Merck). *Lc. lactis* 563 and *Lc. lactis* 565 were grown at 30°C for 24 h on M17 agar (Merck).

Freeze-drying medium
Four different variants of freeze-drying medium were used: reconstituted skim milk (RSM) (10% w/v); RSM (10% w/v) + glycerol (1% v/v); RSM (10% w/v) + gelatin (1.5% v/v); RSM (10% w/v) + glycerol (1% v/v) + gelatin (1.5% v/v). Suspensions of freeze-drying medium were prepared in distilled water and sterilized at 121°C for 10 min before mixing with a volume of washed cells of LAB to obtain initial concentration of 10^9 CFU/ml. These freeze-drying medium were used for the protection of starter cultures or probiotic bacteria in process of freeze-drying due they have not influence on the quality of cheese such as taste, aroma and color.

Freeze-drying
Tested lactobacilli and lactococci were incubated at 30°C for 24 h in MRS and M17 broth, respectively. Flasks containing 30 ml of MRS and M17 broth were inoculated with tested lactobacilli and lactococci, respectively, in final concentration 2 % and incubated at 30°C for 7 h, until early stationary phase. Cells were harvested by centrifugation at 7000 g for 10 min, washed twice with sterile Ringer`s solution and subsequently re-suspended in 30 ml of appropriate drying medium with protectans. Samples of 30 ml were then maintained 1 h at room temperature prior to freezing at -80°C for 24 h, so as to permit equilibration between the cells and drying medium. After overnight storage at the freezer, samples were desiccated in ALPHA 1-4 freeze-dryer (Martin Christ, Osterad am Harz, Germany) under vacuum 0.1 mbar at -15°C and 15°C for the primary (sublimation) and the secondary (desorption) drying stages, respectively.

Viability of tested strains after freeze-drying
The viability of the tested lactobacilli and lactococci, after freeze-drying, were obtained using plate count method. 1 g of sample were homogenized in 9 ml of sterile Ringer`s solution for 1 min with a Vortex mixer. For tested lactobacilli, appropriate dilutions were pour plated in Petri dishes and overlaid with MRS agar and incubated anaerobically for 48h at 30°C. Appropriate dilutions of tested lactococci were pour plated in Petri dishes, overlaid with M17 agar and incubated for 24h at 30°C. The percentage survival of the tested strains after freeze-drying process was expressed as follows: Survival (%) = logNo/logNc x 100, where No is the CFU/g

at the end of freeze-drying and Nc is the CFU/g before freeze-drying (at the end of centrifugation).

Acidification activity

Acidifying activity of strains was determined before and after process of freeze-drying. The strains, before freeze-drying, were revitalized by growing overnight in MRS broth at 30°C and M17 broth at 30°C for lactobacilli and lactococci, respectively. RSM (10% w/v) were inoculated with (1% v/v) revitalized strains and incubated at 30 °C. After freeze-drying of strains, RSM (10% w/v) were inoculated with 1% w/v of freeze-dried samples and incubated at 30°C for 24 h. The pH was measured after 0, 4, 6, and 24 h with a pH meter (Microprocessor pH meter 213, Hanna instruments, Ireland).

RESULTS AND DISCUSSION

Viability of tested strains after freeze-drying

The viability of cells of the tested LAB cultures immediately after freeze-drying was determined. Table 1 shows the viable counts after the freeze-drying process.

Table 1. Viability of tested strains before and after freeze-drying

Tested strains		*Lb. plantarum* 564	*Lb. paracasei* 08	*Lc. lactis* 563	*Lc. lactis* 565
RSM	Before freeze-drying (logCFU/ml)	9,14	9,81	9,15	8,99
	After freeze-drying (logCFU/g)	8,78	9,46	8,91	8,75
	Survival (%)	**96,06%**	**96,43%**	**97,40%**	**97,33%**
RSM+ glycerol	Before freeze-drying (logCFU/ml)	9,14	9,81	9,15	8,99
	After freeze-drying (logCFU/g)	8,68	9,42	8,95	8,76
	Survival (%)	**94,96%**	**96,00%**	**97,81%**	**97,44%**
RSM+ gelatin	Before freeze-drying (logCFU/ml)	9,14	9,81	9,15	8,99
	After freeze-drying (logCFU/g)	8,30	9,30	8,79	8,89
	Survival (%)	**90,80%**	**94,80%**	**96,06%**	**98,88%**
RSM+ glycerol+ gelatin	Before freeze-drying (logCFU/ml)	9,14	9,81	9,15	8,99
	After freeze-drying (logCFU/g)	8,62	9,41	9,00	8,90
	Survival (%)	**94,30%**	**95,92%**	**98,36%**	**98,99%**

Freeze-drying proved effective in achieving high viable cells (> 10^8 CFU/g). The conservation of bacteria by process of freeze-drying has been referred in relation to preserving high cell number that contains more than 10^8 CFU/g (Miyamoto-Shinohara *et al.,* 2000). For tested lactobacilli, the best protection was given by RSM (10% w/v), while for tested lactococci, the best protection was given by combination of RSM (w/v) with glycerol (1% v/v) and gelatin (1,5% v/v). Costa *et al.* (2000) reported that monosaccharide provided very good viabilities of a freeze-dried strain of *Pantoea agglomerans*. Contrasting to this, they also reported that the viability of freeze-drying strains in RSM very low; while results from this study showed the very good viability of tested strains dehydrated in RSM. Generally all tested strains were showed very good viability after process of freeze-drying in all of mediums.

Acidification activity

Lactic acid fermentation is characterized by the bacterial excretion of lactic acid into the milk. To evaluate the acidification activity of tested freeze-dried LAB, the pH values were measured during 24 h in RSM (10% w/v). The pH values of RSM inoculated with spray-dried tested strains were measured after 0, 4, 6 and 24 h. Figure 1. A, B, C, D shows the pH values of RSM inoculated with *Lb. plantarum* 564, *Lb. paracasei* 08, *Lc. lactis* 563 and *Lc. lactis* 565, respectively, before and after freeze-drying..
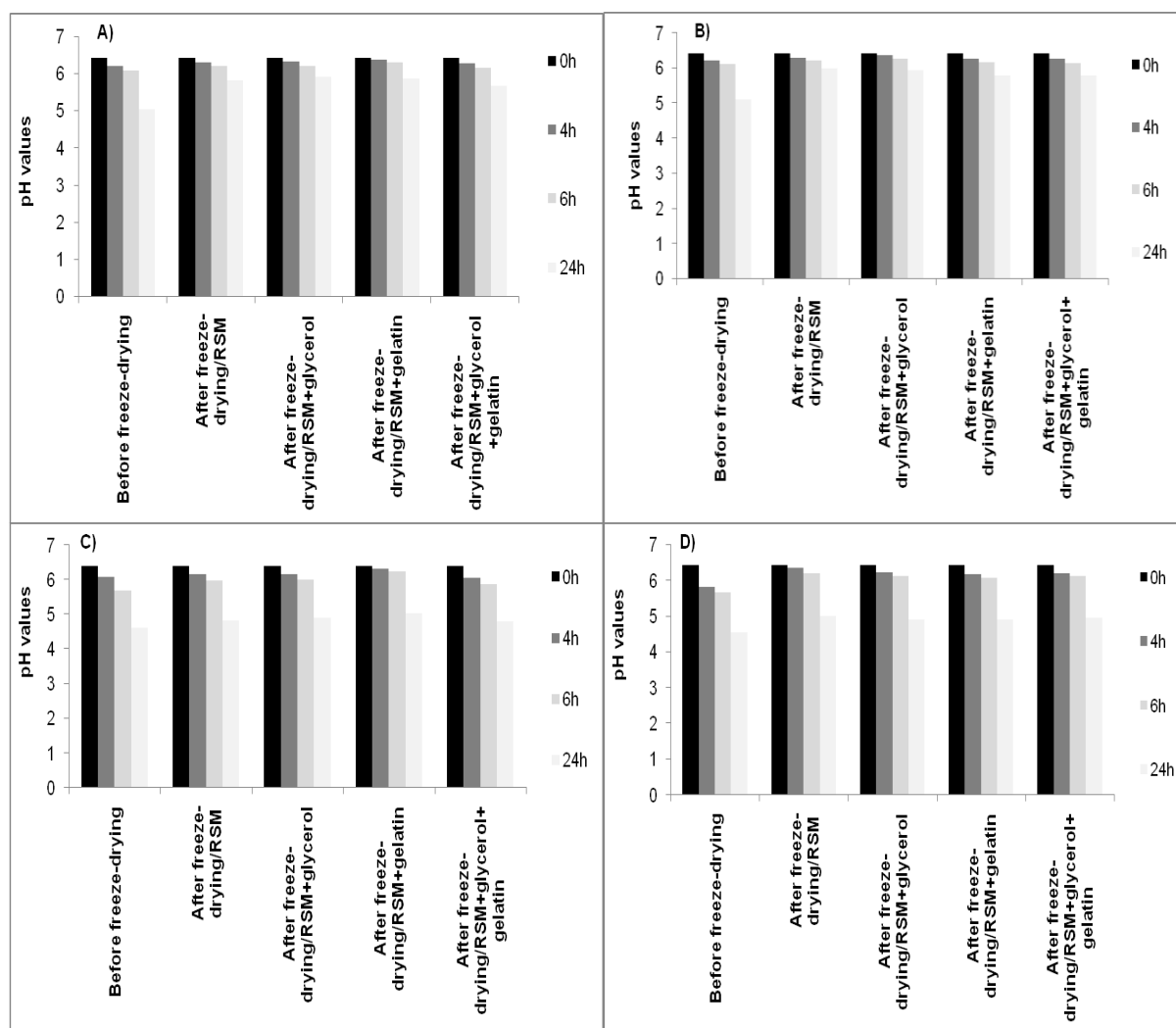
Figure 1. The changes of pH values of RSM (10% w/v) inoculated with: A) Lactobacillus plantarum 564; B) Lactobacillus paracasei 08; C) Lactococcus lactis 563; D) Lactococcus lactis 565

RSM inoculated with cultures before freeze-drying showed typical pH, versus time curves. The pH values of RSM inoculated with *Lb. plantarum* 564 and *Lb. paracasei* 08 were higher than those inoculated with *Lc. lactis* 563 and *Lc. lactis* 565, before freeze-drying. After freeze-drying, tested strains showed reduction in acid production. However, trends of pH changes of RSM inoculated with tested strains before and after freeze-drying were the same. The pH values of RSM inoculated with freeze-dried lactobacilli were 0.6-0.8 fold higher than those inoculated with tested lactobacilli before freeze-dried. There is no differences obtained among the pH values of RSM inoculated with freeze-dried tested strains, depending on the cryoprotectant. An important characteristic for potential starter strains is their ability to acidify their environment rapidly, as the acid production and the accompanying decrease in pH are known to extend the lag phase of food-borne pathogens (Smulders *et al.,* 1986).

CONCLUSION

This study has shown that the survival of selected lactic acid bacteria, *Lb. plantarum* 564, *Lb. paracasei* 08, *Lc. lactis* 563 and *Lc. lactis* 565, when subjected to freeze-drying, are not dependent on the protective medium used. Contrary to these results, Zhao *et al.,* (2005) reported that survival of *Lactobacillus brevis* and *Oenococcus oeni* H-2 when subjected to freeze-drying is dependent of on the protective medium used. Cell number of all tested strains was at the same level before and after freeze-drying contrary to results of Carvalho *et al.* (2003).   Further, freeze-dried *Lc. lactis* 563 and *Lc. lactis* 565 showed satisfactory acidification activity in all protective mediums. Nevertheless, *Lb. plantarum* 564 and *Lb. paracasei* 08 showed reduced acidification activity after freeze-drying in all mediums. Therefore, alternative freezing medium and methods are essential to afford protection and unchanged biochemical properties for these strains.

ACKNOWLEDGMENTS

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FORMULATION AND ANTIOXIDANT CAPACITY OF PLUM AND APPLE JUICES

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ABSTRACT

The aim of this experiment was to determine the total phenolic content (TPC) and antioxidant capacity of clarified plum and apple juices as well as to formulate the optimum proportions of these juices. In addition, the sensory acceptance of formulated juices has been performed.

The juice samples were produced from concentrated fruit juices, diluting to the prescribed dry matter. Total phenolic content was determined using the Folin-Ciocalteu reagent whereas antioxidant capacity was established by two comparative methods (FRAP and DPPH).

Extremely high correlations between TPC and both methods used for determination of antioxidant capacity were observed, as well as between FRAP and DPPH test (R^2 = 0.943).

By sensory analysis the best evaluated juice sample showed significantly higher TPC and antioxidant capacity than apple juice, but significantly lower compared to plum juice. TPC in best sensory evaluated juice was 1162.79 ± 15.02 mg GAE/L, whereas FRAP and DPPH activities were 12043.57 ± 12.37 µmol Fe (II)/L and 3.07 ± 0.02 mmol TE/L, respectively.

The obtained results indicated that a highly acceptable product could be formulated by mixing plum and apple juices thus providing natural sources of phenols with high antioxidant capacity.

Keywords: *fruit juice, apple, plum, total phenols, antioxidant capacity, sensory analysis*

INTRODUCTION

The plums and apples are more widely fruits grown in Serbia commonly used in the production of jams, marmalades, compotes, baby foods, dried fruits, brandy and fruit juices. These fruits are also important sources of natural antioxidants in a human diet.

A large amount of sugar in plums and apples influence their exceptional fruit sweetness. Free organic acids together with the carbohydrate and volatiles compounds contribute to their unique taste affecting the richness of juice.

In addition, these fruits contain a lot of phenolic compounds such as flavonoids exhibited antioxidant capacity (Bermudez-Soto and Tomas-Barberan, 2004; Fu et al., 2011; Wootton-Beard et al., 2011).

Kim et al., (2003) analyzed the different varieties of plums and apples in terms of the content of total phenolics, flavonoids and antioxidant capacity in order to determine their contribution to human health.

Analysis of different varieties of plums, confirmed a high content of flavonoids, total phenolics, antioxidant capacity, and the presence of the following phenols: caffeoylquinic acid, cyanidin, peonidin, quercetin etc. (Chun et al., 2003; Manach et al., 2004).

The phenols found in juice of apple were as followed: chlorogenic acid, caffeic acid, p-coumaric acid, epicatechin, catechin, quercetin etc. (Schieber et al. 2001; Miller & Rice-Evans, 1997; Mullen et al., 2007; Manach et al., 2004).

Positive correlation found between the content of total phenolics and antioxidant capacity suggested that phenols may be responsible for antioxidant capacity of foods and beverages (Fu et al., 2011; Rajić et al., 2012; Bermudez-Soto and Tomas-Barberan, 2004).

It has been proven that *in vivo* intake of juices rich in phenols may contribute to human health (García-Alonso et al., 2006).

Therefore the objective of this experiment was to formulate the clarified juice of plum mixed with apple juice in different ratio. The antioxidant capacity and total phenol content of the obtained fruit juices were tested. The most appropriate sensory combination of these juices has been selected.

MATERIAL AND METHODS

Preparation of juices
Plum and apple juices were obtained from concentrated fruit juice, diluted to a dry matter prescribed by regulation (Official Gazette, Republic of Serbia, 2011). Plum juice was mixture with 80, 60, 40, 20 or 0 % of apple juice (sample B, C, D, E, F respectively), with 100% apple juice (sample A) as a control. The pH was measured by direct potentiometry (pH meter with glass electrode).

Total phenolic content
The total phenolic content (TPC) was determined 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 1000µl of 1:10 diluted Folin-Ciocalteu reagent. After 6 min, 800µl 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 phenolic content was expressed as 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 capacity 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.

DPPH assay
0.1 mM of methanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent was used for DPPH assay. The samples were dissolved in a suitable solvent. In 300 µl of diluted sample 2700µl of methanol solution of DPPH reagent was pured. After a 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm. Results were presented as mmol TE / l juice (Jakobek et al., 2007). Triplicate measurements were taken.

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 and DPPH method) were analysed using ANOVA and Tuckey's HSD test.

RESULTS AND DISCUSSION

Nowadays, it is highlighted that the maintained of acid-base balance is of great importance for the human health. The acidity of the body is increasingly mentioned in a negative context. Therefore, an alkalizing diet rich in vegetables, fruits and fruit juices sould be practiced (Remer and Manz, 1995). The pH of the samples was quite acidic and may contribute to better acid-base balance of the body (Table 1).

Table 1. The pH value of the samples

Samples	Ratio of apple:plum juices (%)	pH values
A	100:0	3.61
B	80:20	3.62
C	60:40	3.64
D	40:60	3.66
E	20:80	3.67
F	0:100	3.68

The total phenol content in the samples, determined using the Folin-Ciocalteu reagent is shown in Figure 1. The values were increased with increasing the amount of plum juice, ranged from 515.52 ± 10.78 mg GAE / l juice for 100% apple juice (A), to 1517.38 ± 44.63 mg GAE / l for 100% plum juice (F).
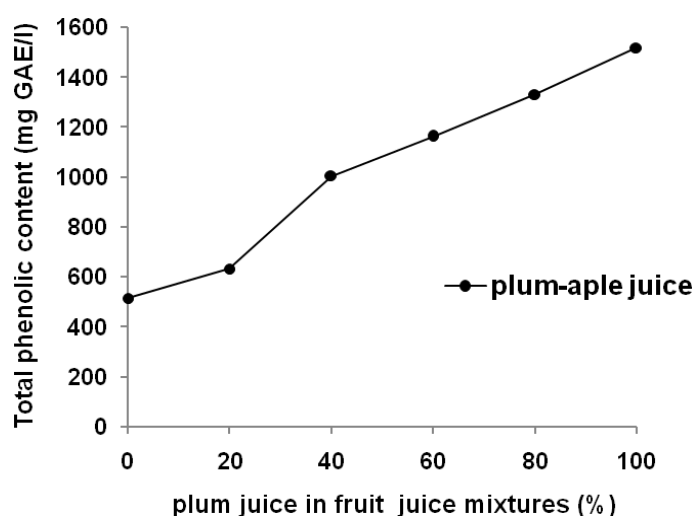
Figure 1.The total phenolic content (TPC) of plum fruit juice blended with apple juice

These values are in accordance with the results obtained for natural squeezed juices of sweet cherry, strawberry, red raspberry and blackberry (Jakobek et al., 2007). In addition, they are related to those reported for the total phenols of different plums cultivars (Kim et al., 2003; Chun et al., 2003; Vinson et al., 2001) as well as with the results obtained for the plums, apples and their juices (Balasundram et al., 2006; Fu et al., 2011; Gardner et al., 2000; Müller et al., 2010).

The antioxidant capacity of the samples, determined by the FRAP and DPPH is shown in Figures 2 and 3, respectively.
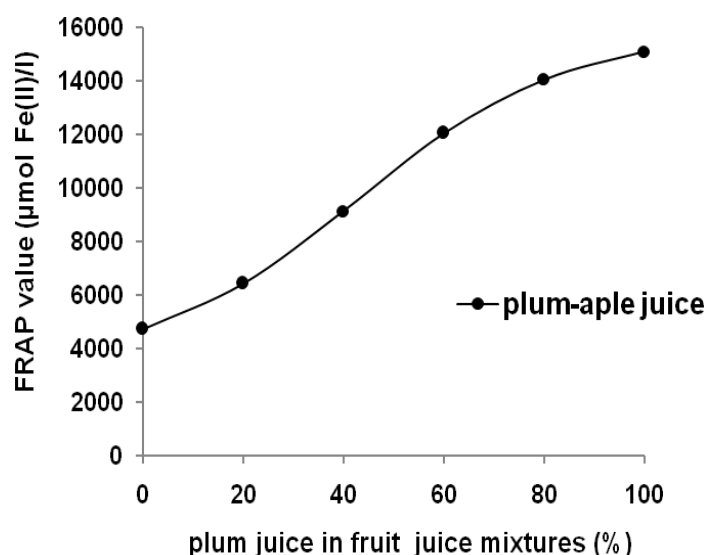
Figure 2. The antioxidant capacity by FRAP assay of plum fruit juice blended with apple juice

The results obtained by FRAP assay ranged from 4731.19 ± 20.62 µmol Fe (II) / l, for apple juice to 15072.86 ± 42.86 µmol Fe (II) / l, for the plum juice, which was in agreement with the values reported by other autors (Pellegrini et al., 2003; Mullenet al., 2007; Wootton-Beard et al., 2011; Fu et al., 2011; Bermudez-Soto and Tomas-Barberan, 2004; Muller et al., 2010).
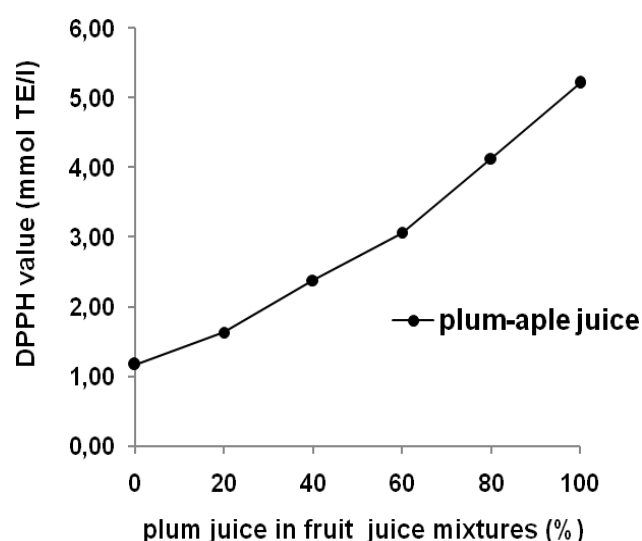
Figure 3. The antioxidant capacity by DPPH assay of plum fruit juice blended with apple juice

Antioxidant activity of formulated juices by DPPH assay is shown in Figure 3. The values ranged from lowest 1.17 ± 0.01 mmol TE / l, for the sample A, to highest value of 5.21 ± 0.08 mmol TE / l, for the sample F. The similar results for plum juice concentrates have been obtained by Bermudez-Soto and Tomas-Barberan, (2004).
The plum juice demonstrated almost three times higher value of total phenols than apple juice, and three or even five times higher FRAP and DPPH values, respectively.
By preliminary sensory analysis the best evaluated juice sample (D) with exceptional aroma showed significantly higher TPC and antioxidant capacity than apple juice (A), but significantly lower compared to plum juice - F (data not shown).

TPC in best sensory evaluated juice was 1162.79 ± 15.02 mg GAE/l, whereas FRAP and DPPH activities were 12043.57 ± 12.37 µmol Fe (II) / l and 3.07 ± 0.02 mmol TE / l, respectively.

The correlations between total phenolic content and antioxidant activity is presented in Figure 4.

a   b   c
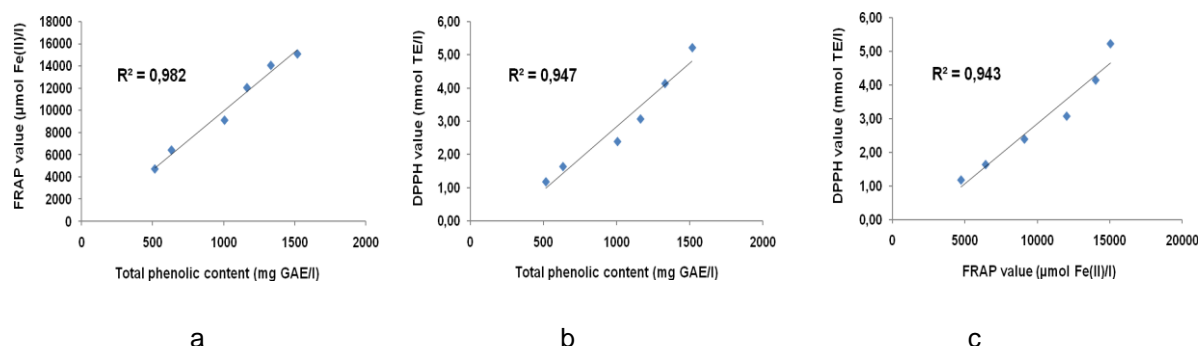
Figure 4. Correlation between the antioxidant capacities and total phenolic content

Extremely high correlations between TPC and both methods used for determination of antioxidant capacity were observed (0.982 and 0.947 for the FRAP and DPPH, respectively) as well as between FRAP and DPPH test ($R^2 = 0.943$).

These high values indicated that phenolic compounds can be responsible for the antioxidant capacity of the tested juices. Also, the methods used for determination of antioxidant activity showed a very high correlation. High correlation between these and other methods used for testing antioxidant capacity of fruits and fruit juices has alredy obtained by Gardner et al., (2000); Fu et al., (2011); Rajić et al., (2012); Chun et al., (2003); Bermudez-Soto and Tomas-Barberan, (2004).

CONCLUSIONS

In order to increase the consumption of plums in our diet, different combinations of plum and apple juices have been formulated. The juices showed harmonious sweet and sour taste and typical plum and apple aroma. The increment of proportion of plum juice in the samples influenced the enhancement of total polyphenols and antioxidant capacity. A high correlation between the methods used indicated that the content of total phenols affected the antioxidant capacity of the juices.

The juice of plum combined with apple juice can be a rich source of antioxidants with health promoting effect.

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SELENIZED YEAST IN PRODUCTION OF SELENIUM ENRICHED *PLEUROTUS OSTREATUS* MUSHROOM WITH GOOD FLAVOUR

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ABSTRACT

The aim of this study was to investigate the potential influence of selenized yeast (Sel Plex, Alltech Inc., Lexington, USA) on chemical composition and flavour of solid state grown mushroom *Pleurotus ostreatus.* Amino acid composition that influences the flavour of selenium-enriched *P.ostreatus P80* (137.84 ppm of selenium in d.w.) and non enriched cultivated strains with particular emphasis on selenomethionine was determined by HPLC method, after complete hydrolysis. Volatile flavour compounds of mushroom cultures were analyzed by GC-MS using Headspace sampler. In mushrooms with high selenium content, selenium in the form of L-selenomethionine was present. High selenium concentration in fruit body did not significantly change the amino acid composition of mushrooms. The major amino acids of fruit body sample were glutamic acid, alanine, aspartic acid and tryptophan. In the fruit body of *P.ostreatus P80* control, 30 volatile compounds were detected, and in selenium enriched sample number of detected compounds was 25. Compounds that were detected in control sample, but not in eriched fruit body are 2-amino-N-ethylpropanamide, 2-methylpropyl pentan-2-yl sulfite, 2,3-pentanedione, heptan-2-one, pentan-1-ol, 2-methyldihydrofuran-3(2H)-one,1-hydroxyacetone, 3-hydroxy-2-butanon, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine and benzaldehyde. Compounds that were detected in enriched *P.ostreatus P80*, but not in control are acetone, ethylacetate, 2-propanol, acetonitrile, 2-methylbenzaldehyde and 2-hydroxyethylmethacrylate. Selenized yeast presents good source of selenium for selenium enriched mushroom production with good flavour, which is probably safety for consumption.
Keywords: *Aminoacids, Flavour, Pleurotus ostreatus, Selenomethionine, Selenized yeast*

INTRODUCTION

Oyster mushroom (*Pleurotus* spp.) has a high nutritional value and may be considered as medical mushroom, since it contains bioactive compounds with immunostimulatory effects and hypocholesterolaemic activities. It is a common edible mushroom, grown commercially around the world for food. Oyster mushroom often has the scent of almonds due to the presence of benzaldehyde. Most of the *Pleurotus* strains samples produce sweet and musty flavour (Jong and Birmingham, 1993).

The aim of this study was to investigate the potential influence of the selenized yeast added into substrate on chemical composition and flavour of solid state grown mushroom *Pleurotus ostreatus.* Selenized yeast was used as a source of Se and N, in order to innovate and develop the biotechnological production of the new natural selenized product with good fragrance. Mushrooms have a different absorption capacity of selenium, which is dependent on the type of substrate for mushroom growing, on the strain of fungi as well as the form and concentration of the selenium which is added to the substrate (Gerely et al., 2006; Savic et al., 2012). The Oyster mushroom is easy to grow and the possibility of accumulation and transformation of selenium in fruit body is easy to be monitored.

Up to date, number of papers has been published regarding selenium absorption in numerous edible and medicinal mushrooms such as *Ganoderma lucidum, Pleurotus sp.,*

Lentinus edodes, Agaricus bisporus, Hericium erinaceum (Gergely et al., 2006; Falandysz, 2008; Malinowska et al., 2009; Zhao et al., 2008). According to the results of those papers, selenium in the fruit body of mushroom mostly exists in the form of selenocysteine, selenomethionine, selenomethylselenocystein, selenite and other compounds which were not identified (Falandysz, 2008). Low concentration of selenium in the growing substrate stimulates protein and amino acid synthesis, while higher concentrations show an opposite acitivy (Zhao et al., 2008). It was also found that selenium is incorporated into the mycelial polysaccharides (Malinowska et al., 2009; Turlo et al., 2010, 2011).

MATERIAL AND METHODS

The *Pleurotus* strain used in this study was comertial *P.ostratus* P80 strain with approximately 137.84 µg Se/g of dry weight. Control samples were obtained on substrate without selenium. Mushrooms were produced by Mycorex Mushroom Limited (Larnaca, Cyprus). The fruit bodies were cleaned, sliced, dried and milled. Sel-Plex® (Alltech Inc., Lexington, USA) was used in the experiments as an organic non toxic source of selenium for mushroom growing. This product, which is produced from yeast *Saccharomyces cerevisiae* CNCM I-3060, contains organically bound selenium in the form of selenomethionine.

Amino acid composition of mushrooms with selenium
Methionine and selenomethionine were determined after hydrolysis of samples with 5M NaOH, at 110°C for 16 h and other amino acids after hydrolysis with 6M HCl, at 110°C for 24 h (Malinowska et al., 2009). The hydrolysate was derivatized with *o*-phthalaldehyde solution containing β-mercaptoethanol. Detection was done using Shimadzu NEXERA UHPLC System with SPD-M20A detector. Supelcosil LC-18-DB column (5µm particles, 250×4.6mm, Supelco Inc., Bellefonte, PA, USA) was used with a linear gradient of 25%–100% solvent B within 48 min, where solvents A and B were 50mM sodium acetate buffer (pH 7.00) and methanol, respectively.

The volatile flavour compounds of mushrooms with selenium
The volatile flavour compounds of Se-enriched and non enriched mushroom powder were analized by GC-MS using Headspace sampler. Components were extracted by the static head-space (HS) in 45°C during 30 minutes followed by needle trap microextraction. A GCMS-QP2010 gas chromatograph (Shimadzu, Japan) coupled with mass detector was used to separate and detect volatile organic compounds desorbed from the needle trap devices for collecting alcohols, organic solvents and fatty acids. Capillary column ZB-WAXplus (30 m, 0.25 mm, 0.25 µm film thickness) from Zebron was used. Tentative identification of components was based on mass spectra and NIST05 version 3.2.4.05 library comparison.

RESULTS AND DISCUSSION

Generally, mushrooms are considered to be a good source of protein (Zhao et al., 2008; Ouzouni et al., 2009). Total contents of free amino acids vary depending on growing substrate and mushroom strain (Yang et al., 2001). One of the goals of this research was determination of the amino acid composition of Oyster mushroom. No differences were found in the amino acid profile of fungi with added selenium compared to control sample, except in the case of silfur-containing amino acids. The major amino acids of the protein in all samples were glutamic acid (15.42-17.41 mg/g dry weight) and aspartic acid (6.81-8.21 mg/g dry weight). Aspartic and glutamic acids are reported to be monosodium glutamate-like components, which gives the most typical mushroom taste (Yamaguchi, 1979). Chen (1986) conducted a series of sensory evaluations on synthetic mushroom extracts and found that alanine, glycine, and threonine were taste-active (sweet taste) amino acids in *Pleurotus spp.* These amino acids were also present in high quantities in both control and enriched

samples. Selenomethionine was detected although its content was low. In selenium enriched fungi, the concentration of methionine was higher compared to selenomethionine (0.8:0.08 mg/g dry weight). Turlo et al. (2007) showed that, for *Lentinus edodes* mycelia rich in selenium, the content of selenium accumulated as seleno amino acids decreases in inversely proportion to the high selenium content of the mycelium. High selenium concentration in fruit body did not cause a significant change in composition of essential amino acids in mushroom fruit body. The results are presented in Figure 1.
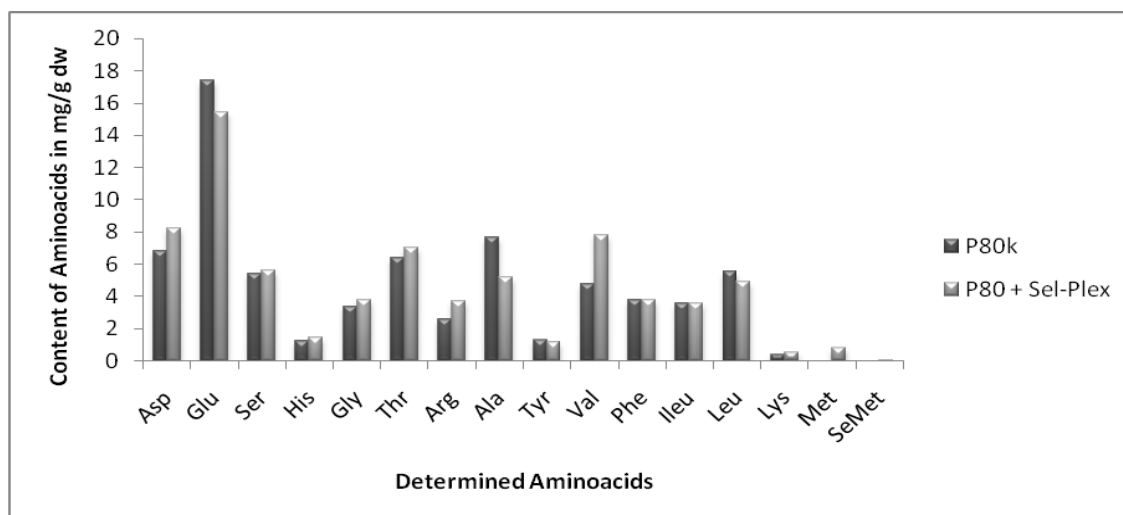
Figure 1. Content of essential aminoacids and selenomethionine (mg/g dry weight) of P.ostreatus P80 control (P80k) and selenium enriched (P80+Sel-Plex) mushroom

In this study, volatiles of mushrooms were separated and detected using the GC-MS method. The needle trap devices for collecting alcohols, organic solvents, and fatty acids were used. Detected compounds include alcohols, aldehydes and esters. Since the scan range was from m/z 35 to 300, non-polar compounds and compounds with molar mass higher than 300 could not be detected. Volatile compounds of non enriched and selenium enriched dry fruit body of *P.ostreatus* mushroom were different. In the fruit body of not enriched *P.ostreatus* P80, 28 volatile compounds were detected and in selenium enriched sample 22 compounds were detected in total. Compounds that were detected in control sample, but not in enriched fruit body are listed in Table 1.

Table 1. The difference in volatile compounds content between P.ostreatus P80 with selenium and control nonenriched sample

P.ostreatus volatile compounds	Retention time (Rt)	Non enriched sample	Selenium enriched sample
l-Alanine ethylamide	1,6	✓	
Acetone	2,67		✓
Ethyl Acetate	3,54		✓
Isopropyl Alcohol	4,29		✓
2,3-Pentanedione	7,02	✓	
2-Heptanone	10,34	✓	
1-Pentanol	12,34	✓	
Coffe furanone	12,59	✓	
Acetyl-methyl-carbinol	13,19	✓	
Diethyl-acetate	13,58	✓	
2,5-dimethyl pyrazine	14,25	✓	
2,6-dimethyl pyrazine	14,40	✓	
Benzaldehyde	19,50	✓	
2-methyl-benzaldehyde	22,48		✓
2-Hydroxyethyl methacrylate	25,91		✓
2-Acetyl-Pyrrole	29,63	✓	

In the selenium enriched mushroom, benzaledehide which gives typical almond flavor was absent. Also, compounds that give mostly floral, chocolate, sweet aroma were lost. In enriched mushroom, compounds that give acetone, hospital-like, ester smell were detected. Furher sensory evaluation of samples is needed in the future. Except the compounds presented in the table, 17 more volatile compounds were detected in both enriched and non enriched mushroom samples. Those were aroma compounds typical for *Pleurotus* mushrooms such as acetaldehyde, 2-methylbutanal, 3-methylbutanal, ethyl alcohol, 2,3-butanedione, hexanal, 1-hexanol, 3-octanol, 2-methylpropyl pentyl sulfite, 2-methyl-1-propanol, 1-butanol, acetic acid, 2,3-butanediol, 4-pentanolide, γ-butyrolactone, ethyl methacrylate and dimethyl sulfone, giving almond, cheese, sweet, creamy, alcoholic and herbal aroma to the mushrooms.

However, the flavors of mushrooms were influenced by compositions of growth medium, growth conditions, and mushroom genetic (Jong and Birmingan, 1993).

CONCLUSIONS

Results of the study provide an opportunity to compare the composition of the volatile components and amino acids in the fruiting bodies of selenium enriched and nonenriched mushroom. Present selenium did not significantly change the amino acid profile of mushroom with the exception of amino acids with sulphur. Selenomethione was present, although in ten times lower concentration compared to methionine. Content of selenomethionine was influenced by high selenium concentration in substrate. Still, essential aminoacid composition which gives monosodium glutamate-like taste and sweet taste to mushroom was unchanged. Selenium enriched and control samples of *P.ostreatus* contained most volatile compounds characteristic for mushroom fruit bodies. However, in selenized mushrooms, less aroma compounds were detected, and the typical compound benzaledhyde was not detected. Due to the changed content of volatile aroma components in enriched Oyster mushrooms, the aroma that derived from unpleasant components could be unequivocally masked by high contents of sweet components such as present amino acids and total soluble sugars.

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DETERMINATION OF ACETAMIPRID RESIDUES IN PAPRIKA

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ABSTRACT

Reversed-phase high-performance liquid chromatography (HPLC) technique was developed to quantify acetamiprid in paprika. Insecticide determination and quantification were performed using an Agilent Zorbax C_{18} column with a mobile phase of acetonitrile/1.5% CH_3COOH (30/70) mixture in an isocratic elution at the flow rate of 1.0 ml/min. The column temperature was maintained at 25 °C and an aliquot of 2.5 µl was injected. The extraction of acetamiprid was done by a QuEChERS method. Validation of the method was performed in accordance with SANCO/12571/2013 criteria. Achieved recoveries at three concentration levels (0.15, 0.30 and 0.45 mg/kg) were between 90-105%. Precisions, expressed in terms of repeatability, (RSDs) of the proposed method were 0.48 and 0.65%. Acetamiprid showed linear calibrations from 0.25–3.0 µg/ml with R^2 of 0.999%. Limit of quantification was established at 14 µg/kg. The obtained matrix effect of 94.52% showed that there were no interference peaks from the sweet cheery matrix on the elution region of the acetamiprid. Described method is applicable for analysis of this insecticide in paprika samples.

Keywords: acetamiprid, residues, paprika, HPLC/DAD

INTRODUCTION

Paprika (*Capsicum annum*) is one of the most important vegetables. The paprika crop is usually attacked by a variety of pests, which multiply rapidly and cultivators apply a combination of several pesticides types to control these pests. For the purpose of spider mite pests control, one of the major pests in paprika cultivation, some pesticides, including acetamiprid have been applied. Acetamiprid belong to the neonicotinoid class of insecticides. Neonicotinoids are derived from nicotine isolated from the tobacco plant and with insecticidal activity and have been used extensively as a commercial insecticide. Neonicotinoid insecticides are potent selective agonists of insect nicotinic acetylcholine receptors (nAChRs) and are used extensively in both crop protection and animal health applications (Millar *et al.*, 2007). Many obvious benefits have been gained from the use of these insecticides in agriculture, but their inappropriate use can result in unacceptably high levels of these compounds in vegetables (Grahovac *et al.*, 2012).

Since the presence of pesticide residues in fruits and vegetables above MRLs can affect consumer health, the regulatory authorities have established maximum residue levels of pesticides for most common vegetables and fruits. MRL for acetamiprid in paprika in EU (500/2013/EC) and in Serbia (Official Gazette, RS No. 29/2014) is 0.3 mg/kg.

The determination of low concentrations of this pesticide in matrices such as vegetables requires the application of an effective extraction followed by chromatographic determination. Neonicotinoids are usually determined by liquid chromatography (LC) with diode array (DAD) detection (Mandić *et al.*, 2005; Wang *et al.*, 2012; Lazić *et al.*, 2014), because direct analysis by gas chromatography (Vilchez *et al.*, 1996; Zhang *et al.*, 2012) is unsuitable due to their low volatility and high polarity. Furthermore, they are determined by LC with electrochemical detector (Rancan *et al.*, 2006), or with mass spectrometer (Zywitz *et al.*, 2003). Enzyme-linked immunosorbent assays have also been surveyed as simple screening method for these compounds (Watanabe *et al.*, 2011). Recently, highly sensitive thermal lens spectrometric (TLS) detection, has been successfully coupled to HPLC and has attracted attention for use in the neonicotinoid and other target analysis of different samples (Guzsvány, *et al.*, 2007).

Several extraction methods have been developed to determine pesticide residues in vegetables. However, in general, all these methods are effective but they are either time-consuming, or solvent-consuming, or require expensive apparatus. QuEChERS (quick, easy, cheap, effective, rugged and safe) method was first introduced (Anastassiades *et al.*, 2003) for the extraction of a wide range of pesticides from fruits and vegetables. This method involves liquid partitioning with acetonitrile followed by a dispersive SPE clean-up with primary secondary amine (PSA) and with or without graphitised carbon black (GCB) (Lehotay *et al.*, 2005). The QuEChERS method proved very popular because it is simple, has a low cost per sample and in fact has all the advantages defined by its name. Today there are two commonly used buffered methods, European committee for standardization method 15662 (EN version 2.2, 2008) and AOAC official method 2007.01.

Considering importance of pesticide residues monitoring in fruit and vegetables, research in this study has been focused on the development of simple and sensitive method for determination of acetamiprid residues in paprika.

MATERIAL AND METHODS

Chemicals and solutions

The certified standard of acetamiprid (purity 98.1%) was purchased from Dr Ehrenstorfer (Augsburg, Germany). Acetonitrile (ACN) of a suitable grade (HPLC) and CH_3COOH were obtained from J.T. Baker (Germany). The dispersive SP extraction (Cat. No. 5982-5650) and clean-up (Cat. No. 5982-5056) kits for QuEChERS sample preparation were purchased as ready-to-use from Agilent Technologies (USA). The water was purified with a water purification system (TKA, Germany). A stock solution of acetamiprid was prepared in acetonitrile at a concentration of 100 µg/ml and stored at -10 °C, in the dark. Calibration solutions for the HPLC analysis were prepared by further dilution with acetonitrile, achieving concentrations in a range from 0.25 to 3.0 µg/ml.

HPLC/DAD analysis

Insecticide determination and quantification were performed by HPLC with diode-array detection (Agilent 1100 Series LC system, United States) and Agilent Zorbax Eclipse C18 column (50 mm × 4.6 mm internal diameter, 1.8 µm particle size). The analysis was done under the conditions described below (Table 1).

Table 1. Conditions for HPLC/DAD analysis of acetamiprid

Mobile phase	A- acetonitrile
	B- ultrapure water with 1.5% CH_3COOH
Mobile phase ratio	30/70
Column temperature	25 °C
Flow rate	1.000 ml/min
Wavelength	254 nm
Injected volume	2.5 µl

Sample extraction

The extraction of acetamiprid from paprika samples was performed using the European Committee for Standardization Method 15662. Homogenized samples of paprika (10 g) were weighed into a 50 ml volume polypropylene tube and 10 ml of ACN was added as an extraction solvent and the tube was tightly capped and vigorously shaken for 1 min. A mix of buffered salts (1000 mg of sodium citrate, 500 mg of sodium hydrogen citrate sesquihydrate, 4000 mg magnesium sulphate and 1000 mg sodium chloride) from separate pouches was added to each tube and immediately mixed for 1 min. After that, the tube was centrifuged at 3000 rpm for 5 min (Sigma, Germany). An aliquot of 6 ml of the upper acetonitrile layer was

transferred to each of 15 ml centrifuge tube containing the sorbent, 150 mg of primary-secondary amine (PSA) and 900 mg of magnesium sulphate.

The tube was vigorously mixed for 1 min and then centrifuged at 3000 rpm for 5 min. An aliquot of the final upper layer was filtered through a 0.45 µm membrane filter and transferred into an autosampler vial for HPLC/DAD analyses.

Validation of the analytical method

Fortified samples were prepared by spiking 10 g of paprika, previously homogenized, with the appropriate volumes of working standard acetamiprid solutions ranged from 0.15-0.45 µg/ml. Method for quantitative analysis of acetamiprid in paprika was validated in terms of linearity, precision, recovery and limit of quantification, in accordance with Document SANCO/12571/2013.

RESULTS AND DISCUSSION

The validation of the studied analytical methods was conducted by using spiked control samples of paprika and was evaluated according to the European SANCO/ 12571/2013 guidelines. HPLC/DAD chromatograms for untreated, controlled sample of paprika and fortified sample are shown in Figure 1 and 2.
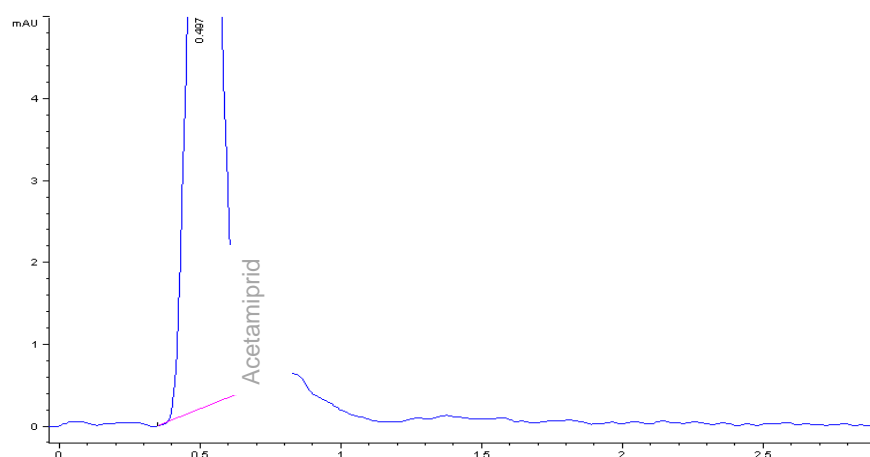
Figure 1. HPLC/DAD chromatogram of untreated, controlled paprika sample
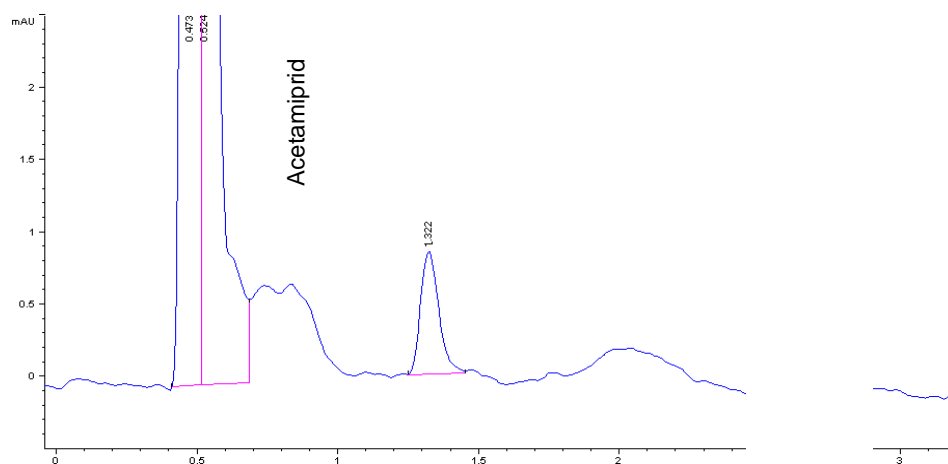
Figure 2. HPLC/DAD chromatogram of acetamiprid in matrix at a concentration of 0.25 µg/ml

The linearity of the detector response was evaluated at a concentration range between 0.25–3.0 µg/ml using five calibration solutions prepared in acetonitrile. Calculations were done using the peak areas. The calibration curves were linear with correlation coefficients of 0.999 and regression equation y=14.31x-0.865.

Limit of quantification (LOQ) for acetamiprid in paprika was estimated from the fortified samples and established as 14 µg/kg.

Precision value was evaluated trough repeatability and expressed as relative standard deviation (RSD). The repeatability was checked by analysis of the same samples at two concentrations of 0.3 and 0.45 µg/ml on the same day, five times. Relative standard deviations (*RSD)* of the peak areas were 0.65 and 0.48%, respectively, fulfilling the criteria of RSD ≤20%.

A critical aspect of pesticide residue analysis is the purification process, which is required to isolate the residues from matrix components and to reduce matrix effects (Lehotay, 2006). Matrix effect, i.e., signal suppression or enhancement, of the studied neonicotinoid insecticide in the paprika matrix, was evaluated. For this study, the matrix effects were examined comparing the slopes of calibration curve of matrix-matched standards (MMC) and solvent-based standards (SC). Acetamiprid standards were prepared in blank matrix extract in five concentrations between 0.125-1.5 µg/ml. Calibration curves were constructed by plotting the peak area against the acetamiprid standard concentrations (Figure 3).
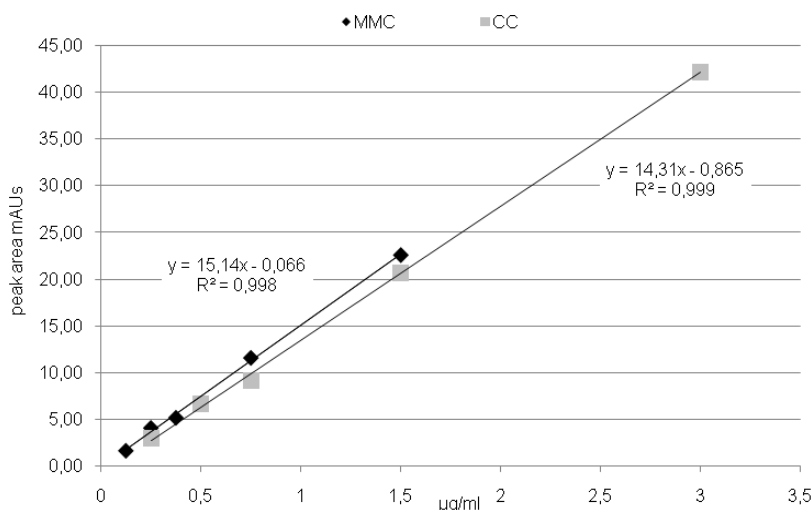
Figure 3. Matrix effect for acetamiprid in paprika sample

The slope of calibration curves were 14.31 and 15.14 for direct calibration and matrix-matched standards, respectively. From equation 14.31/15.14*100, using slope values of line constructed from SC and MMC, matrix effect was calculated. The value of 94.52% indicates that the impact of the paprika matrix on acetamiprid determination can be ignored. The accuracy of the method was carried out using blank sample spiked with a standard solution of acetamipid insecticide at three levels (0.15, 0.30 and 0.45 mg/kg). The mean recoveries were in the range of 90-105%. According to the EU validation guideline for pesticide residues, mean recovery values should be within the range of 70–120%, which was achieved by this method. Results of the recovery achieved in this study confirmed that the optimal recovery was obtained for acetamiprid insecticide in paprika samples.

CONCLUSIONS

In this study, a method for determination of insecticide acetamiprid residues in paprika samples was described. The QuEChERS method of extraction and reverse phase based liquid chromatography were used for determination of acetamiprid in paprika. The obtained

calibration curve displayed good linearity. The good recovery and precision, low *RSD* and LOQ confirmed the suitability of the proposed method for the determination of acetamiprid residues in paprika samples.

ACKNOWLEDGEMENTS

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CANDIDATE PROBIOTIC *LACTOBACILLUS PLANTARUM* STRAIN APPLIED TO PRODUCE FERMENTED TOMATO JUICE

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ABSTRACT

Lactobacillus plantarum PCS26 had been isolated from traditional Slovenian cheese. The data gathered from *in-vitro* tests on human intestinal cell lines indicate its probiotic effects. The suitability of this stain to ferment tomato juice and generate functional non-dairy beverage was studied.

The growth kinetic of the candidate probiotic was examined by measuring its viable count and pH change of inoculated tomato juice during fermentation. The sugars present in the juice and consumed by the bacterium as the main carbon source were also analyzed. Immobilization of *L. plantarum* PCS26 by entrapment in Ca alginate beads was used to improve the growth kinetics and the survivability during shelf life. Supplementation of the medium with whey, as protein enhancer, was studied as well.

L. plantarum PCS26 grew very well on the nutrients provided from the tomato juice reaching growth of 4.3 log cycles during 17 h with maximal viable count of 10^9 cfu/mL and lag phase of just 2 h. Immobilized cells, on the other hand, grew 5.1 log cycles attaining 10^{11} cfu/mL, remaining in the beads still after 29 h, keeping almost ideal immobilization efficiency (η) of 0.998. The pH in both cases decreased to 3.6. Addition of whey into the tomato juice did not improve the growth of *L. plantarum* PCS26 any further.

Keeping in mind the increasing vegetarian and lactose intolerant population, commercialization of such probiotic beverages would be much needed nowadays.

Keywords: *Lactobacillus plantarum*, tomato juice, candidate probiotic, beverage

INTRODUCTION

Fermentation of vegetable juices by lactic acid bacteria has been a very attention-grabbing topic for the researchers in the past decade. Significantly increased awareness for diet related health issues by the general population have made the scientist and managers of the production companies to focus their attention on creating products that will be beneficial for the human health, convenient, and at the same time with good sensory properties (Granato et al., 2010). As vegetables already have high nutritive values; they are reach in vitamins, minerals, dietary fibers and phytochemicals, fermentation with lactic acid bacteria is considered to provide added value in terms of health promoting properties. Laniewska-Trokenheim et al. (2003) have used several lactic acid bacteria to form a starter culture to ferment vegetable juices and create a new assortment of fermented products. The result of this trend is a vast increase in the number of probiotic-containing non-dairy beverages as the demand for the vegetarian probiotic products, especially in the developed countries, is boosting (Betoret et al., 2012).

Survival of the health conferring microorganisms is a main challenge for a number of researchers. According to Gawkowski and Chikindas (2013), a careful strain selection, a proper supplementation of media as well as protection techniques are to be used to create a stable product. Of the numerous starter cultures studied, the genera *Leuconostoc*, *Lactobacillus* and *Pediococcus* have got the primacy in fermenting plant originated foods (Peres et al., 2012).

Tomato juice has been used many times as a nutritive medium for this purpose (King et al., 2007; Koh et al., 2010; Laniewska-Trokenheim et al., 2003; Yoon et al., 2004). Its relatively high amount of fermentable saccharides compared to other vegetables, made tomato along with cabbage, red beet, carrot and spinach an appropriate medium for lactic acid fermentation (Karovicova and Kohajdova, 2003).

The aim of this study was to determine the suitability of *Lactobacillus plantarum* PCS26 to ferment tomato juice. This strain has been isolated from traditional Slovenian cheese (Nissen et al., 2009) and studied for its probiotic properties. Great potential has been noticed in its extracellular metabolites which showed several positive effects on the gut epithelial cells during *in vitro* tests (Dimitrovski et al., 2014). Growth of the culture and sugars' consumption throughout the fermentation process has been followed. Supplementation of the tomato juice with whey and protection of the cells by entrapment in Ca alginate were tested for improving the growth of the culture.

MATERIALS AND METHODS

Preparation of tomato juice

Tomato juice was made from fresh tomatoes in a local fruit and vegetable production company. After washing and visual sorting, the tomatoes were fed to a crusher were smashed to little pieces and then transported to a tank for preheating to about 80 ^{o}C. Two pulping mashing that followed made the tomato mash into homogenous pulp ready for concentration in an evaporator. One-step evaporation was done obtaining soluble solids in the product of around 18 to 19 ^{o}brix after which water and salt were added to create a final tomato juice with 10 ^{o}brix.

For the supplementation of the tomato juice, pasteurized whey in liquid form was obtained from a local dairy production facility and was aseptically added to the juice reaching final concentrations of 10 and 20 % v/v.

Bacterial strain and culture conditions

Candidate probiotic *Lactobacillus plantarum* PCS26 (Deposited at Microbial Strain Collection of Latvia, accession number: PCS 26 (P 975)) (PathogenCombat, 2011) was kept at -20 °C and revitalized by overnight growth in de Man, Rogosa and Sharpe (MRS) broth (Merck, Whitehouse station, New Jersey, USA) at 37 ^{o}C using semi-anaerobic conditions.

Cell immobilization

Overnight culture of *L. plantarum* PCS26 was washed twice with peptone water (0.1 % w/v). Cell suspension (100 μL) was then mixed with 10 mL of 2 % (w/v) Na- alginate (Sigma-Aldrich, St. Louis, Missouri, USA) and extruded through a needle by using a syringe to form droplets. The droplets were dropped into a gently stirred 0.1 M $CaCl_2$ (Merck) solution and kept in the solution for 2 h to obtain Ca-alginate gel beads (ca. 2.2 mm in diameter) with entrapped cells (Velickova et al., 2009). Characterization of the carrier for cell immobilization was done by determination of the immobilization efficiency. The immobilization efficiency, η, represents the ratio of the concentration of the immobilized cells to the concentration of the total cells, immobilized plus free cells.

Inoculation and fermentation

Overnight culture of *L. plantarum* PCS26 incubated on MRS broth at 37 ^{o}C was used as inoculum. Batch fermentations were carried out with 300 mL tomato juice placed in a 500 mL Erlenmeyer flask on a rotary shaker (120 rpm) at 37 ^{o}C for ca. 45 h. Inoculation with 30 μL of probiotic culture was enough to obtain initial viable count of $10^5 - 10^6$ cfu/mL in all fermentations. Around 800 beads were obtained during the immobilization of 100 μL cell suspension and used as inoculum for the fermentations with immobilized cells. Samples were taken aseptically at time intervals of 1 to 2 h and viable count and pH (pH-meter: Sartorius PB-11, Göttingen, Germany) were measured.

Counting of viable cells

Viable count was measured by using the Milles and Misra surface viable count method (Hedges, 2002; Miles et al., 1938). After a series of appropriate dilutions the samples were planted on MRS agar plates and incubated on 37 ^{o}C for 48 h before colony counting. For immobilized cells, ten Ca-alginate beads were dissolved in 0.28 M KH_2PO_4 (Merck) solution with gently shaking for 15 min at room temperature for de-polymerization of the beads and release of the immobilized cells (Zerajic et al., 1990). The number of colonies obtained from these ten beads was used to calculate the viable count in total medium (cfu/mL).

Determination of sugar and acid concentration

Agilent 1200 high performance liquid chromatography system (Agilent Technologies, Inc, Santa Clara, United States) was used for quantification of sugars in the samples during the fermentation. Supelcosil LC-NH$_2$ column, 250×4.6 mm, 5μm particle size, (Supelco analytical, Sigma Aldrich Group, Taufkirchen, Germany) separated the containing sugars using isocratic mobile phase acetonitrile/water = 75/25 % (v/v) at 40 ^{o}C (Muntean and Muntean, 2010). Refractive index detector, also thermostated at 40 ^{o}C, fed the software (Agilent ChemStation) with data for generation of chromatogram. The run time was 15 min at 1.3 mL/min. Concentrations of the measured components were quantified from the peak areas by using appropriate standard curves generated with external standards. The presented data are average values of three independent measurements and the standard deviation are shown as error bars.

RESULTS AND DISCUSSION

Figure 1 shows the growth of the probiotic strain *L. plantarum* PCS26 during fermentation of the tomato juice. The lag phase was relatively short, around 2 h, followed by the exponential phase that lasted until the 17th h during which the cells grew 4.3 log cycles. The viable count after the end of the exponential phase was 1.3·10^9 cfu/mL and remained constant during the next 8 h of the stationary phase. After 25 h of fermentation, the viable cell count decreased.

The pH values decreased from 4.1 to 3.85 at the 17th h. During the stationary phase the pH value continued to decrease and reached 3.5 at the end of the 26th hour of fermentation.

The suitability of various kinds of vegetables, among which tomato, for production of vegetable juices which would undergo lactic acid fermentation was tested by Kohajdová et al. (2006). The authors supplemented the tomato juice with glucose (2% final concentration) and increased its initial pH up to 6.5, by addition of $NaHCO_3$ obtaining optimal conditions for culture growth. It took 72 h for *Lactobacillus plantarum* CCM 7039 to decrease the pH to 3.6 and produce 1.5 % lactic acid. However, no data on the viable count was given. Tomato juice supplemented with fructooligosaccharides was also used as a medium for cultivation of bifidobacteria strains which reduced the pH to 3.51 after only 6 h of fermentation (Koh et al., 2010).
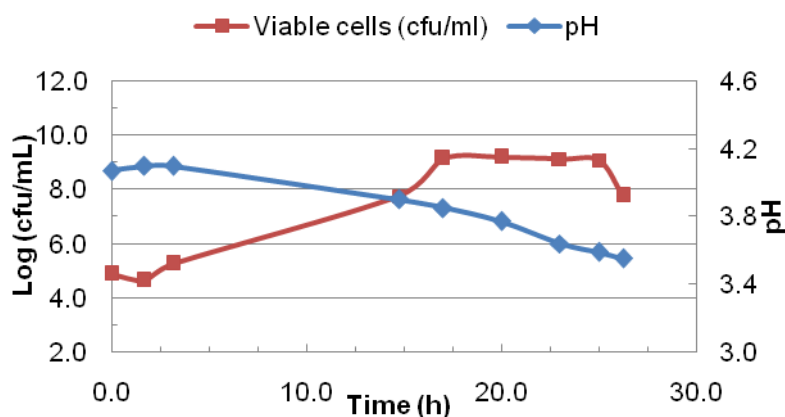
Figure 1: Changes in viable count and pH during fermentation of tomato juice with L. plantarum PCS26

One of the most cited articles when concerning tomato juice fermentation is published by Yoon et al. (2004). The probiotic *L. plantarum* C3 strain reduced the pH to 3.5 and increased the acidity of commercial tomato juice to 1.67 %. The culture grew 3 log cycles reaching $2 \cdot 10^9$ cfu/mL after 72 h fermentation.

Supplementation of the medium with whey, as done by others to promote the growth of lactobacilli (Dalev et al., 2006; Hernandez-Mendoza et al., 2007; Shukla et al., 2013), did not yield positive results for the tomato juice. Whey in concentration of both 10 % and 20 % did not significantly affect the growth of *L. plantarum* PCS26. Probably the tomato juice is providing all necessary growth factors for the culture hence the whey is not contributing to further enrich the medium and increase the growth.

The growth kinetics of *L. plantarum* PCS26 immobilized in Ca alginate beads during tomato juice fermentation is presented in Fig. 2. Immobilized cells started their growth almost immediately after the start of the fermentation and after 17 h reached similar viable count as the free cell fermentation ($3.2 \cdot 10^9$ cfu/mL). However, unlike the free cells, the immobilized cells continued to grow attaining $5 \cdot 10^{11}$ cfu/mL (5.1 log cycles) after 29 h, when the onset of the stationary phase was noticed. During the fermentation, cells proliferated out of the beads and grew in the medium as free cells. They followed the same kinetics pattern of the immobilized cells and reached $1.3 \cdot 10^8$ cfu/mL at 29th h. The immobilization efficiency, as indicator of the cell encapsulation, was almost ideal (η = 0.998) meaning that the methods and the materials used to entrap the bacterial cells were optimal. The pH change was according to the growth of the culture and decreased its value from 4.4 to 3.6 at 29th h.
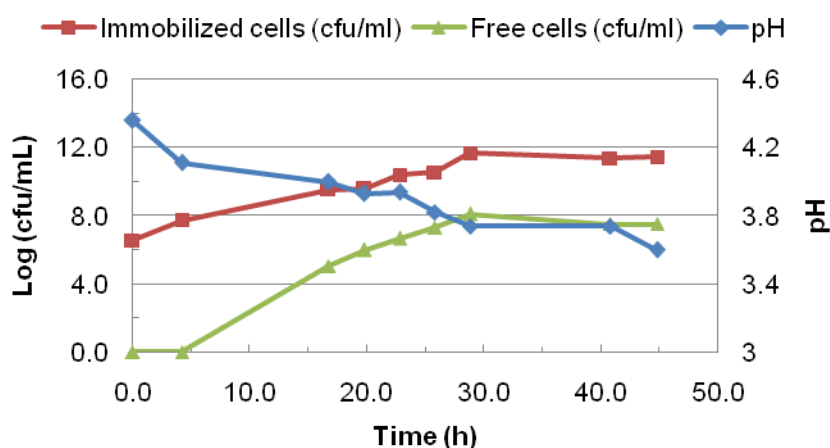
Figure 2: Changes of viable count and pH in tomato juice during fermentation with immobilized L. plantarum PCS26

Sugars were used as carbon source for growth and for energy generation of *L. plantarum* PCS26 throughout the fermentation. Figure 3 presents the concentrations of sugars in the tomato juice during 45 h of fermentation with immobilized cells. The most abundant sugars in the tomato juice were sucrose, fructose and glucose with initial concentrations of 7.9 g/L, 5.2 g/L and 4.6 g/L. As can be seen, fructose was consumed from the very beginning of the fermentation yielding the highest utilization percentage of 48.2 %. Glucose, although generally preferred energy source over fructose and over other carbon sources by many microorganisms, was consumed not only later than fructose, but also at a lower extent of 31.8 %. The larger intake of fructose compared to glucose was also observed in other studies with lactobacilli (Lu et al., 2001; Raccach and Marshall, 1985). *L. plantarum* is classified as a facultative heterofermentative bacterium indicating that sugars can be fermented via the Embden-Meyerhof pathway (EMP) or the phosphoketolase pathway (PKP), leading to homolactic and heterolactic fermentation profiles, respectively (Kleerebezem et al., 2003). The PKP is usually used by lactic acid bacteria to ferment pentose's, and it has a poor energy yield compared to that of the EMP (Arskold et al., 2008).

However, Saier et al. (1996) showed that anaerobic growth of lactic acid bacteria in the presence of fructose induces the synthesis of a phosphotransferase system and glycolytic enzymes that allow fructose to be metabolized via the EMP.
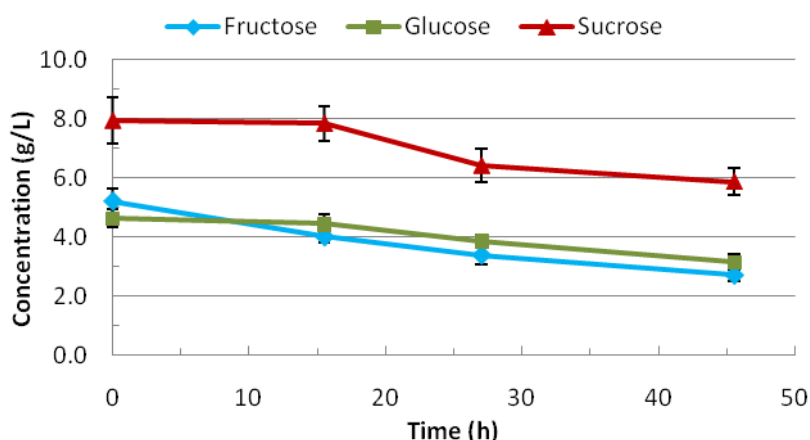
Figure 3: Changes of fructose, glucose and sucrose in tomato juice during fermentation with immobilized L. plantarum PCS26

In the future, sensory quality and survivability of the culture during shelf life have to be studied. Since immobilized cells of *L. plantarum* PCS26 yielded higher cell density, further experiments in terms of beads' diameter and its influence on the sensory quality of the juice should be carried out. All this can eventually lead to commercialization of the fermented tomato juice.

CONCLUSION

Lactobacillus plantarum PCS26, the probiotic effects of which were previously examined and proved, was tested for production of a stable, functional, non-dairy beverage.
L. plantarum PCS26 grew extensively in the tomato juice and yield high viable cell counts in a relatively short time. In the free cell fermentation the bacterium increased its concentration for 4.3 log cycles attaining $1.3 \cdot 10^9$ cfu/mL in 17 h. The entrapped cells in Ca alginate increased the growth for 5.1 log cycles with the viable count of $5 \cdot 10^{11}$ cfu/mL *L. plantarum* PCS26 was proven to utilize tomato juice as growth medium obtaining probiotic non-dairy beverage.

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FUNCTIONAL FOOD PRODUCTS IN THE EUROPEAN LEGISLATIVE FRAME

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ABSTRACT

A series of studies showed increased awareness of importance of healthy eating, which opened a range of possibilities for new types of products, called functional products or functional foods. The concept of functional foods is conceived in Japan thirty five years ago, and today food industry around the world bases its development on this segment.

Functional food products have a very high rate of growth. The trend of a healthy diet based on natural ingredients requires greater transparency and more information about these products and their impact on the health and wellbeing.

In order to place such foods on the market and to emphasize its impact on health, it is of great importance to comply with existing legislation. Many countries in the region still do not have regulations governing this area. Regulation (EC) No. 1924/2006 on nutrition and health claims made on foods (with amendments) and Directive 2009/39/EC on foodstuffs intended for particular nutritional uses, are of high importance for this group of products. Law on food with health claims and food enriched with nutrients (Official Gazette No. 39/13) and the Law for food with particular nutritional purposes (Official Gazette No. 39/13) are currently valid in Croatia.

These regulations include procedures for official notification and scientific studies that confirm the positive effect of these foods on human health. Furthermore, in order to successfully implement, monitor and supervise such food, it is necessary to have an updated Register of approved claims that can be placed on the food declaration. After a long time debate, the European Union managed to regulate the area of food with added values as well as the requirements when and under what conditions these foods can be marketed.

Keywords: healthy eating, healthy food, functional food, health claims, legislation

INTRODUCTION

There is no unique definition of functional food and these foods still not represent specific food category. The definition of functional food products is understood in the wider meaning and includes three main concepts as follows: health benefits, the technological processes and nutritional function. Among its basic nutritional values functional foods are developed and created to assist in prevention of chronic diseases once if it is consumed on the daily basis (Lalor and Wall, 2011). Second concept includes the technological processes used in development of new functional food products. Those could be traditionally used technologies (blending of various formulations, cultivation and breeding) as well as technologies designed to prevent the deterioration of physiologically active compounds (microencapsulation, edible coatings, and vacuum impregnation) and at the end recent technologies aimed to design personalized functional foods (nutrigenomics). The nutritional function refers towards the concept of optimal nutrition. According to Markovina et al. (2011) 40% of young consumers in Croatia are familiar with the concept of functional food. Functional foods could be labelled as fortified foods, enriched foods, altered products and enhanced commodities (Spence, 2006).

According to Euromonitor functional food development is one of the fastest growing food sectors and represents one of the most interesting areas for research and innovation in the food industry (Euromonitor, 2014). Japan and USA are the world's largest markets of functional foods followed by Europe (more in central and northern countries than in Mediterranean) (van Trijp and van der Lans, 2007; Annunziata and Vecchio, 2011). The

United States functional food market had sales of $43.9 billion in 2012, +6.9% over 2011 (NBJ, 2013). Fortified/functional food/beverage category hence is the fastest-growing health/wellness sector worldwide, followed by naturally healthy, organic, and generally better-for-you products (Euromonitor, 2014). The increasing consumer requirements on functional food products might be explained by the huge increase of the healthcare costs, the constant increase in life expectancy and the desire for improved quality of life (Betoret et al., 2011). Consumers demand foods not only to satisfy hunger and to provide necessary nutrients but also to improve physical and mental wellbeing (Robertfroid, 2000). Various factors are related to the expansion of the functional food industry such as innovations in food science and technology, population aging with growing health concerns, an evolving regulatory environment allowing health claims on foods and increased marketing of functional food products (Bigliardi and Galati, 2013a, Bigliardi and Galati, 2013b).

The aim of this paper is to analyse the current situation in the field of manufacturing and marketing of functional foods with special emphasis on the most important EU regulations and the consumers acceptance of functional food products.

TYPES OF FUNCTIONAL FOODS AND INNOVATION

Functional foods have been developed almost in all food industries but the main types of functional foods available on the market are dairy-, soft drinks, bakery- and meat products. The market of functional foods is mainly dominated by probiotics. The main markets of probiotics in Europe are Scandinavia, the Netherlands, Switzerland, Croatia and Estonia. Following functional food products took over the market in the last decade: cereals for the increased cardiovascular diseases, immune protective digestive system developed probiotics, cholesterol-lowering products, hunger reductive shakes/bars, various fruit juices for joint health, immune-boosting dairy beverages, and medicinal teas as well (MSI, 2012). The main trends and types of functional food products are represented in Tables and 2.

Table 1. The Top ten functional food trends in USA (Sloan, 2014)

Functional food trends	Description of consumers priorities
Specialty nutritional - digestive health	Fortified foods – more vitamins, minerals, herbs/botanicals, fish/oil/omega3-s, probiotics
Get real	Organic foods/beverages, absent of artificial ingredients, unprocessed/less processed foods
Beauty-enhancing foods	Natural/organic foods/drinks - energy drinks/shots, sports beverages, 100% juice/juice drinks
The protein evolution	More protein to maintain healthy bones/joints, strengthen immune systems and build muscle strength/tone
Kid specific products	Nutrient and calorie levels specific to kids
Pharma foods	Prevention of heart disease, hypertension, osteoporosis and Type 2 Diabetes i.e. Cholesterol lowering foods/drinks
Alternatives	Free from Foods – gluten free, lactose free, meatless meals (lentils, legumes), dairy free milks (soy, rice, coconut)
Sport nutrition	Sports nutrition supplements, nutrition bars, energy drinks
Weight management	Whole grains, fiber, vitamin D, more calcium, protein, antioxidants, omega-3/fish oil
Millennials food choices	healthier, more natural/organic, less processed, better tasting and fresh food

Table 2. The main types of functional food products introduced into the global market (Prassanna et al., 2014, Figueroa-Gonzalez et al., 2011, Borneo and Leon, 2012, Zdunczyk and Jankowski, 2013)

Functional food products
Probiotics   (*Lactobacillus spp., Bifidobacteria spp.*)
Prebiotics   (inulin, fructo-oligosaccharides, galacto-oligosaccharides, soya-oligosaccharides, xylo-oligosaccharides, isomalto-oligosaccharides, pyrodextrins)
Functional drinks   (weight control/nutrition beverages, energy drinks, sports beverages, ready-to-drink coffee/tea)
Functional cereals (oat based products, barley based products- beta glucans)
Functional meat   ✓ meat products with added functional ingredients (vegetable proteins, dietary fibres, herbs, spices,),   ✓ meat products modificated during processing (production of bioactive peptides during fermentation or curing)   ✓ reformulated meat product - fat reduction, cholesterol reduction, reduction of sodium and nitrite levels, improvement of fatty acid composition
Enriched eggs   Eggs enrichment through supplementation of animal diets with functional ingredients such as LC *n-3* PUFAs, vitamin E, selenium, CLA, lutein

Functional food is a key topic of research&development activities carried out in many member states of the EU. A growing number of research institutions from the public and private sectors are engaged in the development of functional foods (Cooper *at al.*, 2011). Their number is particularly large in Germany and the United Kingdom (Figure 1).
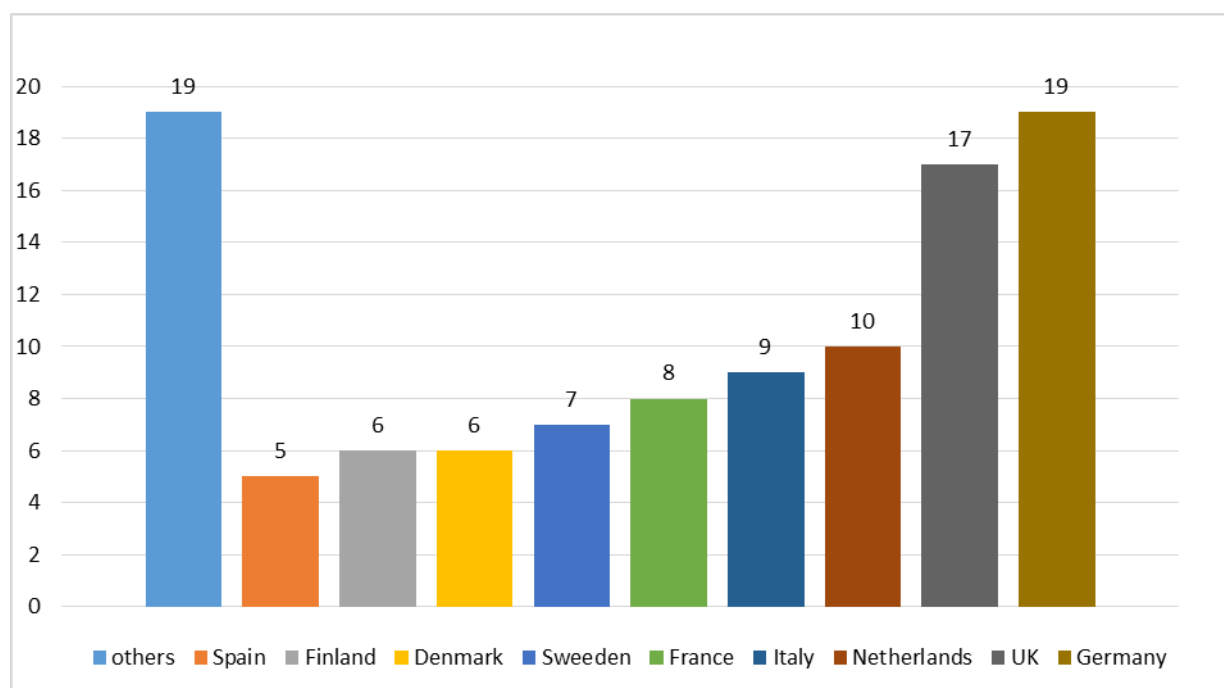
Figure 1: Number of research facilities in the EU active in the field of functional food (Cooper at al., 2011).

Food industry innovations are often aimed at developing important replacement products, following nutritional directions and they are generally new or improved products and services, and can be focused in one area of food technology, product formulation, food qualities or consumer needs.

Among all innovations introduced in food industry, functional food is recognized as one of the most interesting areas of research and innovation (Annunziata & Vecchio, 2011). The ability to capitalize on this source of knowledge will depend upon the organizational structure and approach toward innovation and gatekeepers managing the companies interface with the external environment (Chiaroni et al., 2011). Heterogeneous networks of collaborative partners in developing innovative food products will become increasingly more relevant compared to homogenous network of only one type of partner (Bigliardi and Galati, 2013b). Especially, the role of pharmaceutical and nutraceuticals companies, food ingredient companies, packaging companies, nanotech firms, research institutes are important in functional food development (Khan *et al.,* 2013).

REGULATION SETTING OUT THE FUNCTIONAL FOOD

In the EU there are no specific legal regulations which define the term functional foods. Regulations governing the functional food and functional food ingredients can be classified into any prescribed categories such as: conventional foods, food additives, dietary supplements, medical foods and foods for special dietary needs.

Regulation (EU) 1169/2011 on the provision of food information to consumers was published in the EU Official Journal on 22 November 2011 and entered into force on 12 December 2011 (European Commission, 2011). Regulation establishes the legal framework in the European Union with regard to the information provided to consumers at all stages of the food chain. It defines the responsibilities for mandatory and optional information for food products. Under the new Regulation all packaged foods have to have on declarations nutritional value from the 2016. Until then, nutritional table have to be on food for special dietary uses, fortified foods and foods with nutritional and health claims. Table of nutritional value includes information on energy value, amount of fat, saturated fat, carbohydrates, sugars, protein and salt. Additionally it may be mentioned: the monounsaturated, polyunsaturated fatty acids, polyols, starches, fibres and / or certain vitamins and minerals.

Regulation (EC) No. 1925/2006 on the addition of essential nutrients to foods harmonises the provisions laid down in Member States which relate to the addition of vitamins and minerals and of certain other substances to foods (European Commission, 2006). There is a wide range of nutrients and other ingredients that might be used in food manufacturing, including (but not limited to) vitamins, minerals, amino acids, essential fatty acids, fibre, various plants and herbal extracts. Only vitamins or minerals which are listed below can be added to the food:

✓ Vitamin A (µg)	✓ Vitamin B12 (µg)	✓ Zinc (mg)
✓ Vitamin D (µg)	✓ Biotin (µg)	✓ Copper (mg)
✓ Vitamin E (mg)	✓ Pantothenic acid (mg)	✓ Manganese (mg)
✓ Vitamin K (µg)	✓ Potassium (mg)	✓ Fluoride (mg)
✓ Vitamin C (mg)	✓ Chloride (mg)	✓ Selenium(µg)
✓ Thiamin (mg)	✓ Calcium (mg)	✓ Chromium (µg)
✓ Riboflavin (mg)	✓ Phosphorus (mg)	✓ Molybdenum (µg)
✓ Niacin (mg)	✓ Magnesium (mg)	✓ Iodine (µg)
✓ Vitamin B6 (mg)	✓ Iron (mg)	
✓ Folic acid (µg)		

When food business operators decide to voluntarily put authorised claims on the label, the substance on which a nutrition or health claim is made must be declared. The amount of the substance(s) in question must be stated in the 'same field of vision' as the nutrition labelling. Regulation (EC) No. 1924/2006 on nutrition and health claims made on foods were adopted 2006. and it harmonised rules for the use of nutrition claims such as "low fat", "high fibre" or health claims such as "reducing blood cholesterol" (European Commission, 2006). Nutrition and health claims shall be based on and substantiated by generally accepted scientific evidence. This will enhance the consumers' ability to make informed and meaningful choices. The Commission has established a Register of Nutrition and Health Claims in order to have a comprehensive overview of the permitted nutrition claims and of permitted, rejected and pending health claims (European Commission, 2012). This Regulation respects fair competition and protects innovation in the area of foods and facilitates the free circulation of foods bearing claims. Food companies will be able to use the same claims on its products everywhere in Europe. The scientific proven health claims and afterwards gaining of exclusively rights of using novel ingredients in functional food product has been observed and emphasized as a critical factor in final success of functional food product in the market (Khan et al., 2013). Less/reduced calories and sugar-free were the most frequent health claims advertised by the best-selling new functional foods/drinks in 2013 (IRI, 2014).

Foods for particular nutritional uses (dietetic foods) are specially manufactured to satisfy the particular nutritional requirements of specific groups of people. These could be: Baby foods, Foods for people suffering from gluten or lactose intolerance and Foods for special medical purposes etc. Directive 2009/39/EC established general rules for foods for particular nutritional uses (dietetic foods) aiming to ensure product safety, suitability and appropriate consumer information and to adopt specific directives for certain groups of such foods, if necessary (European Commission, 2009). There are specific regulations for following group of products: Foods for infants and young children, Infant formulae and follow-on formulae, processed cereal-based foods and baby foods, Foods for people with gluten intolerance, Foods for special medical purposes, Foods for sports people and Foods for energy-restricted diets for weight reduction. Some of these products must be notified to facilitate the official monitoring and marketing of innovative products.

The new Regulation No. 609/2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control ('Food for Specific Groups') was adopted on 12 June 2013 and it will repeal Directive 2009/39/EC and abolish the current concept of dietetic foods (European Commission, 2013). The new Regulation will apply from 20 July 2016 and aims to provide a better environment for businesses, better application of rules, and better protect consumers on the content and marketing of these "special" food products e.g. overweight or obese people, people with specific medical conditions, infants and children up to 3 years old , people with metabolism disorders etc.

CONSUMER ATTITUDES TOWARDS FUNCTIONAL FOODS

The functional foods success depends on consumer acceptance and perceptions related to these products. Beyond the detailed nutritional information often found in small print on the back of packaging, marketers often include larger, more prominently displayed health-related claims, such as "all natural" or "low fat", on the front of packaging to boast healthy ingredients. Manny researches showed that respondents tend to be sceptical about the accuracy and credibility of these claims. The survey conducted by Nielsen has shown that the vast majority of respondents (more than two-thirds) indicate that they do not believe food claims, they are never or only sometimes true and that the claims are often unclear (Figure 2) (Nielsen, 2011). Most consumers around the world agree that it is beneficial to eat whole grains and foods with high fibre as part of a healthy diet. Yogurt with acidophilus culture and probiotics are also popular among respondents, and both of these foods are among the top five products for which stresses that are beneficial to health (Figure 3).
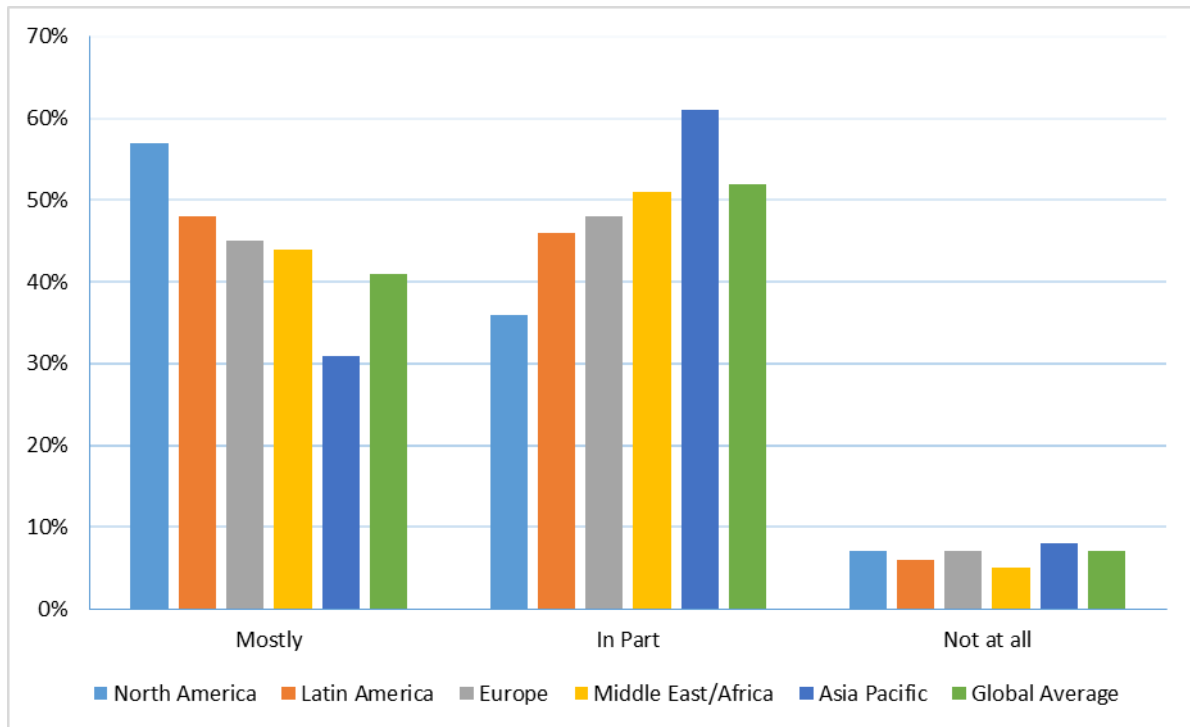
Figure 2: Understanding of the nutritional information panels/labels on food packaging (Nielsen, 2011)
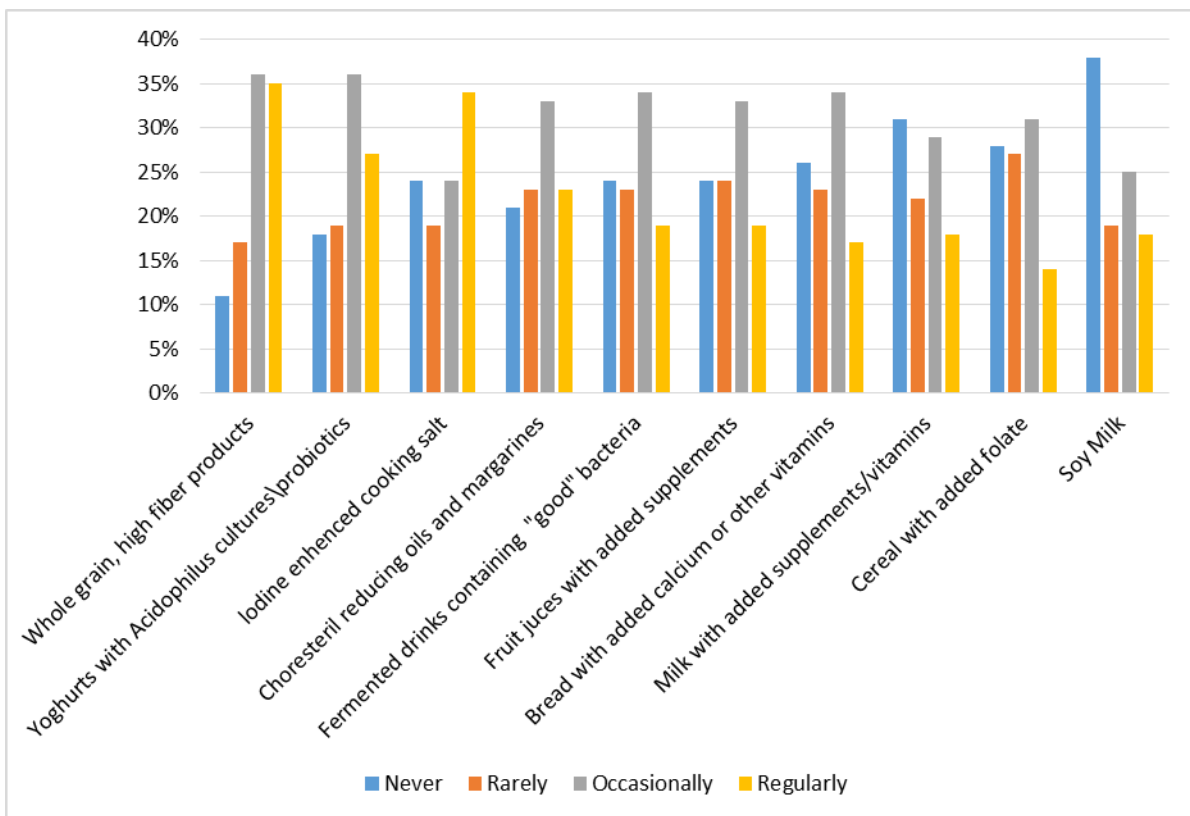

Figure 3: How frequently consumers purchase foods that promote specific health benefits (Nielsen, 2011)

CONCLUSIONS

In recent years in the focus of the public health are problem of obesity and the need to consume healthier foods and reformulation of existing products. Consumers around the world care about healthy eating and manufacturers and retailers have the opportunity to assist in expanding the supply of products that will assist in the implementation and practice of healthy diet, so has intensified pressure to reduce consumption of saturated fat, sugar and salt. Consumer-friendly nutritional labelling can be a powerful marketing tool, but it must take care that information/claims are for consumers easily readable and understandable. Furthermore, it is obvious that there is a need for more education of consumers in order to reduce the scepticism in countries all around the world.

Future knowledge may focus on technological development, testing clinically effects and building a market position through close collaboration with technologically better equipped businesses such as biotech/ pharma, nanotech firms emerging as novel ingredient suppliers and specialized research organizations as Universities and Technological Institutes. Academic institutes and universities offer new scientific and technological knowledge for developing truly innovative food products, what is essential for the food industry where the R&D budget is very scarce.

New EU regulations have created a legislative framework that allows placing on the market new and innovative functional food products under same conditions, but only if those products are adequately declared and provide consumers all relevant information to ensue consumer trust and acceptance.

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RADIOACTIVITY OF MILK IN THE TERRITORY OF VOJVODINA

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ABSTRACT

Milk samples were collected during 2012/2013 from private dairy farms. Total beta-activity was determined in a mineral residue after dry burning using an anti-coincidence system omni guard, Tracerlab (USA), featuring basic activity of less than 1 imp/min. Activity level of ^{40}K in milk samples was determined according to measured total potassium levels, using the specific mass activity of potassium while the method of radiochemical solvent extraction has been used for determining radionuclide ^{90}Sr. Potassium and calcium contents in milk were determined by the method of emission spectrophotometry applying SpectrAA–10, Varian. Caesium and lanthanum were used as ion-suppressors for potassium and calcium, respectively. Results obtained in this research point out that the natural radionuclide ^{40}K was the predominant one in all investigated samples, and the presence of the produced radionuclides, such as ^{90}Sr was also confirmed in all samples. The activity level of radionuclides ^{90}Sr in milk was below 1 Bq dm^{-3} which is slightly lower than in previous few years.
Keywords: *milk, content of K, content of Ca, activity of ^{40}K, activity of ^{90}Sr*

INTRODUCTION

First global assessment of soil fertility and level of hazardous material in the soil in Vojvodina was performed in the period 1992 – 1993 (Kastori, 1997). This study as well as several later researches suggested that Vojvodina is highly suitable region for healthy food production (Čuvardic et al., 2006). The result of abundant research revealed that this region is safe from radioecological point of view and concerning radionuclide pollution as well (Ćupić et al., 2005; Forkapić et al., 2006; Mihaljev et al., 2011; Živkov-Baloš et al., 2011). Soil in many regions in Central and Eastern Europe is contaminated with radioactive material from Chernobyl accident in 1986 (Bikit et al., 1990). At that time, huge amounts of radionuclides were released into the atmosphere and migrated into the food chain. The radionuclides settle on the soils and sediments and incorporate in the plants by deposition and resorption via the root system. Animals and humans are indirectly exposed to harmful effects of radiation through contaminated food of plant origin.

Man and his environment have always been exposed to ionizing radiation arising from diverse natural sources. Natural, "background" radiation has been all along, accompanying the development of organic matter and all living creatures on Earth. Natural radiation is present from the time of birth of the universe and chemical elements, being pretty constant over time but variable with respect to locality (Petrović, 1994). The highest portion of natural radiation is attributed to natural radionuclide potassium-40 (^{40}K), which is present in the mixture of natural potassium isotopes (^{39}K, ^{40}K, ^{41}K) sharing a mass fraction of 0.0119%, whereas its part in the total beta-activity in bio-communities reaches over 90% (Jovanović, 1983).

Use of nuclear energy for peaceful purposes results in the occurrence of produced radionuclides in the biosphere. They significantly increase the total radiation in the environment. Major sources of produced radioactivity are radioactive precipitations following nuclear tests, accidents in nuclear power plants as well as radioactive isotopes applied in medicine, technology, industry, engineering etc. Among the whole range of radionuclides, some of them are significantly more hazardous for humans and animals since present at large amounts, or due to their long half-life and easy transfer throughout the food chain via domestic animals (milk). Moreover, radionuclides are consumed in the food and drinking water. These elements are designated as biologically important radionuclides (BIR) (Mitrović

et al., 1996). Within the group long-lived biologically important radionuclides, strontium-90 (^{90}Sr) is the most toxic with specific interest for foods and the environment. His presence in biosphere is associated with great hazard to living organisms and the environment, since their physico-chemical characteristics and high toxicity significantly contribute to the increase of total radiation risk (IAEA, 1989)..

Foods of animal origin are an important link in the transmission chain of radionuclides, whether naturally occurring or fission products. Amongst foods, milk is particularly important considering its role in human diet (particularly children). Milk is one of the five food products that are consumed daily. Once the fission radionuclides (^{90}Sr) come to the environment, they enter the animal's body very quickly and are subsequently excreted in the milk (Mitrović, 1995).

MATERIAL AND METHODS

Milk samples were collected from a private dairy farms at two-month intervals during 2012/2013. A total of seven samples were collected, whereby each sample implies a composite milk sample for a two-month period.

Collected samples were evaporated, dried in the dryer and then mineralized in a furnace at 450 ± 10^{0}C. Total beta-activity was determined in a mineral residue after dry burning (Stoeppler, 1994) using an anti coincidence system OMNI GUARD, Tracerlab (USA) featuring basic activity less than 1 imp∕min. The efficiency of the measuring device was measured by the method of standard sources. ^{40}K in KCl was used as a standard source for efficiency.

Potassium and calcium contents in milk were determined by the method of emission spectrophotometry applying SpectrAA–10, Varian. Caesium and lanthanum were used as ion-suppressors for potassium and calcium, respectively. Activity level of potassium-40 in milk samples was determined according to measured total potassium levels, using the value of the mass activity of potassium, 31.561 Bq/gK (Eisenbud, 1973). Efficiency of the used counter to beta particles ^{40}K, energy, E_{β}=1.325 MeV was 25%. Other beta-activity ($O_{\beta}A$) was determined from the difference between the total beta activity ($T_{\beta}A$) and the level of activity of potassium-40 ($A^{40}K$). The obtained data were statistically analyzed, and total beta activity and activity of potassium-40 were compared using t-test.

^{90}Sr was determined using extraction method with tri-butyl phosphate (TBP) as a specific reagent (Latimer, 2012) with addition of inactive Y-carrier (Mihaljev, 1998). Count speed (imp/min.) of dried Y-oxalate was measured in anti-coincidence system for measuring low-level beta-activity OMNI GUARD, Tracerlab (USA). The chemical yield of yttrium (measured as Y_2O_3) was over 90%. The same counter was used to determine total beta-activity ($T_{\beta}A$) of the investigated samples.

RESULTS AND DISCUSSION

Total beta-activity ($T_{\beta}A$) and activity of ^{40}K are displayed in Table 1. Total beta-activity in the investigated milk samples ranged from 38.6 Bq/L (IX–X/2013) to 46.0 Bq/L (V–VI/2013). Activity of ^{40}K was proportional to the levels of total beta-activity predominantly resulting from ^{40}K as a natural radionuclide, ranging from 36.6 Bq/L (IX–X/2013) to 43.9 Bq/L (V–VI/2013). Similar results on ^{40}K activity in milk are reported by Vitorović et al. (2010) and Vuletić et al. (2011). This corresponds well with our results expressed as the content of stable potassium in the investigated samples and calculated contribution of ^{40}K in the total beta-activity, which ranged between 89.7% for stable potassium content of 1.30 g/L and 96.1% for stable potassium content of 1.18 g/L. This result is in accordance with the data from the literature (Đorđević, 1982, Radovanović et al., 1997).

Table 1. Total beta-activity, content of total K, activity of ^{40}K and contribution of ^{40}K in the total beta-activity in milk

Sampling period	Content of K [g/L]	A^{40}K [Bq/L]	T$_\beta$A [Bq/L]	O$_\beta$A [Bq/L]	Contribution ^{40}K (%)	Statistical significance
XI-XII /2012	1.19 ± 0.06	37.6 ± 4.9	40.6 ± 3.6	3.0	92.6	p > 0.05
I–II / 2013	1.25 ± 0.06	39.4 ± 5.2	42.7 ± 3.8	3.3	92.3	p > 0.05
III–IV / 2013	1.34 ± 0.07	42.3 ± 5.6	45.1 ± 4.0	2.8	93.8	p > 0.05
V–VI / 2013	1.39 ± 0.07	43.9 ± 5.8	46.0 ± 4.0	2.1	95.4	p > 0.05
VII-VIII/2013	1.30 ± 0.06	41.0 ± 5.4	45.7 ± 4.0	4.7	89.7	p > 0.05
IX–X / 2013	1.16 ± 0.06	36.6 ± 4.8	38.6 ± 3.4	2.0	94.8	p > 0.05
XI–XII / 2013	1.18 ± 0.06	37.2 ± 4.9	38.7 ± 3.4	1.5	96.1	p > 0.05

In the investigated milk samples, there were no statistically significant differences (p > 0.05) between the total beta-activity and activity of ^{40}K indicating that other beta-activity (O$_\beta$A) is due to negligible amounts of other radionuclides.
According to the results presented in Table 2, the activities of strontium-90 measured in the investigated milk samples were very low, being about 20 mBq/L, or less. Similar results on activity of ^{90}Sr in milk are reported by Vićentijević et al.(1998).

Table 2. Activity level of ^{90}Sr, content of Ca and number of Strontium Units (SU) in the investigated milk samples

Sampling period	A^{90}Sr [mBq/L]	Content of Ca [g/L]	[A^{90}Sr/gCa]	SU*
XI-XII /2012	19.8 ± 2.5	1.18 ± 0.06	16.78	0.45
I–II / 2013	11.6 ± 1.7	1.22 ± 0.06	9.51	0.26
III–IV / 2013	17.2 ± 2.1	1.17 ± 0.06	14.70	0.40
V–VI / 2013	19.4 ± 2.4	1.15 ± 0.06	16.87	0.46
VII-VIII/2013	18.0 ± 2.2	1.08 ± 0.05	16.67	0.45
IX–X / 2013	12.6 ± 2.0	1.12 ± 0.06	11.25	0.30
XI–XII / 2013	11.8 ± 1.9	1.16 ± 0.06	10.17	0.28

**1Sr units=37 x 10^{-3}Bq/g Ca*

Basic biological feature of strontium-90 is that it exhibits biochemical behavior almost identical to that of calcium and is thus classified it into the group of osteotropic radionuclides. Strontium ions are deposited in the bone system in the form of hydroxyapatite and bone structures in the body are its target organ. This is particularly important in a view of children's nutrition. Deficiency of dietary calcium will result in easy binding of radioactive strontium to the cells of bone system, as osteocytes do not exhibit ability to discriminate between strontium and calcium ions (Mitrović, 1995). Discrimination between the two elements is expressed through the number of strontium units (SU). In that respect, if the environment is rich in calcium, the binding of calcium will be higher than that of ^{90}Sr (Mitrović, 2001). According some recent research on dairy cows, some 3% of daily intake of Sr-90 is excreted in the milk. As evident from the Table 2, the number of strontium units in the all investigated milk samples was very low and ranged within the interval from 0.26 to 0.46 SU.

CONCLUSIONS

Majority of total beta-activity (T$_\beta$A) of the investigated milk is due to ^{40}K as the most prevalent natural radionuclide. The remaining portion of beta-activity (O$_\beta$A) mainly results from the very low activity of ^{90}Sr, ^{137}Cs and other beta-emitters. Knowing the potassium concentration and its behaviour pattern in the food chain is indispensible in assessing the total radiation load in humans.

In this research, only moderate variations of total beta-activity in the investigated milk from the territory of Vojvodina were established throughout the year representing a periodic function and resulting from dietary regimen, radioactivity level in the feed and season of the year. Furthermore, we can conclude that activity levels of produced of radionuclide ^{90}Sr are lower as compared to the activity recorded during past few years, showing continuous decreasing tendency.

The established numbers of strontium units (SU) and particularly strongly indicates that low contents of ^{90}Sr in the environment result in decreased concentration of this element in particular links of ecological chain. This emphasizes the essential importance of radiation-hygienic control and monitoring of the content radionuclides in milk, which presents an important link in the ecological chain and directly affects radiation hazard and health safety of humans.

The level of radioactive contamination of milk must be systematically monitored with an aim of acquiring better insight in radiation load since milk is considered critical foodstuff in a view of potential presence of radionuclides and their doses.

It is to be emphasized that activity of radionuclides in the investigated milk samples was significantly lower than maximum permissible values set by the relevant legal provisions (Official Gazette of RS, 86/2011). Thus, we can conclude that in a view of radiation hygiene milk can be considered safe food.

ACKNOWLEDGEMENTS

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ANTIBACTERIAL EFFECT OF SELECTED ESSENTIAL OILS AS POSSIBLE DISINFECTANTS IN FOOD INDUSTRY

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ABSTRACT

Aim: Disinfectants used in the food industry have to meet special requirements and therefore the number of usable chemicals is limited. Essential oils (EOs) are organic, natural compounds that can be good candidates for disinfection. In our research, minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of the EOs of thyme, clary sage, juniper, marjoram, cinnamon, and lemon were determined against *Bacillus cereus var. mycoides* 0042, *Bacillus subtilis* 0209, *Staphylococcus aureus* ATCC 43300 (methicillin resistant) and ATCC 25923, *Listeria monocytogenes* 21307, *Pseudomonas putida* 291T and *Escherichia coli* 0582. MIC was determined by micro-dilution method and optical density while MBC was determined by spread plating method.
Basic results: Gram positive and Gram negative bacteria had different sensitivity to the EOs. Best results were achieved by cinnamon oil (0.4-3.2 mg/ml). Lemon EO had no effect at 100 mg/ml. In most cases MRSA had higher MBC values than the not-resistant *S. aureus* strain. Our results suggested that pathogens like *S. aureus* and *L. monocytogenes* were more susceptible to the investigated EOs than spoilage bacteria.
Conclusion: Most of the investigated EOs are promising candidates for use in disinfection solutions.
Keywords: disinfection, essential oils, pathogens, spoilage bacteria

INTRODUCTION

Disinfectants used in the food industry have to meet special requirements and number of usable chemicals is therefore limited, especially in facilities producing organic food. The ability of bacteria to attach to inorganic surfaces like stainless steel, polypropylene, aluminum, ceramic and glass causes severe hygienic problems in the food industry. Attached bacteria are a continuous source of cross contamination and lead to reduced shelf-life of foods. In addition, biofilms formed from attached microbes can reduce the effectiveness of membrane separation operations and cause corrosion (Kumar and Anand, 1998). The objective of our work is to find new, natural compounds which can be used alone or mixed with traditional disinfectants in the food industry. Essential oils (EOs) are obtained by extraction or steam distillation from plant material and have antiviral, antibacterial and antifungal effect (Burt, 2004; Solórzano-Santos and Miranda-Novales, 2012). They can be used for disinfection in liquid or vapor form and rinsing with tap water can be omitted which make this process more simple and cheaper (Oliveire et al., 2010; Valeriano et al., 2012).
In the present study the antibacterial effect of six essential oils was investigated against food related pathogens and food spoilers.

MATERIAL AND METHODS

Bacterial strains

The strains used in this study are from the Szeged Microbiological Collection (SZMC, WDCM 987): *Bacillus cereus var. mycoides* 0042, *Bacillus subtilis* 0209, *Escerichia coli* 0582, *Listeria monocytogenes* 21307, *Pseudomonas putida* 291T, *Staphylococcus aureus* ATCC 43300 (methicillin resistant) and ATCC 25923.

S. aureus and *E. coli* strains were grown in LB broth (10 g NaCl (VWR, Belgium); 10 g bacto peptone (Oxoid, England); 5 g yeast extract (HiMedia, India) in 1000ml). *B. cereus, B subtilis,* and *P. putida* were cultured in TGE broth (10 g glucose (VWR, Belgium); 5 g bacto peptone; 2.5 g yeast extract, in 1000 ml). For *L. monocytogenes* TSB (17 g casein peptone (Merck, Germany); 3 g soya peptone (Oxoid, England); 2.5 g glucose; 5 g NaCl; 2.5 g K_2HPO_4, (Reanal, Hungary), in 1000 ml) was used.

E. coli, S. aureus and *L. monocytogenes* were incubated at 37 °C, *P. putida* at 25 °C, and bacilli at 30 °C for 18-20 hours.

Essential oils

The essential oils of cinnamon (*Cinnamomum zeylanicum*), clary sage (*Salvia sclarea*), juniper (*Juniperus communis)*, lemon (*Citrus lemon*), marjoram (*Origanum majorana)* and thyme (*Thymus vulgaris)* were used in these experiments. EOs were purchased from Aromax Natural Products Zrt. (Budapest, Hungary). Stock solutions (200 mg/ml) of the EOs were made in the corresponding medium containing 1% Tween 40 (Sigma-Aldrich, USA) for dispersing the oil.

MIC/MBC determination

For MIC determination microdilution method was used. In 96 well microtiter plates 100 µl cell suspensions (10^5 CFU/ml) were mixed with 100 µl EO solution in the concentration range of 0.1-100 mg/ml. Negative controls were EOs in sterile medium with Tween 40, positive controls were inoculated media without EO. After 18-24 h incubation at the appropriate temperatures, growth of the bacteria was determined by measuring absorbance at 600 nm (SPECTROstar microplate reader, BMG Labtech) or visually after staining with resazurin. In some cases, EOs added in high concentrations caused turbidity even in the non-inoculated medium making absorbance measurement impossible. In these cases 20 µl resazurin (Cell Titer-Blue® Reagent, Promega) indicator was added to the wells after incubation, and after 1 hour the color was checked visually. Oxido-reductase enzymes of living cells reduce the blue colored resazurin to the pink colored resorufin (Sarker et al., 2007). MIC was defined as the EO concentration where the absorbance decreased below 10% of the positive control, or no change in the blue color of resazurin was observed. For each EO concentration 6 replicates were used.

MBC was detected by tracking plate method (Jett et al., 1997) transferring 10 µl from the cell suspensions, with EO dose corresponding MIC and higher, on non EO treated medium. After 24 hour incubation at appropriate temperature, colony number was counted. MBC was defined as the EO concentration where no colony growth was observed.

RESULTS AND DISCUSSION

Lemon EO showed weak or no effect against the investigated bacteria: MIC values were over 100 mg/ml so lemon EO was excluded from the further study. The other EOs showed strong or medium antibacterial effect with MIC values varying between 0.2 and 100 mg/ml. The most effective EO was cinnamon with MIC and MBC of 0.2 -3.2 mg/ml. Even MRSA showed susceptibility to cinnamon EO (Table 1-2). Generally, MBC was two or more times higher than MIC (Table 2). MRSA and the reference *S. aureus* strain showed different susceptibility to cinnamon, clary sage and thyme EO; generally MRSA was less sensitive.

It seems that effectiveness of the EOs depends on their composition: EOs having aromatic main compounds, like cinnamaldehyde in cinnamon and thymol in thyme, were the best inhibitors. EO components have several target sites in the cell; they can coagulate the proteins in the cytoplasm, damage the cell wall proteins, or disturb the integrity of the cell membrane (Burt, 2004). Due to their hydrophobic character, monoterpene ingredients can penetrate easily into the cell (Nazzaro et al., 2013). The mechanism of action of EOs depends on the cell wall structure of Gram positive and Gram negative bacteria and the reduced sensitivity of Gram negative bacteria to various EOs has been reported repeatedly (Nazzaro et al., 2013; Oussalah et al., 2007; Burt and Reinders, 2003).

Table 1. MIC values of the investigated essential oils (mg/ml)

Gram type	Bacteria	MIC [mg/ml]				
		thyme	cinnamon	marjoram	clary sage	juniper
G-	*Escherichia coli*	1.6	0.2	1.6	60	6.3
	Pseudomonas putida	>100	0.8	6.3	6.3	>100
G+	*Bacillus cereus*	1.6	0.2	1.6	6.3	6.3
	Bacillus subtilis	0.8	0.4	1.6	0.8	3.2
	Staphylococcus aureus	0.8	0.4	3.2	25	3.2
	Staphylococcus aureus (methicillin resistant)	3.2	1.6	3.2	6.3	3.2
	Listeria monocytogenes	1.6	0.8	3.2	100	12.5

In our study, Gram positive bacilli were the most sensitive strains while *P. putida* and *L. monocytogenes* showed much less susceptibility; in some cases MBC values were not detectable within the investigated concentration range.

Because of the different units used for EO concentration, it is difficult to compare our results to those described in the literature. Desai et al. (2012) evaluated the effect of thyme EO against *L. monocytogenes* and found that MBC was 2.5 µl/ml (calculated with the density of the oil, it is in the same range with our results). Oussalah et al. (2007) found that thyme and marjoram were effective against *E. coli*, *S. aureus* and *L. monocytogenes* but the bacteria were more susceptible to thyme.

Table 2. MBC values of the investigated essential oils (mg/ml)

Gram type	Bacteria	MBC [mg/ml]				
		thyme	cinnamon	marjoram	clary sage	juniper
G-	*Escherichia coli*	1.6	0.4	1.6	70	25
	Pseudomonas putida	-	0.8	12.5	12.5	-
G+	*Bacillus cereus*	3.2	0.4	3.2	12.5	6.3
	Bacillus subtilis	1.6	0.4	3.2	1.6	6.3
	Staphylococcus aureus	1.6	0.8	6.3	50	6.3
	Staphylococcus aureus (methicillin resistant)	12.5	3.2	6.3	12.5	6.3
	Listeria monocytogenes	3.2	1.6	6.3	>100	50

CONCLUSIONS

Essential oils used in this study showed strong antimicrobial activity on the investigated food derived pathogenic and food spoilage bacteria. Cinnamon, marjoram and thyme essential oils are the most promising ones to use as potential compounds in disinfectants. It is planned to investigate their effect alone or in combination against bacterial biofilms attached to various surfaces.

ACKNOWLEDGEMENTS

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ANTIFUNGAL ACTIVITIES OF SELECTED ESSENCIAL OILS

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ABSTRACT

Aim: In this study the anti-yeast activities of fifteen essential oils (EOs) were investigated against clinical isolates of Candida species: *Candida albicans*, *Candida glabrata* and *Candida parapsilosis.* Anti-mould activities of the esential oils was also investigated against the plant pathogen and/or mycotoxin producing moulds *Rhizopus microsporus*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Fusarium solani*. Antifungal activity of the EOs was screened by disk diffusion method and minimum inhibitory concentrations (MICs) were determined for the effective ones. To investigate the combination effect of the EOs, fractional inhibitory concentrations (FICs) were defined with the checkerboard method.
Basic results: Strongest antifungal effect with low MIC was obtained with cinnamon, clove and thyme EOs. The most sensitive yeast was *C. glabrata* (MIC: 0.8 mg/ml). Moulds were less sensitive than yeasts with the lowest MIC of 1.6 mg/ml for *A. fumigatus* with cinnamon EO. In most cases combination of EOs resulted in indifferent effect.
Conclusion: Essential oils are possible tools for use as alternative sanitizing or preservation agents.
Keywords: essential oils, antifungal, minimum inhibitory concentration, checkerboard method

INTRODUCTION

Fungi can grow under circumstances, such as low pH and low water activity which are unfavorable for most bacteria. Moulds require oxygen for their growth while fermentative yeasts are able to grow without oxygen. Mould growth on food commodities can cause textural and sensorial changes and/or the production of deleterious mycotoxins. The main mould spoilers associated with fruits, vegetables and cereals are *Mucor* and *Rhizopus* species from Zygomycetes, and *Aspergillus* and *Penicillium* species from Ascomycetes. Yeasts can cause problems in fruit based foods and in dairy products. Milk and dairy products provide a prosperous environment to microbial growth; they are rich in proteins, carbohydrates and lipids. Candida species having lipolytic and proteolytic enzymes; *C. catenulata, C. zeylanoides, C. parapsilosis and C. famata*; are associated with the spoilage of cheeses (Cappa et al. 2001). Candida species in dairy products can be opportunistic pathogens causing infections in immunecompromised patients (El-Diasty et al., 2007). Yeast and mould spoilage results in considerable loss in food supply and contributes to food safety and human health problems.
To avoid fungal contamination of food commodities a lot of different techniques and chemical preservatives are used. Nowadays consumers want to eat their food without synthetic preservatives. This demand initiated the search after natural substances with antimicrobial effects.
Essential oils are plant derived hydrophobic liquids having sometimes more than 50 ingredients. They have been used since ancient times in medicine and cosmetics, and in recent times for food flavoring. They have a broad antiviral, antibacterial, antifungal spectrum and most of them are considered as safe (GRAS) (Burt, 2004). In this study, the antifungal activity of 15 essential oils was tested against various Candida and mold strains.

MATERIAL AND METHODS

Strains and culture conditions
The strains used in this study were from the Szeged Microbiological Collection (SZMC, WDCM 987): *C. albicans* 1363; *C. glabrata* CBS 138; *C. parapsilosis* CBS 604; *A. fumigatus* SzMC 2394 1192/06; *A. terreus* SzMC 2394 2303/06A; *F. solani* I.17 and *R. microsporus* SzMC 13644. Yeast and mold strains were cultured on malt extract medium (0.4% malt extract, 1% glucose, 0.1% yeast extract) at 30°C.

Essential oils
Essential oils of chamomile (*Matricaria chamomilla*); cinnamon (*Cinnamomum zeylanicum*); citronella (*Cymbopogon nardus*) clary sage (*Salvia sclarea*); clove (*Syzygium aromaticum*), eucalyptus (*Eucalyptus sp.);* fennel *(Foeniculum vulgare*); lavender (L*avandula angustifolia)*; lemon (*Citrus limon*), peppermint (*Mentha piperita*); pine (*Pinus sylvestris)*; rosemary (*Rosmarinus officinalis)*; spearmint (*Mentha spicata*); thyme (*Thymus vulgaris)* and tea tree (*Melaleuca alternifolia*) were purchased from Aromax Zrt. (Budapest, Hungary).

Screening for antifungal activity
Agar diffusion method was used. Stock solution (100 mg/ml) was made from the EOs in 50 % ethanol, and 6 µl from these solutions was dropped on paper disks of 10 mm diameter. Disks were placed on agar plates inoculated with spore suspension (10^5 cfu/ml), and after incubation for 24 or 48 hours, diameter of the growth inhibition zones was measured. Negative control was 50% ethanol solution, and positive control, propiconazole (100 µl/ml).

Determination of MIC values
MIC values were determined only for essential oils showing antifungal effect in the above mentioned test. From the stock solution, dilutions were made in twofold increments up to 0.8 mg/ml. Paper disk were prepared as above, and placed on inoculated agar plates. After incubation, inhibition zones were measured. MIC was considered as the lowest EO concentration where inhibition was detected.

Combination of essential oils
For the combination of EOs, macrodilution technique was used. EOs were added to the medium to give MIC, MIC/2 and MIC/4 concentrations. Lemon and citronella EOs were mixed in all possible combinations and added to the medium (checkerboard method). Two ml medium was inoculated with 100 µl *C. albicans* suspension (10^5 cfu/ml). After incubation, growth was checked visually and MIC was considered as the concentration where no growth occurred. Effect of combination was characterized by the fractional inhibitory index (FICI). Fractional inhibitory concentration (FIC) was determined from MIC values determined for an EO alone or in combination: FIC=[MIC in combination]/[MIC alone]. FICI=FIC lemon + FIC citronella. Results are interpreted as synergy (FICI<0.5), addition (0.5≤FICI≤1), indifference (1<FICI≤4) or antagonism (FICI>4) (Lambert and Lambert, 2007; Tserennadmid, 2010).

RESULTS AND DISCUSSION

Screening for antifungal activity
Lavander EO showed no activity against the investigated fungi. Best results were achieved with cinnamon, citronella, clove and thyme.

Determination of MIC values
MIC values for Candida species and molds can be seen in Table 1 and 2. The most sensitive strain was *C. glabrata* with MICs of 0.8 mg/ml. In general, Candida species were more susceptible to the oils than molds. Similarly to the screening results, cinnamon, citronella, clove and thyme showed the strongest antifungal activity with the lowest MICs. Surprisingly,

tea tree oil which is considered as a good antifungal agent showed in our study no effect on Candida species although other authors described low MIC values for *C. albicans* (1.75 – 3.50 mg/ml or 0.5 % (v/v) (Hammer et al., 1999; Rosato et al., 2008), *C. glabrata* (1.75 mg/ml) and *C. parapsilosis* (3.50 mg/ml) (Rosato et al., 2008).

Table 1. MIC values (mg/ml) of essential oils against the investigated Candida species

Essential oil	*C. albicans*	*C. glabrata*	*C. parapsilosis*
Chamomile	>50	>50	>50
Cinnamon	6.25	0.8	0.8
Citronella	25	0.8	6.25
Clary sage	>50	>50	>50
Clove	0.8	0.8	6.25
Eucalyptus	50	-	25
Fennel	12.5	-	-
Lemon	12.5	0.8	>50
Peppermint	-	-	6.25
Pine	>50	-	6.25
Rosemary	0.8	-	-
Spearmint	-	0.8	-
Tea tree	>50	>50	>50
Thyme	0.8	-	6.25

Mucor and Rhizopus species are known to be very insensitive to common antifungal agents due to their excellent breakdown capacity on account of their various hydrolyzing enzymes. In our study, clary sage and clove showed good inhibition activity with MICs of 12.5 and 25 mg/ml. Lopez et al. (2007) measured no inhibition capacity for thyme EO and 17% growth inhibition for peppermint against *Rhizopus stolonifer*. Thyme was also non-effective in our study. Numerous papers describe the antifungal activity of EOs against mycotoxin producing Aspergillus species. Pawar et al. (2006) found that cinnamon bark or leaf EO inhibited the growth of *A. niger*. Cinnamon and thyme EO caused a complete growth inhibition of all investigated Aspergillus species and *Fusarium moniliforme* (Soliman and Badeaa, 2002). Oregano EO inhibited mycelial growth of *A. terreus* at 40 µl/ml and *A. fumigatus* at 20 µl/ml while spore germination of *A. fumigatus* was inhibited at 80 µl/ml (Santos et al., 2008). In our study, clove showed the best MIC results followed by cinnamon but MIC values for thyme were not determined because they were out of the investigated range. Differences versus literature data might have resulted from the different methods used, diversity of EO compounds and different susceptibility of the investigated fungi.

Table 2. MIC values (mg/ml) of essential oils against the investigated mould species

Essential oil[*]	*R. microsporus*	*A. fumigatus*	*A. terreus*	*F. solani*
Chamomile	-	-	-	>50
Cinnamon	-	1.6	25	3.175
Citronella	>50	50	-	12.5
Clary-sage	25	3.175	3.175	25
Clove	12.5	1.6	6.25	12.5
Pine	>50	-	-	-
Spearmint	50	-	-	-
Tea tree	>50	-	-	-

[*] Essential oils which are not presented in the table showed no inhibition effect on the investigated species thus no MIC values could be determined.

Combination of EOs
Combination of lemon and citronella showed indifference thus the EOs have no effect on each other. FIC indices were: 1,5 (indifference)

Table 3. Combination of lemon and citronella essential oils and their effect on the growth of C. albicans

Concentration of citronella essential oil (mg/ml)	Concentration of lemon essential oil (mg/ml)		
	3.13	6.25	12.5
1.6	+	+	+
3.13	+	+	+
6.25	-	-	-

+ growth; - no growth

CONCLUSIONS

Most of the investigated EOs showed antifungal activity and cinnamon, citronella, clove and thyme were very promising candidates for further work. These essential oils have aromatic compounds such as thymol and eugenol which are responsible for their antimicrobial effect. Combination of these EOs with other less effective EOs could lead to lower MIC values due to synergism of the different components of EOs. The investigated yeasts and moulds showed species specific different susceptibility to the EOs. Fortunately the emerging pathogens, *C. albicans* and *C. glabrata* were sensitive to most of the oils thus these EOs could represent alternative or supplementary therapeutically agents. In our further work we will go on with the combination of these EOs and their antibacterial effect will be also investigated.

ACKNOWLEDGEMENT

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RAPESEED (*Brassica napus* L.) SEED USED FOR CONSUMPTION AND INDUSTRIAL PROCESSING

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ABSTRACT

Seed of rapeseed is used for consumption and industrial processing depending on its content. Better understanding of seed components facilitates more precise definition of breeding and utilization goals for individual rapeseed genotypes. Main breeding direction at the Institute of Field and Vegetable Crops in Novi Sad, Serbia is development of „00" genotypes, i.e. without erucic acid and with low glucosinolates content. One of the basic goals in most rapeseed breeding programs is increased content of oleic acid and decreased content of linolenic acid. Industry development also demands creation of rapeseed genotypes with high content of erucic acid. Apart from alterations in fatty acids composition, rapeseed oil quality research also includes increased content of tocopherols, as oxidants. Improved isomeric composition, i.e. relative content of α, β, γ, δ forms, increases oxidative stability of oil as compared to the standard type. Rapeseed meal is used as an ingredient in feed mixes. Its use depends on protein quantity, amino-acid composition and antinutrients quantity and composition.

Keywords: oil, fatty acids, glucosinolates, proteins, meal, rapeseed

INTRODUCTION

Seed of rapeseed (*Brassica napus* L.) is used for consumption and industrial processing depending on its content. Better understanding of seed components facilitates more precise definition of breeding and utilization goals for individual rapeseed genotypes. Such goals regarding enhancement of seed nutritional value and oil quality are determined by very strict demands put forth by the processing and food industries.

Plant oils with elevated content of oleic acid (high oleic = HO) are very attractive to food and processingindustries. Oleic acid methyl ester from rapeseed oil can additionally be used as a component of biodegradable lubricants, additive to mineral fuels (biodiesel), and as a renewable ecologically-acceptable energy source. Most rapeseed breeding programmes incorporate decrease in linolenic acid as one of the basic goals. However, there is also an opposite goal of developing lines with high linolenic acid content, conditioned by requests of a market niche dealing with products containing high content of omega-3 (ω3) fatty acids.

By-product of industrial rapeseed processing is rapeseed meal, which takes third place in the category of oil crops meals in the world. Rapeseed meal used in animal feed mixes is a good protein source (average content 40%), especially regarding high content of amino acids containing sulfur-methionine, cystine and lysine, but its use is limited due to relatively high content of antinutrients (crude fibre, phytic acid, polyphenols and glucosinolates). Glucosinolates include more than 100 forms of organic anions containing sulfur and are the dominant antinutrient present both in rapeseed meal after oil extraction and in full-fat feed obtained from rapeseed seed. Antinutrient nature of glucosinolates is the main reason of long-term research on breeding for reduced content of these compounds in rapeseed, which has resulted in creation and growing of rapeseed with low glucosinolates content („00" cultivarswith glucosinolates content up to 30 μmol/g in unprocessed seed). Despite being undesirable due to their antinutrient nature, glucosinolates, polyphenols and phytic acid are indeed highly antioxidant, provide feed with high oil content and contribute to health benefits, simultaneously decreasing risks of cancer and cardiovascular diseases.

Apart from alterations in fattyacids composition, research on rapeseed oil quality also includes increased content of tocopherols as antioxidants. Improved isomer composition (relative content of forms α, β, γ, and δ) increases oil oxistability compared to the standard type, which saves time and costs for storing rapeseed seed and oil (Hunter and Cahoon, 2007).

CONTENT OF OIL, PROTEIN, FATTY ACIDS AND GLUCOSINOLATES IN NS RAPESEED

Rapeseed is grown for the production of seed with high oil and protein content. The increased demand in raw materials for biodiesel production has resulted in increased production of rapeseed in Serbia. Sixteen rapeseed genotypes grown on two sites, Rimski Šančevi and Sombor, during 2006/07 were analysed in this study. Values of seed yield, seed oil and protein content are presented for each genotype at the given site, while fatty acid and glucosinolate content was presented as a bulk sample for each genotype from both sites. Oil content and quality are conditioned by cultivar genetic potential, i.e. its expression in certain agroecological conditions. Pospišil and Mustapić (1997) reported on the results from micro-trial in Croatia which was set up to determine agronomic and other traits of the new domestic and introduced cultivars „00" type. Oil content in the tested cultivars was very high (44.9-51.8%). Oil content in our trial was somewhat lower, ranging from 36.59% in line NS-L-137 at Sombor site to 49.26% in line NS-L-102 atRimski Šančevi site (Table 1). Line NS-L-102 showed most stable and highest oil content at both sites. Oil content varied at Rimski Šančevi site from 43.11% to 49.26%. Sombor site showed even more pronounce differences in oil content – from 36.59% to 42.70%. Highly significant difference between sites was evident for oil content, and all genotypes had higher values at Rimski Šančevi site.

Table 1. Yield, oil and protein content atR.Šančevi and Sombor sites in 2006/2007

Genotype	Seed yield (%)		Oil content (%)		Protein content (%)	
	R.Šančevi	Sombor	R.Šančevi	Sombor	R.Šančevi	Sombor
Banaćanka	2367	4617	45.04	40.74	18.03	22.37
NS-L-31	2367	4925	45.89	40.63	18.79	24.07
NS-L-126	1717	3392	46.04	42.70	16.49	21.84
NS-L-33	2700	5375	46.40	40.44	16.29	21.51
NS-L-128	2317	3408	45.99	40.44	17.49	23.14
NS-L-129	1900	3442	44.29	39.46	19.01	24.68
NS-L-35	2633	4367	46.68	40.31	15.84	22.32
NS-L-101	2083	4025	43.76	39.78	17.66	23.17
NS-L-132	2133	4350	45.69	39.70	17.20	22.94
NS-L-102	2133	5167	49.26	41.03	16.76	22.71
NS-L-134	1983	5258	46.85	40.07	16.55	22.83
NS-L-34	2467	5325	46.24	39.01	16.80	22.65
NS-L-136	1833	4842	45.67	40.45	18.78	22.67
NS-L-137	1667	5183	43.11	38.35	17.15	21.85
NS-L-138	2033	3308	44.20	36.59	16.65	22.78
NS-L-74	1917	5383	45.90	40.30	16.45	21.41
Average	*2141*	*4523*	*45.69*	*40.00*	*17.25*	*22.68*
	3332		*42.84*		*1.96*	
LSD	0.05	0.01	0.05	0.01	0.05	0.01
Genotype	172.3	229.0	0.60	0.80	0.30	0.40
Site	487.2	647.7	1.70	2.26	0.85	1.13
Interaction G/S	689.1	916.1	2.40	3.19	1.20	1.59

Climate, temperature and available moisture in soil at filling stage and the length of the filling period have the highest effect on oil synthesis (Vrebalov, 1968). Most favourable temperature for oil synthesis ranges between 20 and 25°C, while lower temperatures are more favourable. If temperature exceeds 25°C, and especially 30°C, oil content decreases dramatically because water quantity used for transpiration is larger than that taken up from the soil, resulting in lower turgor pressure and leaf wilting. Such conditions cause oil content to stagnate, and with lower temperature and higher moisture in subsequent phases oil synthesis starts again. When temperature reaches 40°C and relative humidity 90%, oil synthesis ceases (Vrebalov, 1978). Higher oil content under favourable temperature and precipitation was also reported by ChampolivierandMerrien (1996), who state that alterations in oil content and composition depend on seed maturity and filling phase when drought commenced.

Protein content varied from 15.84% in line NS-L-35 at Rimski Šančevi site to 24.68% in line NS-L-129 at Sombor site (Table 1). Line NS-L-129 stands out with stable and high protein content at both sites. Protein content ranged at Rimski Šančevi site from 15.84% to 19.01%. Sombor site showed more pronounce differences in protein content which ranged from 21.41% to 24.68%. Similar to oil content, protein content expressed significant differences between sites and is higher at Sombor site. Extremely dry and hot spring resulted in swift maturation and increased protein content, profoundly so at Sombor site.

Results from the analysis of variance of the studied traits show differences in variability. Square means of the genotypes, site and interaction in the analysis of variance show highly significant variability in seed yield. Oil and protein content had highly significant variability among the studied genotypes and sites (Table 2). Similar results for these traits were obtained in long-term trials (Marjanović-Jeromela et al., 2010).

Table 2.Significance of differences between sites and rapeseed genotypes and their interaction for seed yield and oil and protein content

Source of variation	Degrees of freedom	Square means		
		Seed yield	Oil content	Protein content
Site	1	1.362**	776.1**	709.76**
Genotype	15	1.218**	8.7**	4.29**
Site/Genotype	15	9.177**	2.7	0.73
Error	64	2.283	2.4	0.55

Depending on the intended use of rapeseed, content and composition of oil, protein and glucosynolates can be more important than the actual seed yield. Average content of four basic fatty acids in rapeseed oil (oleic, linoleic, linolenic and erucic) and glucosynolates in seed of the studied rapeseed genotypes are given in Table 3.

Analysis of average glucosinolate content from both sites classified all studied genotypes as „00" and„+0" rapeseed quality types; values that ranged from 9.6 mmol/kg (line NS-L-138) to 16.8 mmol/kg (line NS-L-31) were significantly different.

Oil content in seed and fatty acid composition in oil is determined by plant biology (Aludet al., 1989). Breeding for altered quality often results in lower yields. Genotype with the desired trait does not necessarily yield best, i.e. genotypes bred for more traits can linger after those bred only for yield.Many plant and seed traits are considered in breeding programmes so as to create cultivars of high and stable seed yield which comply with predefined quality standards.

Table 3. Fatty acid and glucosinolate content in bulk seed sample from R.Šančevi and Sombor sites in 2006/2007

Genotype	Oleic acid	Linoleic acid	Linolenic acid	Erucic acid	Glucosinolates
Banaćanka	66.36	16.92	7.09	0.16	16.6
NS-L-31	68.42	15.37	6.41	-	16.8
NS-L-126	65.36	17.19	6.88	0.18	11.4
NS-L-33	66.49	16.16	6.73	0.24	14.2
NS-L-128	64.21	17.76	7.45	0.25	12.1
NS-L-129	67.01	16.24	6.80	0.11	14.5
NS-L-35	66.89	16.76	5.40	-	10.5
NS-L-101	64.33	17.99	6.90	-	15.5
NS-L-132	63.52	16.80	6.90	1.44	12.4
NS-L-102	37.92	12.29	6.56	18.33	11.9
NS-L-134	54.51	14.75	4.02	9.05	11.5
NS-L-34	66.62	16.95	6.07	0.3	11.7
NS-L-36	66.01	16.17	6.23	0.94	15.5
NS-L-137	63.33	18.56	7.42	-	11.2
NS-L-138	65.84	17.30	6.49	0.13	9.6
NS-L-74	67.98	15.35	6.54	0.23	12.5
0.05	1.86	0.93	1.18	0.45	0.97
LSD					
0.01	2.53	1.26	1.61	0.61	1.32

AMINO ACID CONTENT AND COMPOSITION IN NS RAPESEED

The rapeseed meal is viewed by the industry as a by-product of rapeseed production and is used as a feed protein supplement. Rapeseed protein has high levels of indispensable amino acids in general and sulfur amino acids in particular (Weiss, 2006).The values of the protein and amino acid contents of seed are presented for each genotype at both sites. Sixteen types of amino acids were identified. Analysis showed significant effect of the site on protein and amino acid contents (Figure 1). There were significant differences among the genotypes in the basic amino acid content of seed. Several of the genotypes will be included in the breeding program: NS-L-129 (high protein content), NS-L-33, NS-L-128 and NS-L-74 (high lysine content), NS-L-128, NS-L-36 and NS-L 138 (high methionine content) (Figure 2).
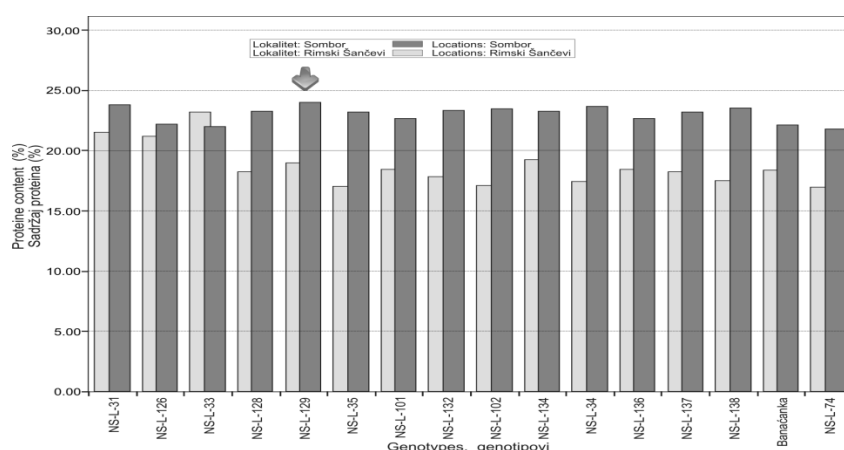
*Figure 1. Protein content in 16 rapeseed genotypes
at Sombor and Rimski Šančevi sites*
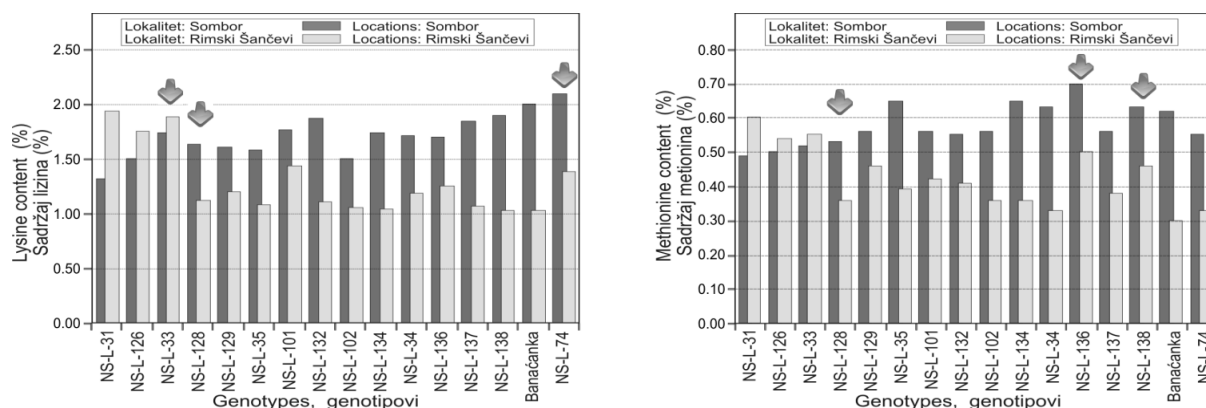
*Figure2. Lysine and methionine content (%) in 16 rapeseed genotypes
at Sombor and Rimski Šančevi sites*

TOCOPHEROL CONTENT IN NS RAPESEED

Tocopherols are natural antioxidants found in plant oils. They are an important component in food and feed. Improved content of tocopherols is a novel and important goal in rapeseed breeding. Tocopherols exist in four forms (α-, β-, γ-, and δ-tocopherol), which are diverse in molecular structure and biological role. Rapeseed oil mostly contains α- and γ- tocopherols with ratio of α / γ tocopherols about 0.5(Marwede et al., 2004).

Eighty-eight rapeseed lines were used as material in this study, taken from the collection of Institute of Field and Vegetable Crops Novi Sad, Serbia(Figure 3). The lines were chosen because they differed in oil quality (fatty acid content), besides other traits, and were grownin field conditions. Tocopherol content was determined using HPLCmethod.
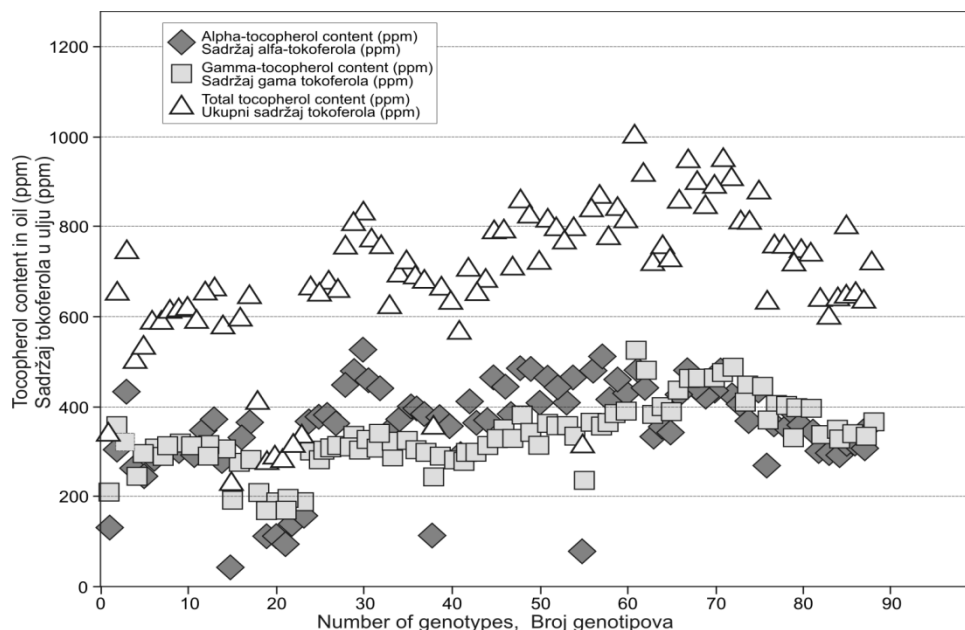
Figure3.α-tocopheroland γ-tocopherol content in 88 NS lines of rapeseed

Content of α-tocopherol ranged from 106 to 524 ppm, and content of γ-tocopherol ranged from 211 to 525 ppm. Correlation between α- and γ-tocopherols was not determined, nor between tocopherol and oil content. Content of an individual tocopherol can be increased without any effect on other tocopherols or other main quality components.

CONCLUSIONS

Based on the presented results, perspective lines stand out intended for various breeding goals, as requested by rapeseed producers and processing industry demands for high-quality domestic raw material. There were significant differences among genotypesregarding basic fatty acid and tocopherols content in oil, as well as glucosinolates and amino acid content in seed. Severalprospective genotypes will be included in the breeding programme: NS-L-33 (high seed yield), NS-L-102 (stable and high oil content), NS-L-129 (stable and high protein content), NS-L-31, NS-L-129 and NS-L-134 (high content of oleic acid), NS-L-128 and NS-L-138 (high content of linoleic and linolenic acid) and NS-L 138 (low glucosinolate content).

ACKNOWLEDGEMENTS

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PRODUCTION OF BIOGENIC AMINES BY LACTIC ACID BACTERIA ISOLATED FROM "ZLATAR" CHEESE

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ABSTRACT

"Zlatar" cheese is one of the most significant representatives of national traditionally manufactured white cheeses in brine. The authenticity of cheeses from the mountain Zlatar comparing to other cheeses from the region, has been based on characteristic autochthonous microflora, consisting dominantly of lactic acid bacteria (LAB), which are responsible for milk fermentation and ripening. It is known that certain LAB, especially enterococci and lactobacilli, are particularly active in the production of biogenic amines (BAs). Therefore, these microorganisms naturally present in raw materials, when introduced through the processing, or added as starter culture, can critically influence BA production during the manufacture of fermented milk products.

The aim of this study was to monitor production of seven biogenic amines (Cadaverine – CAD, Putrescine – PUT, Spermine – SPE, Spermidine – SPD, Histamine – HIS, Tyramine – TYR and Tryptamine – TRY) in selected 96 lactic acid bacteria (LAB) strains. A total of 96 LAB, isolated from "Zlatar" chease, including *Lactococcus lactis* ssp. *lactis* (43 strains), *Enterococcus faecalis* (31 strains), *Lactobacillus plantarum* (11 strains), *L. garvie* (4 strains), *Enterococcus faecium* (2 strains), *Lactobacillus sakei/Lactobacillus curvatus* (2 strains), and *Leuconostoc mesenteroides* ssp. *mesenteroides* (3 strains) were used in this study.

The decarboxylase activity of the microorganisms was studied in growth medium after 24h cultivation. The ability of 96 LAB strains cultivated in MRS (de Man,Rogosa, Sharpe) broth and M17 broth supplemented with 0.5 % glucose, to produce biogenic amines was assessed using liquid chromatography tandem mass spectrometry (LC-MS/MS).The concentrations of six amines in each sample were below 250 µg/L, except tyramine with average concentrations of 4 mg/L, confirming that analysed LAB strains do not pose health risk when used in food production in respect to biogenic amines formation.

Keywords*: "Zlatar" cheese, lactic acid bacteria, biogenic amines, liquid chromatography tandem mass spectrometry*

INTRODUCTION

One of the most important representatives of autchthonous white cheese in brine from Serbia is "Zlatar" cheese. It is made from uncooked whole cow's milk near Nova Varoš, at the foothills and mountain slopes of Zlatar (Vesković Moračanin et al., 2012). Its specificity is mostly related to the climate, geography, soil conditions, water, botanical composition of natural meadows and pastures, breeds and breeding of dairy cattle, as well as traditional customs and habits of local people (Ostojić and Topisirović 2006).

Besides geographical and social characteristics, diversity of large number of different LAB species (and also the strains within one species) plays important role in overall properties of the product as well. Metabolic activity of these strains leads towards the development of the final product and its specific taste. Therefore, propper selection of autochthonous LAB microflora (technological and protective species or strains) which might be used in household craftsmanship, represents a significant tool from the aspect of safe and quality-standardized product (Radulović et al., 2008, Mijačević and Bulajić, 2007, Vesković Moračanin S. et al., 2012, Barros et al., 2008).

Biogenic amines (BAs) are group of biologically active molecules that play functional roles in the nervous system and in the regulation of blood pressure (Santos,1996, Russo et al.,

2010). BAs can be formed in food due to enzymatic decarboxylation of corresponding amino acids by microorganisms. This reaction is catalyzed by substrate-specific enzymes, decarboxylases, originating from microbiota in the food environment. Thus, the monoamines, histamine (HIS), tyramine (TY) and tryptamine (TRY), arise from histidine, tyrosine and tryptophane, respectively. Similarly, the diamines, putrescine (PUT) and cadaverine (CAD), are formed from ornithine and lysine, respectively. Putrescine is a precursor for formation of the polyamines, spermidine (SPD) and spermine (SPE) (Kalač et al., 2000).

In general, BAs can be found in various foods and beverages such as fishery products, meat, dairy, vegetables, fruits, nuts, chocolate, wine and beer (Santos, 1996, Spano et al., 2010). Production of BAs in food depends on precursors presence, i.e. amino acids, as well as microorganisms that poses decarboxylation activity. In order for this process to be successful, favorable conditions are required for microbial growth and their enzymatic activities (Suzzi and Gardini, 2003).

Amino acid decarboxylases are present in many microorganisms of food concern. They have been found in species of the genera *Bacillus* (Rodriguez-Jerez et al., 1994), *Pseudomonas* (Tiecco et al., 1986), as well as in genera of the family *Enterobacteriaceae*, such as *Citrobacter, Klebsiella, Escherichia, Proteus, Salmonella* and *Shigella* (Durlu-Özkaya et al., 2001) and *Micrococcaceae*, such as *Staphylococcus, Micrococcus* and *Kocuria* (Leuschner et al., 1998). Furthermore, many LAB belonging to the genera *Lactobacillus, Enterococcus, Carnobacterium, Pediococcus, Lactococcus* and *Leuconostoc*are are able to decarboxylate amino acids (Butturini et al., 1995, Lonvaud-Funel, 2001).

Many analytical techniques have been used for determination of BAs in the past decades, especially histamine, having in mind its importance in scombroid poisoning and regulatory requirements for its content in fish and fishery products. Thin layer chromatography being the first quantitative technique, has nowadays historical, rather than practical value. However, the majority of analytical methods for determination of BAs in various matrices are based on reversed-phase high performance liquid chromatography with either UV or fluorescence detection after pre-column or post-column derivatisation (Hungerford, 2010). Liquid chromatography tandem mass spectrometry (LC-MS/MS) has been also employed in analysis of BAs recently, although availability of such instrumentation is still rather limited and cost of such analysis relatively high, especially for screening of large number of samples. However, high selectivity and sensitivity of LC-MS/MS makes this technique more than adequate for analysis of multiple BAs in various matrices.

The aim of this paper is to determine ability of LAB isolated from traditional "Zlatar" cheese to produce biogenic amines (histamine, tyramine, cadaverine, putrescine, spermidine, spermine and tryptamine). The established LAB collection with determined technologically positive properties would be the base for further research with the aim of potential production of national starter cultures.

MATERIAL AND METHODS

2.1. Strains and growth conditions

A total of 96 strains of LAB, isolated from "Zlatar" cheese, were used in this study (Table 1).

At different production stages of traditional "Zlatar" cheese, LAB were previously isolated using conventional microbiological techniques (Sharpe, 1979, Veskovic Moracanin et al., 2013) and have been molecularly identified by sequencing their 16S rDNA (Vesković Moračanin et al., 2013a).

LAB strains (*Lactobacillus* and *Leuconostoc* spp.) were kept frozen at −20 °C in de Man-Rogosa-Sharpe (MRS) broth (Merck, Germany) supplemented with 20 % glycerol, while *Lactococcus* and *Enterococcus* spp were kept frozen at −20 °C in Difco™ M17 Broth (BD Company, USA) supplement with 0.5 % glucose. Prior to use, the microorganisms were subcultured twice in 10 mL of MRS/M17 broth (1 % inoculum, 24 h, 30 °C). LAB strains were then cultivated for 24 hours in MRS/M17 broth, and 10 mL of liquid cultures were decanted in 10 mL polypropylene centrifuge tubes immediately after incubation. One half of the samples were analyzed on the same day, while other half was frozen and analyzed on the next day

after quick thawing in order to avoid subsequent formation of biogenic amines. Both batches contained an aliquot of pure MRS/M17 broth in order to assess biogenic amines content in broth itself and prevent misinterpretation of results.

Table 1. The strains of LAB isolated from "Zlatar" cheese

LAB - genus	Number of isolates	LAB – species	Number of isolates
*Lactococcus*spp.	47	*Lactococcuslactis* ssp. *lactis*	43
		Lactococcusgarviae	4
Enterococcus spp.	33	*Enterococcus faecalis*	31
		Enterococcusfaecium	2
Lactobacillus spp.	13	*Lactobacillus plantarum*	11
		Lactobacillus sakei/Lactobacillus curvatus	2
Leuconostoc spp.	3	*Leuconostocmesenteroides* ssp. *mesenteroides*	3
The total number of isolates of LAB			**96**

2.2. Analysis of biogenic amines produced by LAB

Analytical method for determination of biogenic amines in MRS and M17 broth using LC-MS/MS is modified procedure originally proposed by Sagratini et al. (2012) for determination of biogenic amines in fish.

2.2.1 Materials and standards

Analytical standards of biogenic amines (histamine, cadaverine, putrescine, spermine, spermidine, tyramine, tryptamine) were purchased from Sigma-Aldrich (USA). Trichloroacetic acid (TCA) and ammonium acetate was obtained from J.T. Baker (The Netherlands). HPLC-grade acetonitrile (ACN), methanol (MeOH) and water was supplied by Sigma-Aldrich (USA). Ammonium hydroxide, ammonium acetate, formic acid and glacial acetic acid were purchased from Merck (Germany). Solid phase extraction (SPE) cartridges "Strata X 33 µm" polymeric sorbent were purchased from Phenomenex (USA). SPE manifold with 24 ports "Visiprep Standard Vacuum Manifold" was obtained from Sigma-Aldrich (USA).

2.2.2 Sample preparation

Aliquot of 5mL from each LAB strain culture was transferred using automatic pipette to the polypropylene centrifuge tube of 50 mL. 15 mL of 5% TCA was added and the mixture was homogenised for 1 minute at maximum speed using Bibby Scientific Ltd (UK) vortex, model SA-8. In order to prepare homogenate for SPE, pH value was adjusted to 11 using 25% ammonium hidrohyde solution and indicator stripes (Merck, Germany).

SPE cartridges were conditioned with 4 mL of MeOH, followed by 4 mL of water using vacuum manifold system. Then, 4 mL of the sample were loaded onto the cartridges allowed to pass at flow rate of approx. 1 mL/min under gentle vacuum. Rinsing of cartridges was accomplished using 4 mL of MeOH/H2O mixture (5:95, v/v) and cartridges were dried under vacuum in order to remove excess of water. Graduated centrifuge tubes were placed into the manifold and biogenic amines were eluted with 4 mL of the mixture MeOH/acetic acid (99:1, v/v). Eluted solution was evaporated under gentle stream of N2 to dryness, reconstituted in one mL of 1% TCA, filtered into the autosampler vials and 10 µL was injected into the LC-MS/MS system.

LC-MS/MS analysis was carried out using Waters Acquity system with Waters TQD detector (Waters, USA). Separation was performed on Thermo Scientific Hypersil Gold (Thermo Scientific, USA), 100x2.1 mm, 3µm. Mobile phase was 10 mM ammonium acetate in 0.1% formic acid (mobile phase A) and ACN (mobile phase B) at flow rate of 0.3 mL/min. The gradient program was 0 min 20% B, 0-10 min 85% B, 15-25 min 20% B. Mass spectrometric analysis was performed in multiple reaction monitoring (MRM) mode. Positive electrospray (ESI+) was used for obtaining molecular ion. Temperature of the ionisation source and dessolvation gas (N_2) were set at 120°C and 400°C respectively. Cone gas and dessolvation

gas flow were 50 L/h and 550 L/h. Cappilary voltage was 3500 V. MRM conditions for each biogenic amine are given in Table 2.

Table 2. Multiple reaction monitoring (MRM) conditions for the tested biogenic amines

Biogenic amine	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)	Dwell time (ms)
HIS	112	95	10	12	150
PUT	89	86	10	12	150
CAD	103	72	10	12	150
SPE	203	112	10	12	150
SPD	146	112	10	12	150
TYR	138	121	10	12	150
TRY	161	144	10	12	150

Detection limit of the method was experimentally determined and is 0.05 mg/L for all biogenic amines while limit of quantification was 0.1 mg/L

RESULTS AND DISCUSSION

The results from the analysis of 96 LAB strains concerning BAs content generally showed very low content of all seven of the BAs. HIS, CAD, PUT, SPE, SPD and TRY were found in all samples either in quantities below detection limit of the analytical method (0.1 mg/L) which was the case with HIS, SPE, SPD, PUT, TRY, or in quantities that are above limit or detection, but below the quantities found in the growth media (CAD). TYR was the only amine with measured concentrations in the range from 0.54 mg/L to 5.3 mg/L, while majority of investigated strains contained 4 mg/L of TYR. All concentration values, including TYR are very low and are obtained due to high sensitivity of analytical technique used in the experiment. Presence of five compounds (HIS, SPE, SPD, CAD, PUT) in the samples was only due to their presence in the MRS and M17 broth which were analyzed in each sample batch in order to establish the origin of BAs. Both of the media composition consists of meat and yeast extracts that are rich with amino acids. At some point of production and distribution, low quantities of some BAs were formed, and were recorded during the analysis, having in mind that all BAs are thermally stable compounds and can survive temperatures of sterilization (Taylor, 1986). Therefore, the interpretation of results has to be conducted by subtracting intensities of each BA found in the samples from the values found in MRS broth or M17 broth depending on the microorganism. This was the only viable approach in determination of BAs content in media already containing these compounds, in order to present accurate results. Figure 1 shows chromatogram of the MRS broth without LAB cultures. Chromatogram shows quantifying transition product of CAD (103 Da>86 Da), with very high signal confirming the presence of CAD in the medium.
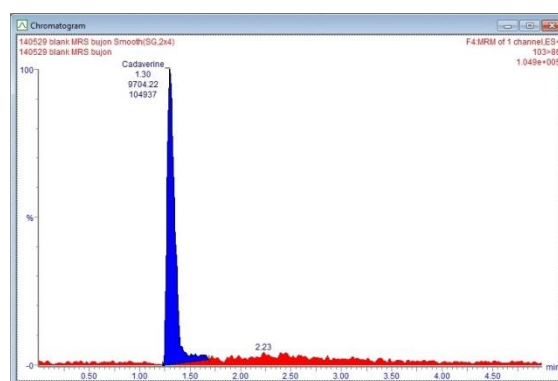

Figure 1. Chromatogram of one transitional product (103 Da>86 Da) of CAD in MRS broth

Figure 2 shows calibration curve of TRY. High coefficient of determination was achieved (0.9809) which was the case with all analyzed compounds.
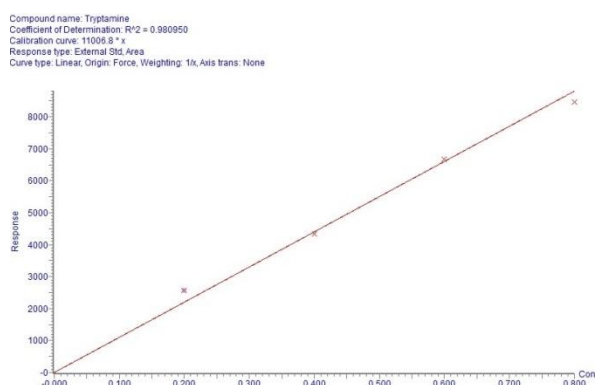
Figure 2. Four-point calibration curve of TRY

Figure 3 shows TYR content in one sample (*Enterococcus faecalis*) containing 4.92 mg/L. This strain has grown in M17 broth, and corresponding chromatogram of pure M17 broth shows TYR content below detection limit of the analytical method. From the assessment of the chromatograms it can be concluded that TYR is the only biogenic amine whose presence does not originate from growth medium, but from the microbial activity. However, its concentrations are still low enough not to pose health risk in cheese manufacturing process.
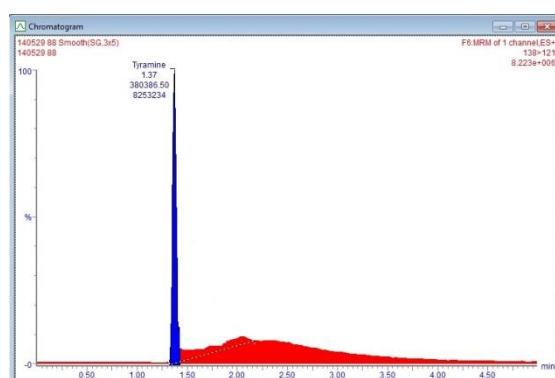
Figure 3. Chromatogram of one transitional product (138 Da>121 Da) of TRY in M17 broth

The obtained results are in accordance with the findings of other authors (Bover- Cid and Holzapfel, 1999, Bover-Cid et al., 2001, Kučerová et al., 2009). *Enterobacteriaceae* and certain LAB, isolated from fermented foods (including strains of protective and starter cultures), especially enterococci and lactobacilli, are particularly active in the production of BA, particilarly TYR. Enterococci isolated from raw milk (O'Brien et al. 2004), have also produced certain amounts of TYR. *Enterococcus* spp. isolates (*E. durans, E. faecalis and E. casseliflavus*) were also found to be the producers of TYR in dutch type semi hard cheese, (Komprda et al. 2008).

CONCLUSIONS

Natural isolates of LAB from "Zlatar" cheese are not potential producers of biogenic amines (*in vitro*) in significant quantities, and therefore are suitable in manufacturing of this product. Enterococcus faecalis was one of the LAB strains that produced TYR. However, its concentrations are still low enough not to pose health risk in cheese manufacturing process.

Besides other favorable properties of LAB isolates (their protective and technological roles), application in "Zlatar" cheese manufacture is possible from the safety aspect in respect to biogenic amines production.

ACKNOWLEDGEMENTS

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CONVERSION OF HAZARDOUS PESTICIDE PACKAGING WASTE TO NON-HAZARDOUS BY TRIPLE RINSING TECHNIQUES

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ABSTRACT

Application of pesticides is common operation for mass production of food, feed, fibre and feedstock in agriculture. Nowadays, it is not possible to achieve food security without application of pesticides. The principles of sustainable agriculture, perform crop protection using possible minimum of chemicals.

However, the intensive use of pesticide generates a large amount of packaging waste, which, along with lack of users education ends up disposed on the fields, nature or municipal landfills. Hazardous waste represent one of the greatest dangers to the environment. Due to the lack of adequate waste treatment and sustainable waste management, the amount of waste that surrounds us is increasing and threatening. As a result contamination of soil and water with hazardous residual contents of discarded packaging occurs.

The aim of this research was to present the use of specially designed pesticide packaging containers and techniques of rinsing, as a simple method for converting hazardous to non-hazardous packaging waste. Rinsing of packaging should occur immediately after pesticide deployment in to the sprayer mix tank because pesticide residual sets quickly and after that, it is almost impossible to remove them. In this research three rinsing techniques will be presented: triple rinsing, pressure rinsing and integrated rinsing, as well as comparison between them. Based on experience and available data from developed EU countries, properly rinsed packaging doesn't exceed limit values for leftovers, legally defined, for non-hazardous classification of packaging which is 3 % for toxic and 0.1 % for very toxic active ingredients contained in pesticide. This simple technique gives great opportunity in developing successful waste management scheme, and different solutions concerning disposal and recycling.

Keywords: pesticide, packaging, waste

INTRODUCTION

Hazardous waste represents one of the biggest threats to the environment. Due to the lack of adequate methods for waste disposal and sustainable waste management the amount of waste that surrounds us increases and threatens. Hazardous substances have direct and indirect impacts on human health and other living organisms, the condition of land, water and air, and the consequences of their harmful effects are manifold (Radusin, 2014)

As the world population increases, the demand for food products also increase (up to 70%) (FAO, 2008). Less availability of agricultural land and the need for the utilization of crops grown for energy and food, sets an enormous challenge to achieve maximum yield on less available farmland (Popp et al, 2014) (Verger and Boobis, 2013). This trend involves higher consumption of pesticides because organic production, despite higher prices and lower yields, and all the positive impacts cannot meet the needs of the growing market of primary agricultural production.

One of the challenges are the waste disposal of pesticide packaging. Disposal of plastic packaging is becoming a growing problem in the area of handling waste. The intensive use of pesticides generates large amounts of waste packaging (Huyghebaert et.al, 2003) which, along with the lack of user education leads to pesticide packaging waste dumped beside the fields, in nature or in municipal landfills. As a consequence, there is a risk of contamination of soil and water with residual contents of discarded hazardous packaging (Ruzic and

Poznanović, 2009). It is estimated that the annual improper and uncontrolled depositing about 50 tons of discarded pesticides is 10,000.000 packaging units. In developing countries, yet the majority of the waste, including pesticide packaging waste is disposed of in an inappropriate manner (Veiga, 2013) (Agamuthu, 2013).

EU countries have accepted the obligation to recycle or incinerate at least 60% of plastic packaging. Practices are very different. Some countries promote recycling, some burning, and some also the most expensive method, incineration or co-incineration (Levitan and Barros, 2003) (Achilias et.al, 2007). The applied method depends on how the packaging waste pesticide is treated, as hazardous or non-hazardous.

Contamination of packaging with various substances (especially those that fall into the poisonous), is an obstacle for the safe storage and recycling. The goal of this investigation was to present the conversion techniques of hazardous to non-hazardous pesticide packaging waste. Based on experience and available data from developed EU countries, properly rinsed packaging doesn't exceed limit values for leftovers, legally defined, for non-hazardous classification of packaging giving the opportunity for solving the issues of pesticide packaging waste in the same manner as municipal waste (Radusin, 2014)

MATERIAL AND METHODS

Based on available sources this research was conducted to provide the optimal solution for solving hazardous pesticide packaging waste for Serbia. Part of this research was based on experiences of other EU countries. Comparison of results and solutions from the litereture are described together with the sugestions of optimal solutions for Serbia in results and discussion

RESULTS AND DISCUSSION

Empty pesticide packaging that have not been properly rinsed present potential threat to public health and environment. Rinsing is one of the most important steps in pesticide management scheme. Rinsing of packaging should occur immediately after pesticide deployment in to the sprayer mix tank because pesticide residual sets quickly and after that, it is almost impossible to remove them. After (during) rinsing, rinsate should be added to the sprayer mix tank. There are three rinsing techniques:

1. Triple rinsing.
2. Pressure rinsing.
3. Integrated rinsing.

Triple rinsing
Triple rinsing is technique adequate in the lack of proper rinsing equipment. Triple rinsing is suitable for smaller types of plastic containers (small enough to be shaken by hand).

Triple rinsing technique for small type of packaging is implying few actions that are specified as followed: after emptying content, packaging should be drained for at least 30 seconds above the sprayer mix tank, 30 % of the packaging should be filled with clean water; after that the closure is restored back to the packaging and intensively shaken for several seconds; after removing the closure rinsate is added to the sprayer mix tank. This procedure must be repeated at least three times. After rinsing procedure, packaging is being damaged so it can't be used again.

Figure 1. Triple rinsing
(http://www.secpa.rs/index.php/teme-i-dogadaji/zbrinjavanje-ambalaznog-otpada)

For larger packaging (up to 200 liters) these actions are implying filling of drum with water to 25% of capacity, replacing and tighten bungs, tip drum on to its side and roll it back and forth, ensuring at least one complete revolution, for 30 seconds. Stand the drum on its end and tip it back and forth several times to rinse the corners and turning the drum over on to its other end and repeat this procedure. Carefully empty the rinsate into the spray can and repeat the procedure at leas two more times. The base of the drum has to be damaged with a drill so that it cannot be reused. (http://interstateagplastics.com/rinising-containers-pressure-and-triple-rinse-videos)

Pressure rinsing
Pressure rinsing equipment uses water under pressure in the form of spray jet for cleaning internal surface of the packaging. Pressure rinsing equipment usually includes sharp device which penetrates the packaging for rinsing procedure, and at the same time make packaging unusable. This equipment should be used in accordance with manufacturer's instructions.
This technique involves the installation of pressure rinsing equipment – pressure rinse nozzle, to a hose connected to a water supply, after which the packaging content is empted and drained in to the sprayer mix tank. Bottom or side of the packaging is penetrating with the pressure rinse nozzle, and the packaging is placed above sprayer mix tank, water flow is released and packaging is rinsed for at least 30 seconds.
For larger type of packaging that is too heavy to be lifted above the sprayer mix tank, for example 200 liter drums, a suction/rinse probe can be used with the packaging standing upright. The packaging is slightly tilted so the remaining contents gather in a corner at the bottom and suck these into sprayer mix tank. The pressure rinse nozzle is turned on, while suction the rinsate into the sprayer mix tank, and rinsing is applied for at least 5 minutes. After turning the pressure rinsing nozzle is turned off and suction of the rinsate continues into the sprayer mix tank. The packaging can be tilted to enable all the rinsate to be sucked into the sprayer mix tank.

Figure 2. Pressure rinsing *equipment*

Integrated rinsing

Integrated rinsing is the most efficient technique of rinsing. Integrated rinsing equipment is usually part of every modern sprayer. Equipment consists of a static nozzle inside the induction hopper of the sprayer. It uses water under pressure (of typically three to five bar) in the form of jet spray.

Figure 3. Examples of sprayer induction hopper with static rinsing nozzle

Integrated rinsing equipment is easy to handle and provides high level of operator's safety. After adding packaging content to the induction hopper, packaging is over the static nozzle and pressed down, after that cleaning mechanism is activated. The packaging is held for 30 seconds above the static nozzle. The rinsate should be automatically added to pesticide mixture inside the sprayer. After draining, the packaging is inspected for residues and damaged as prevention of packaging re – use.

Figure 4. Procedure of integrated rinsing

Comparison of rinsing techniques

Clean packaging without pesticide residuals is goal achieved by all three rinsing techniques. However, there are some differences regarding procedure time, special equipment, rinsing efficiency etc. Advantages and disadvantages of all three presented techniques is presented in Table 1. (Anonym, 2005) According to European waste catalogue (EWC), pesticide packaging can be classified as non-hazardous waste if concentration of active ingredient doesn't exceed the threshold limit defined with this catalogue. The threshold limits in the EWC, notable for pesticide packaging classification are:

– One or more substances classified as very toxic at a total concentration > 0.1 %.
– One or more substances classified as toxic at a total concentration > 3 %.
  If defined threshold is exceeded, pesticide packaging must be classified as hazardous waste. Closure always must be classified as hazardous waste.

Table 1. Comparison of rinsing techniques

Rinsing technique	Advantages	Disadvantages
Triple rinsing	– No special equipment is required – Can be used in all situations – No cost	– Residues are influenced by operator technique – Additional operating time is required – Some risks of endangering the operator – Is difficult for non – rigid packaging – The rinsate is not automatically added to the sprayer mix tank
Pressure rinsing	– Residues of less than 0.01 % – Prevents containers from being reused – Low cost	– Special equipment is required – Additional operating time is required – Some risks of endangering the operator – The rinsate is not automatically added to the spray mix
Integrated rinsing	1. Residues of less than 0.01 % 2. Used for both rigid and flexible packaging 3. Simple operation 4. With correct use of equipment, negligible risk to the operator 5. Rinsate is automatically added to sprayer mix tank	

European Crop Protection Asociation (ECPA) members companies conducted several mayor experiments to provide evidence that primary pesticide packaging could be properly rinsed with available rinsing techniques (Anonym, 2007a). Every experiment has confirmed that remains of active ingredient were much bellow threshold of 0.01 % for very toxic and 3 % for toxic substances set by the EWC (Table 2)

Table 2. Resulting level of pesticide residues at packaging

Country of management scheme	Number of samples/Year	Analytical lab	Number of samples with average T+ > 0.1% and T > 3%
Germany	40 samples in 2006.	RTP, US	None
Germany, France, Poland, UK, Canada, Brazil	47 samples in 2007.	RTP, US/Brazil	None
Germany, France	90 samples in 2008.	Institute Kuhlmann (Germany)	None
France	22 samples in 2008.		None

CONCLUSIONS

All three techniques presented in this research are relatively different, and all have some advantages and disadvantages compared to other two, but final results (clean packaging) is achieved with all three of them. The most important advantages of this technique could be classified as economic and environmental advantages. Economic advantages are that properly rinsed packaging ensures maximum utilization of original content and can be classified as non hazardous waste. This classification has the mayor influence on the cost of management scheme. A management schemes that deals with non hazardous waste can be up to three times less expensive then the schemes that deals with hazardous waste. Environmental advantages: properly rinsed packaging minimizes contamination of soil, ground and surface water with pesticides, eliminates possibilities of user exposure to pesticide and and enebles recycling.

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INFLUENCE OF DIFFERENT PACKAGING SOLUTIONS ON TEXTURAL PROPERTIES OF TOMATO UNDER CONTROLLED STORAGE CONDITIONS

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ABSTRACT

Packaging of fresh fruits and vegetables before its exposure at the market is becoming a common practice applied in order to increase shelf life of the products. Application of different packaging solutions for fresh tomato enables prolonging its shelf life at the market from former several days up to 2-3 weeks. The aim of this research was to determine the influence of different packaging solutions based on conventional polymer packaging materials on postharvest shelf-life of fresh tomato hybrid "Brooklyn", under commonly applied conditions at the market, regarding the changes in textural properties. Tomato fruits were packed in packaging units presenting packaging solutions and using packaging materials that are the most frequently used in tomato supply chain. Sealed packaging units were formed from polyethylene, cellophane and polystyrene trays covered with polypropylene stretch film. Comparatively perforated packaging units were made from polyethylene and cellophane. The main changes in textural properties (hardness, gumminess and resilience) were exhibited during the first week of storage regardless of packaging material and solution applied. During later storage periods, only slight changes of textural properties were exhibited with better preservation of hardness and gumminess in the case of tomato fruits packed in sealed packaging units. Texture parameters are strongly dependent on ripening process causing changes in the structure of cellulose, hemicelluloses and pectin that are the constituents of the fruit cell wall structure.
Keywords: Tomato, packaging, postharvest, shelf-life

INTRODUCTION

Fresh produce in general, including tomato, is characterized with rapid quantity, quality and safety attributes during its exposure at the market resulting in short shelf life and high losses. The direction and the intensity of the changes depend on temperature and relative humidity conditions and permeability properties of the materials used for tomato packaging. Green market departments in supermarkets, in which temperature in range 12-15°C that enables long preservation of products' quantity, quality and safety properties is maintained, are becoming the most usual distribution channels for fresh produce. This temperature is favorable for extension of tomato shelf life due to the fact that it is low enough to enable retention of freshness and extension of shelf-life by reduction of the respiration rate and speed of biochemical changes, but still above the temperatures which may induce chilling injuries which may affect the quality of fruit (Aguayo *et al.* 2004; Batu 2004; Fonseca *et al.* 2002; Mangaraj et al. 2009).

Packaging of fresh fruits and vegetables before its exposure at the market is becoming the common practice applied in order to increase shelf life of the products by minimizing weight loss, contributing to preservation of sensory properties and increasing the safety of the products with variety of packaging solutions using different packaging materials available and utilized for packaging of fresh produce including tomato (Irtwange 2006; Abdullah Farhan-Ul-Haq Saeed 2010).

The most frequently used polymeric materials for packaging of fresh tomato are conventional packaging materials like polypropylene (PP), polyethylene (PE), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephtalate (PET) and others. Cellophane is the most common cellulose based polymer used in packaging of fresh fruits and vegetables.

Perforation of packaging material in order to prevent formation of off-odors and quick spoilage is one of the packaging solutions available as the possibility to expand shelf life of many commodities including tomato (Mangaraj *et al.* 2009; Kantola and Helén 2001). Modified packaging atmosphere (MAP) which is commonly used for highly perishable, high value commodities (Fonseca *et al.* 2002; Irtwange 2006; Gorris and Peppelenbos 1992; Mangaraj *et al.* 2009; Suparlan and Itoh 2003) is rarely applied for fresh tomato due to its relatively low price and relatively short shelf life. However, the equilibrium modified atmosphere (EMA) packaging is primarily used for the packaging of fresh fruit and vegetables as a low cost technique implying simple sealing of the products in the package unit and subsequent reaching of equilibrium atmosphere inside the package as the consequence of respiration of packed produce and packaging material permeability (Irtwange 2006).

The aim of this research was to determine the influence of different packaging solutions based on conventional polymer packaging materials and different storage time on textural properties of fresh tomato under conditions commonly applied at the market.

MATERIAL AND METHODS

Fully ripe tomato fruits (*Solanum lycopersicum* L.) hybrid "Brooklyn" produced in the glasshouse in late production with application of good agriculture practice (irrigation, controlled fertilization, bumblebee pollination, etc) were used for the experiment. Picked tomato fruits were transported from the glasshouse to postharvest pilot plant. Temperature was maintained at 13 °C in transport vehicle cargo bay and during all further manipulation.

Tomato fruits were packed in packaging units presenting packaging solutions and using packaging materials that are the most frequently used in tomato supply chain. Sealed packaging units were formed from polyethylene (PE), cellophane (CELL) and polystyrene (PS) tray covered with polypropylene (PP) stretch film. Comparatively perforated packaging units were made from PE and CELL by punching of holes with 0.5 cm diameter at the distance of 10 cm in the material. Packaging materials were purchased locally and their thickness was 40 µm for PE and 10 µm for CELL. All measurements were performed also on unpacked tomato stored in plastic boxes. For each packaging solution eight packaging units containing 4 tomato fruits were formed and stored for 3 weeks under controlled storage conditions (temperature 13 °C (± 1 °C) and 95 % RH (± 5%)).

Texture Analyzer TA.XT Plus (Stable Micro Systems, England, UK) equipped with 30 kg load cell was used for texture profile measurements. During the TPA (Texture Profile Analysis) tests tomato fruits were compressed twice with 100 mm diameter stainless steel cylinder (P/100) with compression speed of 5 mm/s to the distance of 10mm. This test provides complete texture profile including hardness, cohesiveness, adhesiveness, chewiness, gumminess and resilience of measured samples.

RESULTS AND DISCUSSION

The hardness value is the peak force of the first compression of the product and it relates to the consumers' impression of fruit hardness with consequent categorizing too hard fruits as immature and too soft fruits as senescent. Regardless of applied packaging solution the hardness of tomato fruits (Fig. 1) almost decreased by half during the first week of storage with the most intensive drop in hardness in the case of unpacked fruits. During the second week hardness remained for all investigated packaging solutions almost constant. The differences among hardness of fruits packed in different packaging units emerged after the third week. More intensive drop of hardness after three weeks of storage was recorded for fruits packed in perforated packaging units as well as for the unpacked fruits and fruits packed in sealed PE package. From the aspect of long lasting harness preservation, sealed cellophane and PS tray with PP stretch film provided the best results.
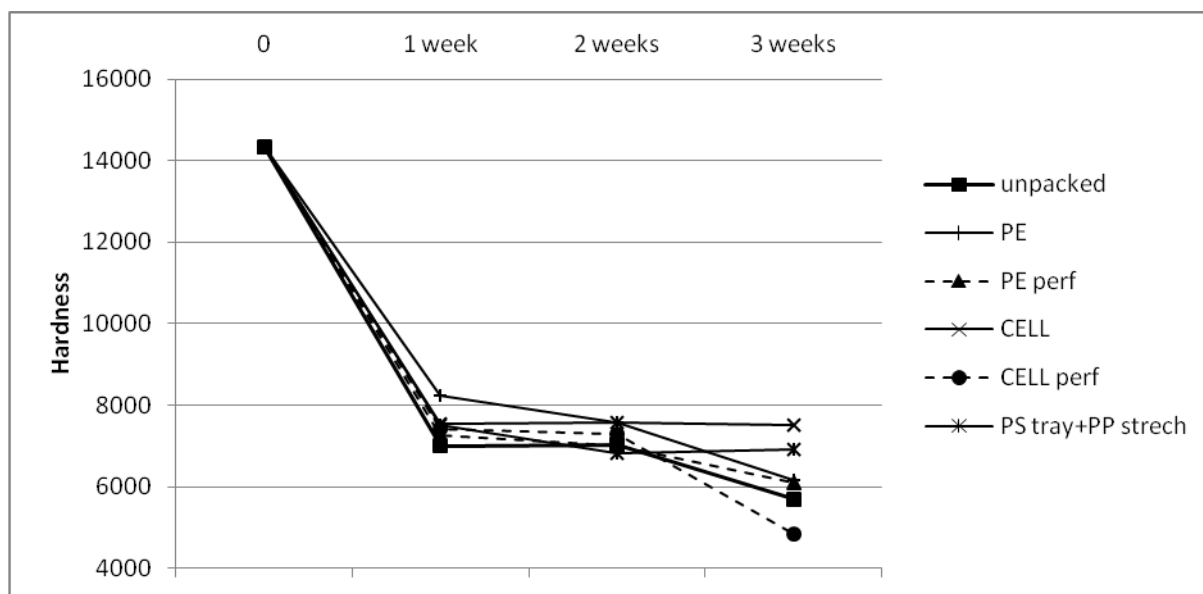
Figure 1. Influence of storage time and packaging solution on hardness of tomato fruits

Resilience shows the ability of tested sample, in this case tomato fruit, to regain the initial shape that it had before the compression. The better the fruit regains its initial shape resilience is closer to 1 and oppositely, lower values of resilience indicate that the fruit after compression stays deformed. Deformation of the fruits after compression will result in consumers' impression of overripe fruit.

Fresh tomato fruits are characterized with resilience slightly above 0.30 (Figure.2). After one week reselience drops to the level from 0.20 to 0.23. After two weeks of storge resilience remains at the same level but slight differences among fruits packged in different packaging solutions disapear. Suprisingelly, after three weeks resilience is somewhat improved but no regularities related to the type of packaging solution could be noticed.

Figure 2. Influence of storage time and packaging solution on resilience of tomato fruits
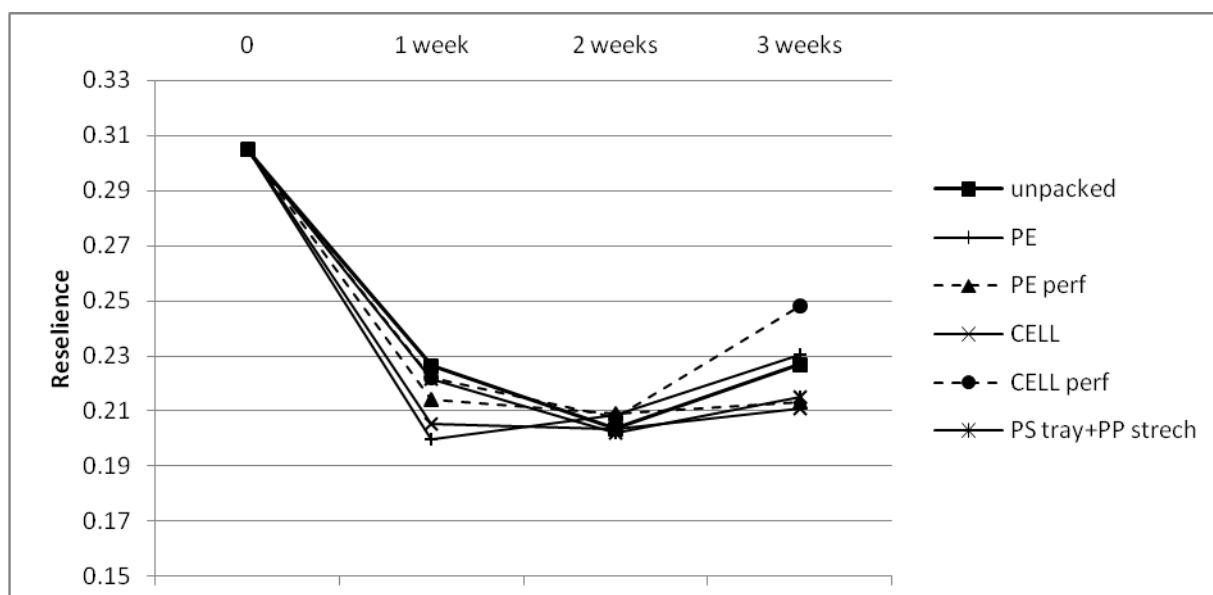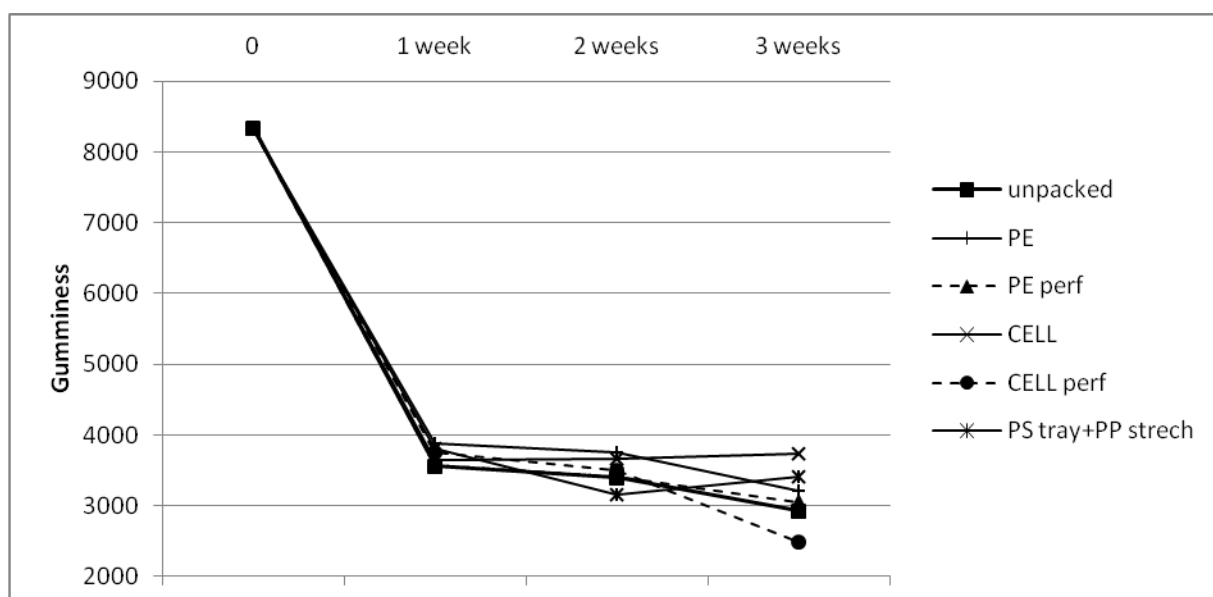
Figure 3. Influence of storage time and packaging solution on gumminess of tomato fruits

Finally, by pressing the tomato fruit for the second time consumers get impression whether the pressure affects the cohesiveness of the fruit and based on that impression conclude about the integrity of the fruit after application of operations normally used in tomato consumption or utilization in food preparation: cutting, peeling, slicing etc. Gumminess of the fruits as the complex indicator of cohesiveness and hardness is the best indicator of this aspect of tomato fruit textural properties. Gumminess (Fig. 3) measured with TPA test decreases by half, in the case of all tested packaging solution after one week of storage. This drop is an expected outcome of the registered drop in fruit hardness. Prolonged storage results in differentiation of fruits from different packaging solutions regarding gumminess. Fruits packed in sealed packaging units are characterized with somewhat higher gumminess values in comparison to unpacked fruits and those packed in perforated packaging units.

It is evident that, during the first week of storage, tomato fruits underwent the most expressed textural changes that are primarily the consequence of decrease in fruit hardness. After the first week, the equilibrium state concerning the textural properties was achieved but during longer storage period of two, and especially of three weeks differentiation among the tested packaging solutions can be noticed. Textural parameters are strongly dependent on ripening process causing changes in the structure of cellulose, hemicelluloses and pectin that are the constituents of the fruit cell wall structure (Kantola and Helén 2001, Seymour et al. 1993). It has been reported that as the ripening process progresses, depolymerisation or shortening of chain length of pectin substances occurs with an increase in pectin stearase (EC 3.1.1.11) and polygalacturonase (EC 3.2.1.15) enzyme activities (Yaman and Bayoindirli 2002; Kantola and Helén 2001). For the unpacked samples and perforated films textural properties decreased slightly more than for the sealed samples as the storage intervals increase indicating that for the sealed packed samples these processes were probably stopped or reached the equilibrium state.

CONCLUSIONS

The most significant changes in texture of tomato fruits occured during the first week of storage regardless the type of the packaging material and solution used for packaging. Hardness, as the most important indicator for consumers, remained in acceptable ranges when fruits were packed in PE and CEL, while control fruits and fruits packaged in perforated PE and CEL became undesirably soft. Although different packaging materials were tested,

the differences in hardness and resilience could be spotted only between the perforated and non-perforated materials.

Differences in texture started to appear between the second and the third week of storage. Sealed packaging solutions showed advantage in comparison to the perforated ones in the case of tomato fruits packaging.

ACKNOWLEDGEMENTS

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MOLECULAR DETECTION OF *ALTERNARIA* SPP. ON ORGANIC SPELT WHEAT

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ABSTRACT

Molds of genus *Alternaria* are important contaminants of small grains and their products causing considerable losses to growers and food processing industry. Negative impact on quality and safety of food and feedstuffs is closely related with toxigenic potential of some *Alternaria* spp. There is a growing interest in spelt wheat as high quality grain suitable for organic production. Considering the importance of *Alternaria* spp. and increasing demand for spelt wheat products, aim of this study was to identify the most frequent *Alternaria* species on spelt kernles. Since detection based on mophological characterisation is laborious, time-consuming and might not detect molds on the species level with high certainty, molecular approach by PCR assays has been used. ITS sequence analysis indicated that *Alternaria tenuissima* was dominant species on spelt wheat kernels followed by *Alternaria infectoria.* Accurate identification of *Alternaria* spp. is a crucial phase in further detection of their toxic metabolites, thus PCR method may be applied to screening agricultural commodities for the presence of mycotoxin producers.
Keywords*: Alternaria spp., spelt wheat, PCR*

INTRODUCTION

Fungi of genus *Alternaria* are cosmopolitan saprophytes, plant pathogens, mycotoxin producers and allergens, so early detection and accurate identification of *Alternaria* spp. is the cornerstone of successful food safety management. The ubiquitous nature of small spored *Alternaria* species makes them important in a broad range of agricultural commodities, including cereals and cereal based products. As saprophytic, they can spoil food and feed products by deterioration and by production of biological active metabolites (Pitt and Hocking, 1985). As plant pathogens, they cause yield losses in the field and spoilage in storage. Moreover, some species are mycotoxin producers that might be harmful for human and animal health (Ostry, 2008). In addition to toxic capacities, *Alternari*a spores are related to allergen release, particularly with asthma (Pulimood et.al, 2007) and skin infections (Dubois et al.,2005). Because of their negative impact on yields, quality and food safety correct identification of *Alternaria* spp. is essential and necessary.
Spelt wheat (*Triticum aestivum* ssp. *spelta* L. Thell) is one of the oldest cultivated hulled grains which was about to disappear, however it was resurged due to its valuable nutritious properties and high suitability to unfavorable environmental conditions. Nowadays, spelt is considered as health-promoting food and interest for spelt wheat based products is constantly growing particularly in organic food market. Along with expansion of demand for spelt products, scientific interest in this crop is growing too. Literature survey mostly reveal data concerning genetic diversity (Bertin *et al.* 2001; An *et al.* 2005, Onishi *et al.* 2006), growth characteristics (Ruegger *et al.* 1993, Troccoli and Codianni 2005, Konvalina *et al.* 2010, Ugrenović 2013), nutritional and technological properties (Zanetti *et al.* 2001; Bojnanska and Frančakova 2002; Schober *et al.* 2006; Abdel-Aal 2008; Abdel-Aal *et al.* 2008; Bodroža-Solarov *et al.* 2011, Filipčev *et al.* 2013, 2014, Filipović *et al.* 2013, Kohajdová and Karovičová 2008; Bodroža-Solarov *et al.* 2014) and potential utilization of spelt by products in renewable energy systems (Brlek *et al.* 2012).

However, resistance on mycobiota and their detection have just been marginally reported (Kema 1992; Kurowski et al., 2012; Wachowska 2012). Higher tolerance to fungal pathogens is associated with adherent hulls which protect kernels from fungal infection (Riesen *et al.* 1986; Bodroža-Solarov *et al.* 2010) and contamination of mycotoxins (Vučković et al. 2013). Since spelt has genetic disposition for production in organic farming which is conducted without conventional chemical treatments, occurrence of mycobiota particularly with toxic potential is an issue of high concern. Thus, aim of this work was to present polymerase chain reaction (PCR) as rapid detection method for prevalent *Alternaria* spp. on spelt kernels.

MATERIAL AND METHODS

Collection of isolates
Spelt wheat kernels were collected from commercial fields and local market including four different genotypes with organic certificate. Samples were at least 1 kg in size. To isolate internal fungi, from each sample 100 kernels were analyzed after superficial disinfection with 0,4% NaClO solution for 2 minutes followed by rinsing with sterile water then dried over a filter paper in petri plates in four repetitions (25 kernels per plate). After 7 days incubation in darkness at room temperatire the health of the spelt kernels was observed under a stereomicroscope. Phytopathological isolations of *Alternaria* spp. were carried out on PDA (Potato Dextrose Agar) and incubated at 25°C for 7 days under an alternating light/dark cycle (12h photoperiod). For further researches, 12 monosporial isolates were choosen, 3 from each genotype.

DNA extraction
Fungal fragments were transferred onto 100 mL autoclaved medium containing potato dextrose broth and shaken in a 250mL Erlenmeyer flasks at 25 °C under continuous light for 3 days. Mycelium was collected by vacuum filtration and freeze dried for extended storage. Total DNA of each isolate was extracted from the mycelia by the DNA Isolation Kit (Agilent Technolgies, USA) according to the instructions of the manufacturer.

PCR amplification and sequencing
The ITS region (ITS 1, 5.8S, ITS2) of rDNA was amplified by polymerase chain reaction (PCR) using the primer pair ITS1 and ITS4 (White *et al.* 1990). 50μL PCR reaction mixture contained 1 μL genomic DNA, 1 μL of each primer (ITS1 and ITS4), 25 μL Paq 5000Hotstart PCR Master Mix (Agilent, Technologies) and 22 μL PCR H_2O. The PCR reaction was carried out in thermal cycler (Sure Cycles 8800; Agilent Technologies, Santa Clara, CA, USA) programmed for the following parameters: an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 20 s at 95 °C, 20s at 54 °C and 30s at 54 °C, and a final extension step of 5 min at 72 °C. Successful amplification was checked by lab-on chip electrophoresis on Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). Determination of size of DNA fragments and DNA fragments quantification were determined Using Agilent DNA 1000 kit. PCR products purification was carried out with the Qiaquick PCR Purification Kit (Quiagen, Hilden, Germany) and sent to sequencing in both directions to Macrogen Europe Inc. (Amsterdam, Netherlands).

Data analysis
BLAST alignment program was used to search identity of sequences to those available in GenBank database. Sequences were multiple aligned with referent isolates *Alternaria tenuissima* CBS 918.96 and *Alternaria infectoria* CBS 210.86 using Clustal W software (Larkin *et al.* 2007) with some manual gap improvements.

RESULTS AND DISCUSSION

Mycological observations of spelt kernels showed high infection by *Alternaria* spp. on all four genotypes, indicated that fungi of genus *Alternaria* are widely distributed on spelt wheat in Serbia (Fig 1).

Figure 1. Alternaria on spelt kernels

In order to identify prevalent *Alternara* spp. the ITS1, 5.8S and ITS2 DNA regions of 12 *Alternaria* isolates were amplified. Succesful PCR reactions resulted in a single band observed on gel. Amplified fragments of isolates were in the range of 569-579 bp with clear separation of 2 isolates yielded in length of 613-618 bp (Fig 2).

Figure 2. Gel electrophoresis of PCR amplification products of DNA fragments.Lanes 1-4, 6-8 and 10-11: A.tenuissima; Lanes 5 and 9: A.infectoria. Lanes M: DNA marker (1000bp)

This result showed distinct amplification products which indicated different *Alternaria* species. Further, ITS sequence analysis and comparison with available sequences in the GenBank database revealed 100% identity with the *Alternaria tenuissima* as predominant species followed by *Alternaria infectoria.* After aligment of the ITS sequences with referent isolates *Alternaria tenuissima* CBS 918.96 (EF031053) and *Alternaria infectoria* CBS 210.86 (FM958526) All sequences generated in this study have been accessioned in GenBank (accession no. KM516075-KM516086). Separation of amplified products of different species on gel was confirmed by sequencing, which indicates that PCR assay in combination with electrophoresis might be helpful for rapid detection of mycobiota. This method appears to be accurate and sensitive for diagnosis of fungal diseases when compare with classical culture based identification, which requires a lot of expertise in morphology-based taxonomy and is time consuming. Also, DNA could be used as an indirect marker of the presence of *Alternaria* mycotoxins in raw and processed cereal products (Edwards et al., 2002; Niessen, 2007). The most frequent *Alternaria* spp. detected on common wheat worldwide were *A. alternata* and *A. tenuissima,* followed by *A. infectoria* and *A.triticina*, so our results are in accordance with reported data (Logriecco *et al.* 2003; Kosiak *et al.* 2004; Lõiveke *et al.* 2004; Patriarca *et al.* 2007; Bensassi *et al.*, 2009), Zur *et al.*, 2002; Nicolaisen *et al.*, 2014). Along with occurrence on kernels, some *Alternaria* spp. such as *A.triticina* (Prasada and Prabhu 1962;

Perello´ and Sisterna 2006) and *A. triticimaculans* (Perelló *et al.* 1996) may infect leaves and result significant yield losses in India and Argentina. A new species, *A. hungarica* has been recently reported by Toth *et al.* (2011) as a minor foliar pathogen of wheat. However, no data on molecular detection of *Alternaria* spp. on spelt wheat is available, so this is the first report of that kind.

CONCLUSIONS

This study suggested that PCR method is suitable for rapid screening of grain in field samples or in commercial practice. Presence of *A.tenuissima* and *A.infectoria* on organic spelt kernels indicated that despite of relative resistance of spelt to fungal pathogens, monitoring is essential part of disease management. Considering a specific mycotoxin profile of different *Alternaria* spp, precise identification is highly important for risk assessment and development of targeted interventions aimed at implementation of food safety strategies.

ACKNOWLEDGEMENTS

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DETECTION OF TETRACYCLINE-RESISTANT LACTIC ACID BACTERIA IN "ZLATAR" CHEESE

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ABSTRACT

Artisanal products prepared from either raw meat and milk may act as carriers for antibiotic-resistant bacteria. This issue is currently of great concern in food safety. In this study, a collection of 96 lactic acid bacteria, mostly including genera *Lactobacillus* and *Enterococcus* was recovered from "Zlatar" cheese and was subjected to a polyphasic molecular study with the aim of detection of tetracycline resistance genes. Using 16S rRNA sequencing technique, lactic acid bacterial isolates were identified as *Lactobacillus plantarum*, *Lactobacillus sakei, Enterococcus faecalis*, *Enterococcus faecium*, and *Lactococcus lactis* subsp. *lactis.* Interestingly, a total of 5 *Lactococcus garvieae* strains known to be implicated in subclinical mastitis in cows were also identified which raised suspicion to plausible tetracycline resistance. Subsequently, all isolates were screened for presence of gene encoding ribosomal protection proteins (RPP) using conventional PCR. A total of five presumptive tetracycline-resistant strains (*L. plantarum*, *E. faecalis* and *L. garviae*) were further tested on presence of tet(M) and tet(K) genes resulting in four tet(M) positive strains while one strain was neither tet(M) nor tet(K) positive. This molecular study indicates that taxonomically and genotypically diverse lactic acid bacteria from traditional cheese can be a host for tet genes.

Keywords: lactic acid bacteria, tetracycline resistance, cheese

INTRODUCTION

The Zlatar cheese, manufactured in Nova Varoš municipality and Mt. Zlatar region of Serbia has been one of the most important representatives of artisanal white cheeses in brine. It has been produced using unpasteurized raw cow milk and rennet or previously coagulated milk followed by curd cutting, salting and ripening in brine during 1-60 days. Apart from influence of ecological factors, authenticity of Zlatar cheese is also result of activity of indigenous microflora, primarily lactic acid bacteria (LAB), in dairy fermentation and cheese ripening (Veskovic et al., 2012).

Although LAB are essential in selection of significant technological and protective strains which could be used as autochthonous starter cultures in manufacturing of safe and standardized products these could be also a threat to human health due to carriage of antibiotic resistance-protein encoding genes. There are scarce evidences on antibiotic resistance regarding non-pathogenic bacteria ("normal" flora) in food available, although they may act as reservoirs of resistance genes. Antibiotic resistant coagulase-negative staphylococci (CNS) and LAB commonly found on the body of animals may contaminate milk or meat and are subsequently to be found in fermented food made with raw material (Teuber et al., 1996). Furthermore, the CNS and LAB were suggested to be a reservoir of antibiotic resistance genes which can be transferred to *Staphylococcus aureus* (Perreten et al., 1997). A streptomycin-, tetracycline-, and chloramphenicol-resistant *Lactococcus lactis* subsp. *lactis* was isolated from a raw milk soft cheese ($2{\times}10^8$ CFU/ g) containing a conjugative plasmid coding for the three resistances (Perreten et al., 1997). Also other lactic acid bacteria were reported to be resistant to antibiotics (Charteris et al.,1998; Orberg and Sandine, 1985; Vidal and Collins-Thompson, 1987; Raccach et al., 1985; Reinbold and Reddy, 1974; Olukoya et al., 1993; Katla et al., 2001).

Tetracyclines have been used extensively since their introduction in the early 1950's. They are the second most used group of antibiotics after the penicillins and they still have different applications in various fields mostly for the treatment of infections in poultry, cattle, sheep, and swine. In some cases, e.g. for therapeutic treatment of large numbers of poultry, the antibiotics are added directly to feed or water or can be administered in aerosols. According to data compiled by the European federation of animal health (FEDESA [http://www.fedesa.be]) tetracyclines are the most frequently used antibiotics in animal husbandry (66% of the total amount, corresponding to 2294 tons/year).

Bacterial resistance to tetracyclines was first reported in *Shigella dysenteriae* in 1953, shortly after their discovery (Roberts, 1996). Prior to this, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines, as illustrated by the finding that among 433 different members of the *Enterobacteriaceae* collected between 1917 and 1954, only 2% were tetracycline resistant (Hughes and Datta, 1983). The emergence of resistance has followed the introduction of these agents for human, animal, and agricultural use. Tetracycline resistance (Tcr) has now become widespread in both Gram-negative and Gram-positive species due to acquisition of tetracycline resistance genes (tet genes) located on transposons or plasmids.

Currently, two tet genes are considered to belong to the same class and are given the same gene designation if they have ≥ 80% of their amino acid sequence in common (Levy et al., 1999). A total of 29 classes of tet genes and four classes of oxytetracycline resistance (otr) genes have been described and characterized. There is no intrinsic difference between a tet and an otr gene. The otr genes were first identified in oxytetracycline-producing *Streptomyces*, and thus the nomenclature reflects the organisms first shown to carry the particular gene. Resistance to tetracyclines is primarily due to acquisition of tet genes rather than to mutation of existing chromosomal genes. Ribosomal protection is the most widespread of the Tcr mechanisms. Ribosomal protection proteins (RPP) are cytoplasmic proteins (72-kDa) which protect the ribosomes from the action of tetracycline, doxycycline and minocycline. They confer a wider spectrum of resistance to tetracyclines than is seen for bacteria carrying tetracycline efflux proteins. The RPP have homology to elongation factors EF-Tu and EF-G (Taylor and Chau, 1996). Current data suggest that the ribosomal protection proteins bind to the ribosome. This causes an alteration in ribosomal conformation which prevents tetracycline from binding to the ribosome, without altering or stopping protein synthesis. TetM and TetK proteins demonstrate similar mechanism of tetracycline resistance (Gevers, 2000).

Aim of this study was to detect presence of tetracycline resistance genes in LAB previously identified using molecular approach.

MATERIAL AND METHODS

Cheeses - Lactic acid bacteria were isolated from white brine Zlatar cheeses at different periods of ripening. The cheese was manufactured by adding the rennet to raw unpasteurized milk immediately after milking and curding. Subsequently, curd was cut into smaller pieces and salted. The thickness of the selected slices was between 0.5 and 1.0 cm avoiding slices having dumps inside the cheese body. For ripening cheese slices were transferred into a small wooden barresl and poured over with the whey brine created by self-pressing of cheese. Barrels were finally closed using wooden cover and left on for the ripening. The ripening time was between 60 days at 10 to 15°C.

For microbiological analysis 20 g of each sample was taken from the cheese and homogenized with 180 mL sterile 2% (w/v) sodium citrate solution in a sterile conical flask. Decimal dilutions of the homogenates were prepared with sterile 0.85% (w/v) sodium chloride and were plated on media most suitable for isolation of LAB: a) for presumptive lactobacilli, on MRS agar pH 5.7 (Merck GmbH, Darmstadt, Germany) at 30°C and 45°C for 72 h in aerobic conditions, and in anaerobic conditions in anaerobic jars with Anaerocult A (Merck, Germany) for 5 days; b) for presumptive lactococci on M17 agar pH 7.2 (Merck, Germany) at 30°C for 72 h.

Approximately thirty colonies per sample were randomly taken from both MRS (30°C and 45°C) and M17 (30°C) agar plates corresponding to the highest dilution at which growth occurred. The cell morphology of all strains of LAB was determined by microscopy (Zeiss, Germany). After microscopic observations, the colonies were sub-cultured to purity on MRS or M17 medium. Gram-positive and catalase-negative isolates were stored in milk at + 4°C and also frozen at − 20°C in M17 (for cocci) and in MRS (for rods) broth containing 15% of glycerol (v/v). Overall 96 isolates were isolated and after catalase test, Gram staining and microscopy, were chosen for further analyses.

Gram-positive and catalase-negative isolates of LAB were identified to genus level, by tests as follows: colony morphology and pigmentation, growth at 30 and 45°C in MRS and M17 broth, growth at 4.0 and 6.5% (w/w) NaCl in MRS and M17 broth, production of carbon dioxide from glucose by sub-culturing the isolates in tubes with MRS broth Durham's tubes, L-arginine and esculin hydrolysis and citrate-utilization.

For the sequencing of the 697 bp long 16S rRNA region, total DNA from all 96 isolates was used as a template for PCR amplifications with P1V1 (5'-GCGGCGTGCCTAATACATGC-3'') and P4V3 (5'-ATCTACGCATTTCACCGCTAC-3') primers, using AmpliTaq Gold DNA Polymerase with Gold Buffer (Invitrogen, USA). Reactions were carried out in thermocycler Applied Biosystems AB2720. The obtained PCR product was purified by QIAquick PCR Purification Kit (Qiagen, Germany), and sequenced by GATC Biotech, Germany. The sequence was aligned in the NCBI database using the standard nucleotide–nucletide homology search BLAST (http://www.ncbi.nlm.nih.gov /BLAST).

After identification, isolates were submitted for PCR-based detection of genes encoding resistance to tetracycline (tet genes). In the first PCR assay, tet genes encoding tetracycline resistance through ribosomal protection proteins (RPP) were detected using universal primers. If positive for RPP genes, additional PCR test were performed using gene-specific primer for tet(K) and tet(M). The genomic DNA of tested strains was isolated by using MasterPure Complete DNA and RNA Purification kit (Epicentre, USA) and amplification procedure was performed in thermocycler (Applied Biosystems AB2720). The PCR reaction mixture consisted of Amplitaq Gold PCR Master Mix 2x (Invitrogen, USA), 400 nm each of the primers tested and 100 ng of respective DNA. Primer sequences, annealing temperatures and amplicon sizes are listed in Table 1. PCR-based detection of tet genes was performed under the conditions given in Table 2. Amplification products were detected by electrophoresis in 1% agarose gel (Invitrogen, USA), stained with ethidium bromide and as a final step were visualized and documented by using UV transluminator and GelDoc system (Eppendorf, Germany).

Table 1. Primers and PCR conditions for selected antibiotic resistance genes

Resistance gene	Primers	Ta (°C)	Amplicon size (bp)	Sekvenca	Reference (s)
rpp	rpp-f rpp-r	45	1083	5'- GAYACNCCNGGNCAYRTNGAYTT-3' 5'- GCCCARWANGGRTTNGGNGGNACYTCA -3'	Clermont et al. (1997)
tetM	tetM-f tetM-r	55	1513	5'- GAYACNCCNGGNCAYRTNGAYTT -3' 5'- CACCGAGCAGGGATTTCTCCAC -3'	Clermont et al. (1997)
tetK	tetK-f tetK-r	55	384	5'- TTATGGTGGTTGTAGCTAGAAA - 3' 5'- AAAGGGGTTAGAAACTCTTGAAA -3'	Collard J.M. (2000)

Table 2. Program for multiplex PCR assay

Initial denaturation		95°C	5 min
30 cycles		95°C	30 s
		45*/55°C	60 s
		72°C	120 s
Final elongation		72°C	7 min

*45°C annealing temperature for RPP encoding gen

RESULTS AND DISCUSSION

Using 16S rRNA sequencing technique, lactic acid bacteria isolates were identified and 3 most dominant genera were:

- *Lactococcus* (*Lactococcus lactis* subsp. *lactis* and *Lactococcus garvieae*) - 47,9% of isolates,
- *Enterococcus* (*Enterococcus faecalis*, *Enterococcus faecium)* – 36,5% of isolates and
- *Lactobacillus (Lactobacillus plantarum*, *Lactobacillus sakei)* – 13,5% of isolates.

Interestingly, a total of 5 *Lactococcus garvieae* strains known to be implicated in subclinical mastitis in cows were also identified which raised suspicion to plausible tetracycline resistance.

Subsequently, all isolates were screened for presence of gene encoding ribosomal protection proteins (RPP) using conventional PCR. A total of five presumptive tetracycline-resistant strains (*L. plantarum*, 3×*E. faecalis* and *L. garviae*) were further tested on presence of tet(M) and tet(K) genes resulting in four tet(M) positive strains while one strain was neither tet(M) nor tet(K) positive. Further study should be conducted to investigate plausible presence of other tet genes.

CONCLUSIONS

Our findings showed that in artisanal unpasteurized milk products, beside enterococci also lactobacilli could be a host for acquired resistance genes and may spread their resistances through bacterial populations. The problem of antibiotic resistance in human medicine will not be solved if there is a constant inflow of resistance encoding genes into the human microflora via the food chain. With the established genetic mechanisms for exchange of DNA between bacteria, the normal flora is capable of donating drug resistance genes to pathogenic counterpart species. However, the basic scientific knowledge of the mechanisms of antibiotic resistance in LAB and its transmissibility still remain very limited. Because LAB are extensively used for food and feed, including starter cultures for fermentation, probiotic cultures as food and feed ingredients, and protective cultures to inhibit specific spoilage organisms, this knowledge is becoming increasingly important with regard to food safety issues.

ACKNOWLEDGEMENTS

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PASTING PROPERTIES AND FERMENTATION CAPACITY OF PURE OAT FLOUR AND WHEAT-OAT FLOUR MIXTURE

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ABSTRACT

Oat grain (*Avena sativa*) is suitable for human consumption since it contains valuable nutrients such as proteins, unsaturated fatty acids, vitamins, minerals and phytochemicals, as well as soluble fibers, especially β-glucans. Higher lysine content in oat than that in other cereals contributes to better nutritional value of oat grains. Due to high lipid concentration lipolytic enzymes present in oat products could cause hydrolysis followed by oxidation and formation of rancidity. Therefore, in order to prevent the development of bitterness and off-flavours, hydro-thermal inactivation of lipolytic enzymes before milling is necessary.

The purpose of the present work was to evaluate the effect of oat flour addition to wheat flour on the pasting properties of the obtained slurries, as well as the dough fermentation characteristics. Two different fractions of oat flour (particle sizes 250-180 and 180-150 µm), obtained from non-treated and hydro-thermally treated oat grains were selected for the study. Their pasting properties were determined as well as in the mixture with wheat flour of optimal pasting characteristics for baking purposes. The obtained results indicated that utilization of oat pre-treatment had opposite effect on the torque of the flour slurry. Slurry containing 100% pre-treated oat flour demonstrated higher torque in comparison to non-treated oat flour, whereas the oat pre-treatment influenced lower peak torque in the mixture with wheat flour. Regardless of oat pre-treatment, slurries prepared from flour blends containing 30% of oat flour fraction <180 µm expressed higher torque than those containing 30% oat flours of 250-180 µm particle sizes, as a result of higher starch content in the oat flour fraction. The same trend was noticed in the slurries prepared from pure oat flour fractions. In addition to fermentation capacity, measured by rheofermentometer, all tested doughs showed decreased maximum dough height, whereas doughs containing pre-treated oat flour exhibited higher value than that of non-treated oat flour. Doughs prepared from oat flours of 250-180 µm particle sizes demonstrated higher value of total volume of CO_2 produced during fermentation in comparison to dough containing oat flour of 180-150 µm particle sizes.

Keywords: oat flour, pasting, fermentation capacity

INTRODUCTION

Oat grain (*Avena sativa*) is considered to be suitable for human consumption because it is rich in valuable nutrients such as proteins, unsaturated fatty acids, vitamins, minerals and phytochemicals (Head et al., 2010). The most dominant protein fraction in oat grain is salt-soluble globulin, while prolamins are concentrated in a small amount (Flander et al, 2008). Oat globulin fraction contains higher level of lysine and better amino acid composition than other cereal proteins, which are considered to be limited in lysine (Salehifar and Shahedi, 2007). Oat grain is characterized by significant amount of vitamin E and most of the B vitamins, especially thiamin, niacin, biotin and pantotenic acid. Among other cereals oat is characterized with the highest amount of lipids (6-11%); where 80% of oat lipids are unsaturated. Due to high percentage of oleic and linoleic acid, favorable ratio (2:2) of polyunsaturated to saturated fatty acid is achieved. Naturally present antioxidants in oat, such as tocopherols and phenolic components, contribute to the stability of intact, undamaged oat grain (McEwan et al., 2005; Salehifar and Shahedi, 2007). However, during storage of oat products lipolytic enzymes which are naturally present in the grain could cause lipid degradation starting with hydrolysis and followed by oxidation. As a result of the lipid deterioration, rancidification occurs which shortens period of storage and renders oat

products unusable. Therefore, in order to prevent the development of bitterness and off-flavours, hydro-thermal inactivation of lipolytic enzymes before milling is required (Ekstrand et al., 1993; Lehtinenet al., 2003).

Oat seed, in addition to its nutritional value, has demonstrated positive health benefits which rely mainly on the total dietary fiber, such as cellulose, arabinoxylans and especially, mixed-linked (1-3),(1-4)-β-D-glucans (Drzikova et al., 2005). Potential benefits of β-glucan include lowering of serum blood cholesterol, glucose metabolism regulation and improving digestion diseases. Also, it is considered that desirable carbohydrates present in high amount in oat grain are related to reduction in the risk of different types of cancers (Salehifar and Shahedi, 2007; Hüttner et al., 2010). Moreover, oat could be suitable for consumption by most people suffering from celiac disease, as well as non-celiac gluten sensitivity, which have gained growing attention in recent years (Volta et al., 2013). There is increasing interest in implementing alternative cereals in a human diet in terms of better nutritional aspect and health demands (Mariotti et al., 2006; Peymanpour et al., 2012). Products made of oat, such as bread, are considered to have mild, nutty, pleasant aroma and better nutritional quality. Excellent moisture retention properties of oat also contribute to longer freshness of the products (Flander et al., 2007).

The aim of the present paper was to evaluate the pasting properties and fermentation capacity of pure oat flour (raw and hydro-thermally treated) and wheat-oat flour mixture in order to perceive its potential to be used as raw material in bread baking.

MATERIAL AND METHODS

Materials
Dehulled oat grains and wheat flour were purchased from a local market. Wheat flour was chosen on the basis on its optimal pasting characteristics for bread baking purposes. Samples of oat grains, both non-treated and hydro-thermally pretreated were milled using a hammer mill (ABC Engineering, Serbia) and sieved (Bühler sieving machine) to obtain the samples with two fractions (250-180 and <180μm particle sizes). The moisture, ash, protein, and starch content of the fractions were determined according to standard ICC methods (110/1, 104/1, 105/1 and 123/1, respectively). Fat and sugar content, as well as acidity were determined according to the approved methods of AOAC (945.16, 939.03 and 939.05, respectively).

Pasting characteristics
Pasting properties of slurries prepared from pure oat flour fractions, as well as from wheat-oat mixtures (wheat flour : oat flour = 70:30) were measured using the HAAKE MARS rheometer (Thermo Scientific, Germany) according to Pojić et al. (2013).

Fermentation properties
The Rheofermentometer F3 (Chopin, Villeneuve-La-GarenneCedex, France) was used to study the dough development and gas release of dough made of oat flour fractions. 300 g of flour, 2,5% yeast and 2% salt were mixed with water in Brabender Farinograph mixing bowl to obtain dough of consistency of 500 BU plus 1 minute after reaching the dough consistency. After mixing, 315 g of dough was placed in a fermentation basket of the rheofermentometer with a 2000-g cylindrical weight. The cover of the vat was fitted with an optical sensor and the test was run for 3 h at 30 °C.

The recorded parameters were: Hm-maximum dough height (mm); T1 - the time at which dough attains the maximum height (h); V_{CO_2}- total volume of CO_2 (ml) produced during 3 h of fermentation and the gas retention coefficient (%) (the measure of the proportion of CO_2 retained in the dough).

Statistical analysis

All determinations were made in duplicate, and the average results with the standard deviations are presented. Analysis of variance and Tukey's multiple range tests were performed using Statistica 10.0 software (Statsoft, Tulsa, OK) at 5% significance level.

RESULTS AND DISCCUSION

Chemical compositions of four oat flour fractions used in this study are presented in Table 1. The obtained results indicated that oat flour fractions with <180 µm particle sizes were characterized with higher starch content due to presence of internal parts of endosperm rich in starch. Oat flour fractions with 250-180 µm particle sizes were characterized with higher ash, fat and sugar content compared to that of particle sizes <180 µm as a result of the presence of the outer parts of the grain obtained by milling process. It is noticeable that acidity greatly decreased in oat flour fractions which were hydro-thermally pre-treated, regardless oat flour particle sizes.

Table 1. Chemical characterization of oat flour fraction

Parameters	Non-treated fractions (µm)		Pre-treated fractions (µm)	
	250-180	<180	250-180	<180
Moisture (%)	7.8	7.8	7.7	7.5
Ash (% d.m.)	1.0	0.8	1.0	0.9
Protein (% d.m.)	13.4	12.1	14.4	12.1
Starch (% d.m.)	66.6	71.8	63.4	72.4
Fat (% d.m.)	5.2	4.4	7.2	6.2
Sugar (% d.m.)	1.2	0.7	2.3	0.9
Acidity	12.4	11.7	3.8	4.1

All investigated oat containing samples, in terms of pasting properties, expressed higher maximum torque compared to control wheat flour sample (Table 2). The increase in the torque could be result of lower amylase activity in oat flours, thus improving pasting characteristics of the investigated slurries. The utilization of hydro-thermal pre-treatment affected the increase of maximum torque of slurries containing pure oat flour. However, the opposite effect was observed with the hydro-thermally treated wheat-oat mixtures. Regardless of application of pre-treatment, slurries prepared from flour blends containing finer fraction of oat flour (<180 µm) exhibited higher maximum torque value than those of coarser oat flour particles (250-180 µm). The same trend was noticed for the slurries prepared from pure oat flour fractions. This increase in maximum torque value could be explained by higher starch content of oat flour fractions <180 µm than those of particles between 250-180 µm. Moreover, the pre-treatment could influence starch pre-gelatinization and thus increase of maximum torque of the investigated slurries.

The results describing fermentation properties of analyzed dough samples are summarized in Table 3. In general, all oat containing dough samples demonstrated decrease of maximum dough height (Hm) compared to control wheat dough. Moreover, dough samples containing hydro-thermally pre-treated oat flour expressed higher values of Hm than those of non-treated oat flours. This decrease could result in a bakery product of lower volumes in comparison to product made from pure wheat flour (Abd El Hady et. al., 1999).

Table 2. Pasting properties of investigated slurries

Samples	Particle sizes (µm)	Pre-treatmen	Torque M (µNm)
Control	-	-	6587±14.14[a]
Oat flour	<180	N	16915±21.21[f]
Oat flour	<180	Y	22600±141.4[g]
Oat flour	250-180	N	14440±141.4[e]
Oat flour	250-180	Y	22545±205.1[g]
Mixture	<180	N	13455±120.2[d]
Mixture	<180	Y	10900±±127.3[b]
Mixture	250-180	N	12385±106.1[c]
Mixture	250-180	Y	10685±148.5[b]

N - Hydro-thermal pre-treatment is not employed, Y-Hydro-thermal pre-treatment is employed
Values are the mean±standard deviation. Values followed by the same letter in the column are not significantly different (p>0.05)

It is also evident that this decrease is less pronounced for dough containing thermally treated oat flours than for non-treated ones regardless of flour particle size fraction. Dough containing non-treated oat flours demonstrated decrease of T1 values, while time needed to achieve maximum dough height was approximately at the same level for thermally treated oat flour doughs as for the control dough. Hence, dough prepared with non-thermally treated oat flours should have shorter fermentation time.

Table 3.Fermentation properties of investigated doughs

Samples	Dough development curve		Gaseous release curve	
	Hm (mm)	T1 (h)	Vco$_2$ (ml)	Gas retention coefficient (%)
Wheat flour	46,4	2,18	1449	54,1
Mixture of wheat flour and oat flour <180 µm	32,4	1,28	1207	56,4
Mixture of wheat flour and oat flour 250-150 µm	33	1,18	1261	54,3
Mixture of wheat flour and pre-treated oat flour <180 µm	35,8	2,30	1235	53,8
Mixture of wheat flour and pre-treated oat flour 250-150 µm	35,5	2,16	1287	53,5

Total volume of CO_2 produced during fermentation was higher in dough prepared from pre-treated oat flour in comparison to the non-treated, probably due to dextrinisation of starch during hydro-thermal treatment. Moreover, it is evident that higher sugar content was determined in pre-treated flour samples than in non-treated (Table 1). It is also evident that coarser fraction of oat flour (250-180 µm) had higher total sugar content in comparison to that of finer fraction (<180 µm) which was consequently reflected in higher total volume of CO_2 produced during fermentation and increase in maximum dough height (Table 3). However, gas retention coefficients did not followed the gas production process being lower for dough containing pre-treated oat flour. This observation could be related to the influence of heat treatment on oat protein complex making it more rigid and less extensible.

CONCLUSIONS

According to obtained results it could be concluded that slurries prepared from pure oat flour, as well as from oat-wheat flour mixtures demonstrated higher maximum torque values compared to wheat flour samples. The utilization of oat pre-treatment influenced the decrease in the values of maximum torque during the pasting measurements in slurries of oat-flour mixtures in comparison to that containing non-treated oat – wheat flour mixtures. Concerning fermentation properties, dough containing pre-treated oat flours exhibited higher maximum development reached by the dough than that of non-treated oat flour. Moreover, dough samples containing coarser oat flour fractions (250-180 µm) expressed higher value of total volume of CO_2 produced during fermentation in comparison to dough containing finer oat flour fractions (<180 µm) regardless of pre-treatment. Therefore, oat flour could be used in the special cases of wheat flour processing; e.g. processing of wheat flour with high amylase activity taking into account other dough quality parameters affected by oat flour addition. The application of oat flour pretreatment could be chosen on the basis of expected final goal: higher maximum torque or shorter fermentation time.

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WORLD WIDE SPREAD OF *SALMONELLA ENTERICA* SEROTYPES, HARBORING DIFFERENT MECHANISMS OF RESISTANCE

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ABSTRACT

Salmonellae are one of the most common foodborne pathogens. Intensive trades, traveling and therapeutic use of antibiotics present significant risks for worldwide dissemination of multiple resistant *Salmonella*. In this work we briefly describe some biological and genetic features of clonally distributed *S*. Typhimurium, *S*. Swarcengrund, *S*. Kentucky and *S*. Infantis. They have acquired various mechanisms of resistance and developed important genetic traits that enable them to survive in the environment for years. *S*. Typhimuirum var. Copenhagen, *S*. Typhimuirum DT12/193 and S. Kentucky, develop three to four point mutations in topoisomerase genes. All these isolates have clinical resistance to fluoroqinolones (MICs from 4-32 mg/L). *S*. Enteritidis and S. Infantis resistant to nalidixic acid and teteracycline are the most prevalent serotypes in Serbia. The resistance to quinolones in existing clones of *S*. Infantis was attributed to single point mutation in *gyrA* gene (Ser83→Tyr). In several strains increase of MIC to CIP (2μg/ml) was noted and second point mutation in *parC* gene was found (Ser80→Arg) (Velhner et al., 2014). The prudent use of antimicrobial agents is the most important way to prevent resistance development in bacteria.
Keywords: *Salmonella, resistance, fluoroquinolones, clonal spread*

INTRODUCTION

Detection of salmonella in clinical specimens and food samples is an important task for microbiologists and epidemiologists. Applying molecular typing methods, resistotyping and detection of resistance genes enables sourcing the origin of infection as well as possible spreading means during salmonella infection. Therefore, active monitoring systems are helpful in characterizing outbreaks caused by salmonella. Additional to genes for invasiveness, salmonella may harbor dangerous resistance genes and, as a consequence, the therapeutic options in both humans and animals are often limited. Chromosomally encoded or plasmid mediated resistance mechanisms and efflux pump system prevalently contribute to resistance to antimicrobial agents in salmonellae (Giraud et al., 2006, Velhner and Stojanović, 2012).

Animal source foods are the main origin of human infection with salmonella. Homemade meals are the most frequently implicated in small outbreaks in Serbia, as reported by Petrović et al., (2005). This has been related to a long lasting practice of preparing homemade cakes, mayonnaise and other foods which require usage of row eggs. Restaurant outbreaks have become less common in Serbia because of improvements made in safe food handling. It was experimentally documented that domestic kitchen presents important place of cross contamination when poultry meat was used to prepare meals. *Salmonella* spp. was isolated from dishcloth and counter top, inferring possibilities of cross contamination in domestic households along with restaurants and other public places for food preparation (Gorman et al., 2002). In this work we have described worldwide spread of serotypes *S*. Typhimuirum, *S*. Swarcengrund, *S*. Kentucky and *S*. Infantis and the resistance genes they have acquired during the years.

Clonal spread of Salmonella Typhimuirum STDT 104 and STDT204

Multiple-resistant *Samonella* Typhimuirum DT104 (STDT104) was firstly reported from cattle in England and Wales in early 1990 and presently it spreads around the globe, causing infections in humans and farm animals (Threlfall, 2000). This salmonella has emerged as a multiple resistant clone harboring resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (ACSSuT, R type). Salmonella genomic island 1 (SGI1) has been found in STDT104 and is composed of integron InC and InD containing resistance genes. This region is bounded by 25 bp inverted repeats and flanked by direct 5 bp duplications. Such genetic construction implicates that SGI1 was inserted by transpositional mechanism (Carattoli et al., 2002).

Additional resistance to quinolones and/or trimethoprim was detected in the multiresistant STDT104 in 1992 (Threlfall, 2000).The major mechanism of resistance to quinolones has been attributed to target mutations of chromosomal DNA, mainly in the *gyrA* gene with the Asp87→Asn substitution as the most frequent non-silent mutation. Because of the extensive use of enrofloxacin (a fluoroquinolone antibiotic) in farm animals, the STDT104 with decreased susceptibility to CIP was found most frequently in turkeys, chickens and cattle. This resistotype was also isolated from 11 patients who were hospitalized in Denmark and who did not respond to therapy with ciprofloxacin. Evidently, the STDT104 represents the multiple resistant clone that has spread internationally and causes epidemics in many countries (Threlfall, 2000).

In the year 1990, clonal spread of *Salmonella* Tyhpimurium var. Copenhagen (definite phage type 204) resistant to fluoroquinolones was reported by Heisig et al. (1995). The isolates were sampled from cattle and sporadically from humans in Germany. Some years later Guerra et al., (2003) had found that S. Typhimuirum var. Copenhagen harbors four point mutations distributed in *gyrA* (Ser83→Ala, Asp87→Asn), *gyrB* Ser464→Phe and *parC* gene (Ser80→Ile), with 32µg/mL of MIC value to CIP. Because of the occurrence of STDT204 highly resistant to fluoroquinolones, the urgent measures in reducing selective pressure by antibiotics were initiated.

Elevated MIC to NAL ($\geq$ 32 mg/L) in STDT but also in ST var. Copenhagen is frequently being detected from slaughterhouse and diagnostic isolates from turkeys and it has been found also in cattle, pigs, poultry, cats, dogs and horses in the USA. These serovars carry class 1 integrons and harbor different resistance types depending on a animal species and samples tested (diagnostic or slaughter) (review article by Zhao et al., 2005). *S.* Typhimuirum var. Copenhagen had also caused a large outbreak in a nursing school in Finland due to the consumption of contaminated lettuce imported from Spain. These isolates have been characterized by similar pulsotype and resistotype, implicating that the infection commenced from the same source (Takkinen et al. 2005).

In *Salmonella* Typhimurium DT204, the AcrAB-TolC efflux pump plays very important role in resistance to fluoroqinolones. It was elucidated that if AcrAB efflux pump is inactive the other pump AcrEF is overexpressed. The insertion sequence IS*1*or IS*10* with the promoter region leads to overexpression of the AcrEF genes and therefore activates the alternative efflux system. In summary, resistance to fluoroquinolones is being developed due to several unrelated mechanisms of resistance in STDT204 (Giraud et al., 2006).

First report of CIP[r] S. Swarcengrund in the USA and its intercontinental spread

First report on fluoroqinolone resistant *Salmonella* Schwarzengrund in the USA was published in 2001 by Olsen and coworkers (Olsen et al., 2001). It was isolated from several residents in the nursing homes (termed nursing home A and B) in Oregon. Oregon Health Department and Center for Disease Control and Prevention had conducted a comprehensive survey to establish the source of infection and spread means in two counties in the State of Oregon. It was evident that *S.* Schwarzengrund was "imported" to USA by a patient who had traveled and been hospitalized in Philippines in 1995. Upon his return to USA the patient was admitted to the hospital in New York and *S.* Schwarzengrund was isolated from the insertion site of the urinary catheter. This patient was resident in the nursing home A and he apparently had transmitted infection to other residents. Epidemiologists have found that

infection with CIP[r] *S*. Schwarzengrund was also disseminated to a hospital X and to other nursing home. Therefore, *S*. Schwarzengrund CIP[r] was most likely transferred from Philippines to USA and during several years caused nosocomial outbreaks in one hospital and two nursing homes in Oregon.

The PFGE profiles of these resistotypes were similar and distinctive point mutations on topoisomerse genes were found (Ser83→Phe and Asp87→Gly). All isolates were resistant to ampicillin (AMP), ciprofloxacin (CIP) and trimethoprim-sulfamethoxazole (STX) and in some strains resistance had been attributed to other classes of antibiotics as well. It has been postulated that easy accesses to antimicrobial drugs in developing countries contributes in occurrence and spread of CIP[r] clones of the Far East countries (Cui et al., 2008).

Clonal spread of *S*. Infantis harboring resistance to quinolones

S. Infantis may have also become significant contaminant in the environment and potentially harbors different mechanisms of resistance. Clonal spread of *Salmonella* Infantis resistant to quinolones was reported from Japan (Shahada et al., 2006), Hungary (Nógrády et al 2007), Israel (Gal More et al., 2010), Germany (Hauser et al., 2012) and Serbia (Velhner et al., 2014). As postulated by Hauser et al. (2012), *S*. Infantis did not accumulate various genetic rearrangements over time and a clonal strain with probable recent ancestor circulates among broiler chickens. *S*. Infantis is the most prevalent serotype in Japan while the most frequent resistotype is attributed to streptomycin (STR), sulphamethoxazole and TET. However, increasing number of isolates resistant to ofloxacin is being reported (Shahada et al., 2006).

The most frequently detected resistotype in Hungary was NAL, STR, sulphonamides (SSS) and TET resistant. The PFGE genotyping revealed B2 as the most common pulsotype. Two multiple resistant isolates of *S*. Infantis from Hungary harbored resistance to ciprofloxacin as well (Nógrády et al., 2007). Similar types of multiple resistant S. Infantis were also identified in Germany (Hauser et al., 2012).

Research from Israel provided the evidence that the "old" isolates of *S*. Infantis had different genetic profile comparing to more recent strains (Gal More et al., 2010). The most common resistance pattern in their strain collection was to nitrofurantoin, NAL and TET. Clonal dissemination of *S*. Infantis in Germany is reported by Hauser et al., (2012). Some of the registered clones were multiresistant, showing fifteen different resistance profiles. Multiresistant clones most frequently originate from broilers or broiler meat. *S*. Infantis sampled in Serbia has unique NAL-TET resistant resistotype. It has been regularly detected in broilers but it is also found recently in layer chickens (unpublished data). It is the third most frequent serotype registered in humans in Serbia. The clonal spread was determined on the basis of resistotype and pulsotype along with the point mutations in the *gyrA* gene. It is interesting to note that in strains with increased MIC to CIP (2 µg/mL), additional to mutation in *gyrA* at Ser83→Tyr that have occurred in all SI isolates representing the collection, second point mutation in *parC* (Ser80→Arg) was found as well (Velhner et al., 2104).

World wide spread of Salmonella Kentucky

Multiple resistant *Salmonella* Kentucky harboring resistance to fluoroquinolones has caused attention since its frequent finding in travelers returning from African continent in France (Weill et al., 2005). In the following years it was evident that *S*. Kentucky has internationally spread and that most of the strains confer the same multi locus sequence type 198 (MLST 198) and pulsotype cluster X1 (Le Hello et al., 2011). Resistant *S*. Kentucky harbors genetically different SGI1 backbones carrying different gene cassettes. Several SGI1 variants have been described and they have potential of horizontal gene transfer (Le Hello et al., 2012). The oldest clone ST198-X1, isolated from West Africa in 1961, harbors resistance to amoxicillin, gentamicin, sulfamethoxazole, spectinomycin, streptomycin and tetracycline. Some year's later integration of SGI1-K was recognized and Ser83→Phe mutation in the *gyrA* gene was detected (Table 1). High resistance to fluoroquinolones since 2004 onwards is attributed to second point mutation in the *gyrA* gene (Asp87→Asn, Gly or Tyr transition) (Le Hello et al., 2011). Since 2009, S. Kentucky ST198–X1 has developed resistance to extended spectrum β lactamase, plasmid encoded cephalosporinase or carbapenemase.

Some isolates also harbor resistance to aminoglycosides, trimethoprim-sulphamethoxasole and azithromycin. There is urgency for screening of genes encoding carbapenemase production due to its possible occurrence in livestock industry worldwide (Le Hello et al., 2013a).

Table 1. Mutational polymorphism in Salmonellae resistant to quinolones and fluoroquinolones

Salmonella serotype	Origin/ref*	Transition on *gyrA*	Transition on *gyrB*	Transition on *parC*	Transition on *parE*	MIC to CIP µg/mL
S. Typhimuirum DT12/193	Japan/17	Ser83→ Phe, Asp87→ Asn	Not found	Ser80→ Arg	Not found	≥24
S. Typhimuirum var. Copenhagen (PT 204)	Germany/5	Ser83→ Ala, Asp87→ Asn	Ser464→ Phe	Ser80→ Ile	Not found	32
S. Schwarzengrund	USA/15	Ser83→ Phe Asp87→ Gly	Not found	Not found	Not found	>4, >32
S. Infantis	Serbia/20	Ser83→ Tyr	Not found	Ser80→ Arg	Not found	2
S. Kentucky	France/10, 12,13	Ser83→ Phe Asp87→ Asn, Tyr,Gly	Not found	Ser80→ Ile	Not found	4-16

*ref/source of information

CONCLUSION

It is evident that in particular four serotypes of salmonella (*S.* Typhimuirum, *S.* Swarcengrund, *S.* Infantis and *S.* Kentucky) have spread around the world, harboring different mechanisms of antimicrobial resistance. Analysis and comparison of earlier and more recent strains clearly demonstrate that these serotypes have acquired various resistance patterns over the years. It is evident that the main origin of infection in humans is animal source foods. It is impossible to prevent *Salmonella* dissemination due to different management practice on farms, intensive trade as well as travelers, who once infected with exotic *Salmonella* may "import" new pathotypes to their home countries. Salmonellae originated from foreign countries have been only periodically reported in Serbia but it is necessary to minimize the occurrence of the local spread of clonal *S.* Infantis resistant to NAL and TET and *S.* Enteritidis in Serbia (Kozoderović et al., 2011, Velhner et al., 2014).

ACKNOWLEDGMENT

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THE INFLUENCE OF SEX ON SLAUGHTER CHARACTERISTICS AND MEASURES OF CARCASS IN GOAT KIDS OF DOMESTIC BALKAN BREED

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ABSTRACT

The paper presents the investigation of slaugher results, i.e. meat production results, and establishs relationships of traits of individual measures on the carcass and the halves in 96 goat kids of the domestic Balkan breed (with a sex ratio of 50:50).
Goat kids were slaughtered at the age of 90 days. Subsequent to slaughtering and primary carcass treatment, weight of warm carcass with head and offals was registered. After cooling period of 24 h, at the temperature of 0 to + 4°C, weight of cold carcass with head and offals was determined, as well as without head and offals. Following linear carcass measures (length and width of the carcass) were taken after cooling: Calcaneum – Ischium (pin bone), tail head – neck, Articulation genus – Articulation humeri, tail head – Atlas, width of both legs/thighs, breast width, scapula width and breast depth. After cutting of the carcasses into halves, the following measures were taken on the left half: Pubis – Talus ankle/joint, Pubis – I rib, Pubis – Atlas, Pubis – Articulatio genus.
Statistical analysis was performed using appropriate software procedures (Proc Means, Proc Corr, Proc REG) in the statistical software package SAS (SAS 9.1.3, 2007).
Higher values of dressing percentage/meat yield were determined for male goat kids, as well as linear measures on carcass and halves, compared to female goat kids. However, statistically significant differences at the level of $p<0.05$ were determined in mass of cold and warm carcass with head and offals, as well as without offals, whereas statistically significant differences at the level of $p<0.01$ were determined for certain values of carcass linear measures (breast width and scapula width) and measured lengths on carcass halves (Pubis – Talus ankle/joint).
Keywords: *goat kids, domestic Balkan breed, dressing percentage, slaughter results*

INTRODUCTION

Production of goat meat in the world, although it is four times smaller than the meat of sheep, is of great importance for many countries, especially in Asia, Africa and South America (Dubeuf *et al.,* 2004). In EU countries the production of goat meat is of much less importance and scope, especially in countries where dairy goat breeds, in which the meat is a by-product, are grown. It is estimated that the goat meat in Europe is about one tenth of the total quantity of sheep meat. Greece, Spain, Italy and France are the largest manufacturers of these types of meat products because they produce two thirds of the total quantity of goat meat in Europe (Webb *et al.,* 2005).
Goat meat is widely consumed in the developing countries. According to FAOSTAT (2008), total meat inventory is about 280 million MT. Goat meat represents only 2% of this total. The total amount of goat meat produced in 2008 was 4.9 million MT. The developing countries produce approximately 97% of this amount, reflecting the great importance of goat meat to feed millions of people in these countries. The top ten countries producing goat meat are all from Asia and Africa. China leads the world in producing goat meat, accounting for 38% of the world total goat meat produced. Goat meat production has been increasing from 2.65 million MT in 1990 to 4.93 million MT in 2008.
Due to the long-standing prohibition of keeping goats, which was in force from 1954. (unique law in the world that has been adopted in the former Yugoslavia), on the territory of the Republic of Serbia has not been paid special attention to the production of goat meat, nor to statistical data on the number of goats. Although a goat milk is the primary product on the

territory of Serbia , meat production should not be ignored. High biological potential of goats for good fertility should be properly utilized (Memisi *et al.,* 2001). Goat is known as the most prolific ruminant, which many breeders use to increase the caprine meat production by forcing goat fertility and creating a race that in a year give average 2-3 young goats. This ability of goats can be used very well due to sequential yeaning, and this is where the caprine meat production is more profitable than milk production and processing, (Memiši and Bauman, 2002 and 2007; Memiši *et al.,* 2004)**.**

Goat meat has about the same nutritional value and digestibility as sheep meat (to put it more exact: more protein and less fat compared to sheep meat). It is still less appreciated because of specific smell and flavor, especially if the animal is older. On the basis of chemical composition, goat meat, in terms of nutritional and biological value, is not inferior to other types of meat.

MATERIAL AND METHODS

Research was carried out in herds of Balkan goats kept on individual farms. In 96 kids of Domestic Balkan breed (gender ratio 50:50), the production of meat was studied in a semi-intensive rearing system.

Goat kids were slaughtered at the age of 90 days. Subsequent to slaughtering and primary carcass treatment, weight of warm carcass with head and offals was registered. After cooling period of 24 h at the temperature of 0 to + 4 ^{o}C, weight of cold carcass with head and offals was determined, as well as without head and offals.

Linear carcass measures

Following linear carcass measures (length and width of the carcass) were taken after cooling: Calcaneum – Ischium (pin bone), tail head – neck, Articulation genus – Articulation humeri, tail head – Atlas, Width of both legs/thighs, Breast width, Scapula width and Breast depth. After cutting of the carcasses into halves, the following measures were taken on the left half: Pubis – Talus ankle/joint, Pubis – I rib, Pubis – Atlas, Pubis – Articulatio genus.

Statistical analysis

Statistical analysis of data was done by application of several program procedures (ProcMEANS, Proc CORR, Proc REG) of the statistical program package SAS (SAS 9.1.3, 2007).In the regression analysis, independent variable value was pre-slaughter body weight of goat kids, whereas slaughter and carcass traits were dependent variables.

RESULTS AND DISCUSSION

Descriptive statistical indicators of slaughter traits and carcass measurements of Balkan breed goat kids are presented in Table 1. Higher values of dressing percentage/meat yield were established in male goat kids, as well as linear measures on carcass and carcass halves, compared to female goat kids. However, statistically significant differences at the level of p<0.05 were determined in mass of cold and warm carcass with head and offals, as well as mass of cold carcass without offals, whereas statistically significant differences at the level of p<0.01 were determined for certain values of carcass linear measures (breast width and scapula width) and measured lengths on carcass halves (Pubis – Talus ankle/joint).

Data obtained on slaughter traits of Domestic Balkan goat can be compared to results obtained by other authors, but there are certain discrepancies considering the diversity in breeds, body masses and ages of goat kids included in trials. Similar values for dressing percentage of the warm carcass in two groups of goat kids of Serbian White breed, slaughtered at the age of 70 days, are reported by Žujović *et al.* (2008). In lighter goat kids of average pre-slaughter body mass of 12.654 kg (from 8.8 to 15.0 kg) and medium heavy kids of average body mass of 17.61 kg (from 15.1 to 20.0 kg), established the dressing percentage of 57.21% and 55.01%, respectively. In this study higher values of dressing

percentage/meat yield and of certain carcass linear measures were determined in male kids compared to females, which is in complete agreement with results obtained by other authors (Peña *et al.,* 2007; Domingo *et al.*, 2008). In addition, expression of observed traits greatly depends on goat genotype as well as management of animal nutrition and care.

Table 1. Descriptive statistical indicators of slaughter traits and carcass measurements of Balkan breed kids

	Sex		t-value	df	p
	Male	Female			
Pre-slaughter body weight (kg)	13.51	13.12	1.82	94	0.0723
Weight of warm carcass with head (kg)	7.86	7.62	2.37	94	0.0197*
DP of warm carcass with head and offals (%)	58.26	58.13	0.50	94	0.6148
Weight of cold carcass with head and offal (kg)	7.53	7.29	2.40	94	0.0183*
DP of cold carcass with head and offals (%)	55.83	55.66	0.69	94	0.4923
Weight of cold carcass without offals (kg)	6.10	5.92	2.43	94	0.0169*
DP of cold carcass without offals (%)	45.24	45.15	0.32	94	0.7482
Calcaneum – Ischium (pin bone) (cm)	24.63	24.14	1.90	94	0.0608
Tail head – neck (cm)	48.15	47.51	1.84	94	0.0690
Articulatio genus – Articulatio humeri (cm)	58.21	57.62	1.95	94	0.0537
Tail head – Atlas (cm)	65.40	64.60	2.02	94	0.0458*
Width of both legs/thighs (cm)	12.56	12.20	2.41	94	0.0180*
Breast width (cm)	12.67	12.28	2.73	94	0.0077**
Scapula width (cm)	12.69	12.35	2.70	94	0.0081**
Breast depth (cm)	21.75	21.32	1.60	94	0.1130
Pubis – Talus ankle/joint (cm)	28.09	27.48	2.63	94	0.0099**
Pubis – I rib (cm)	53.31	52.73	2.26	94	0.0259*
Pubis – Atlas (cm)	64.28	63.68	2.04	94	0.0441*
Pubis – Articulatio genus (cm)	16.65	16.29	1.81	94	0.0735

p-level: * P<0.05, ** P<0.01; DP- dressing percentage

Obtained results for mass of warm and cold carcass with and without head and offals, as well as value of dressing percentage of warm carcass of Balkan goat, are also similar to those established by Becerril-Herrera *et al.* (2006), in kids of Mexican Creole goat breed (59.27%). Marichal *et al.* (2003), reported similar values of dressing percentage in kids reared on Canary islands. Results obtained for certain slaughter traits (mass of warm carcass and dressing percentage/meat yield) established in kids of Domestic Balkan goat breed, were at the level of those presented by Ekiz *et al.* (2010) for kids of Turkish Saanen and Maltese breeds of average pre-slaughter weight of 13.31 and 14.48 kg with mass of warm carcass of 7.22 and 7.68kg and dressing percentage of 54.26 and 53.01 %.

CONCLUSIONS

The paper presents the investigation of slaugher results, i.e. meat production results, and establishs relationships of traits of individual measures on the carcass and the halves in 96 goat kids of the domestic Balkan breed (with a sex ratio of 50:50).

Higher values of dressing percentage/meat yield were determined for male goat kids, as well as linear measures on carcass and halves, compared to female goat kids. However, statistically significant differences at the level of $p<0.05$ were determined in mass of cold and warm carcass with head and offals, as well as without offals, whereas statistically significant differences at the level of $p<0.01$ were determined for certain values of carcass linear measures (breast width and scapula width) and measured lengths on carcass sides (Pubis – Talus ankle/joint).

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HOUSE FLORA IN PROCESSING UNITS DURING PRODUCTION PROCESS OF *PETROVSKÁ KLOBÁSA*

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ABSTRACT

Production of traditional dry-fermented sausages relies on natural contamination by environmental flora. This microbiota is usually referred to as "house flora". This contamination occurs during slaughtering and increases during manufacturing. This paper reviews the diversity of microbiota in small-scale processing units, during production process of the traditional fermented dry sausages - *Petrovská klobása*. Test samples were collected in two village households in Bački Petrovac, where the preparation of *Petrovská klobása* samples was performed in a traditional manner. Examination included total of 43 samples. Generally, before stuffing, *Listeria monocytogenes* and *Staphylococcus aureus* were detected in 6.97 and 9.30% of the samples, respectively, while *Escherichia coli* was enumerated in 18.60%. Sausage samples at the end of the storage period (270. day) were safe with presence of bacteria populations from the working environment, such as: aerobic bacteria, *Micrococcaceae*, Lactic acid bacteria and *Enterococcus spp*. Examination of the hygienic status of the processing environment, equipment, raw materials and final product provides an overview of growth trends and the disappearance of bacterial populations.

Keywords: *Petrovská klobása, house flora, processing environment, growth trends*

INTRODUCTION

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 in typical and regional food. *Petrovská klobása*, traditional and autochthonous dry – cured sausage, presents a part of gastronomic heritage of Slovaks in Vojvodina. Nowadays, they are producing it in traditional way according to the original recipe of their ancestors, without the use of nitrate/nitrite, glucono delta-lactone (GDL) and microbial starters. In rural households, in the Municipality of Bački Petrovac, this sausage is made by the end of November and during December. *Petrovská klobása* is made by mixing partly cooled (cca 4 h p.m) or cold (cca 24 h p.m) medium chopped lean pork and fat (up to 10 mm) with addition of powdered red hot spicy paprika, salt, crushed garlic, caraway and sugar. A well-mixed filling, which is prepared within 15-30 minutes by using a unique technique of manual mixing with kneading and overturning, is stuffed into natural casings consisting of the rear part of pig intestines (colon), forming units 35-45 cm long and 4.5-5.0 cm in diameter. After stuffing, the sausages are left to drain for a while and then they are smoked by a cold process for about 10-15 days with pauses, using specific kinds of wood (cherry wood in particular). When a smoking process is finished, the sausage is kept in a dry and well ventilated place to dry and ripen, until it achieves an optimum quality, which takes about four months (Tasić, 2012; Janković, et al., 2013; Šojić et al., 2014). *Petrovská klobása* is a product with a protected designation of its geographical origin, under number 44, based on the order issued by the Republic Bureau for Intellectual Property, number 9652/06 G-03/06, on 21/05/2007. In order to achieve a recognizable product of standardized supreme quality which will be continually produced in the controlled conditions, the aim of this study was to determine the parameters of typical house flora during the production process of *Petrovská*

klobása, which is crucial because of the safety (pathogenic flora), acceptability (spoilage flora) and sensorial quality (technological flora) (Leroy et al., 2010).

MATERIALS AND METHODS

Samples
Test samples were collected from two village households (A and B) at Bački Petrovac, where the preparation of *Petrovská klobása* samples was performed in a traditional manner. Testing included examination of 43 samples, such as: swabs - workers' hands (n=11), working surfaces (n=1), equipment before beginning the operation (n=9), equipment after the operation (n=9), and other swabs from the working area - wall, drain etc. (n=7) and samples of fillings (n=2) and sausages at the end of the storage period (n=4). All samples were kept refrigerated and analyzed within 2 h.

Microbiological tests
Each sample was tested on the presence of the following bacteria: (1) total viable count (SRPS ISO 4833-1), Plate Count Agar -PCA, Oxoid incubated at 30°C for 72h; (2) total count of bacteria of the *Micrococaceae* family, Manitol salt phenol - red agar, Oxoid, incubated at 30°C for 72 h; (3) total count of *Enterobacteriaceae* (SRPS ISO 21528-2), Violet Red Bile agar with glucose - VRBG, Oxoid, incubated at 30°C for 72 h; (4) total count of β - glucuronidase positive *E. coli* (SRPS ISO 16649-2), Tripton Bile x Glucuronide agar (TBX), Oxoid, incubated at 44°C for 24 h; (5) *Enterococcus* spp. on Bile esculin azide agar, Biokar diagnostics, incubated at 37°C for 48 h; (6) total count of coagulase positive staphylococci (SRPS ISO 6888 - 1), Baird Parker, Oxoid, incubated at 37°C for 24 h; (7) *Pseudomonas* spp., on Pseudomonas Selective Agar - Cetrimide Agar, Merck, incubated at 35°C for 48 h; (8) total count of sulphate-reducing bacteria which grow in anaerobic conditions (SRPS ISO 15213), Iron Sulfite Agar, Oxoid, incubated at 37°C for 48 h; (9) total count of *Clostridium perfringens* (SRPS ISO 7937), Sulfite cycloserine Agar, Oxoid, incubated at 37°C for 20 h; (10) *Salmonella* spp. (SRPS ISO 6579), on modified Rappaport Vasilidis Soft Agar incubated at 42°C for 24 h, Rambach, Merck, incubated at 37°C for 24 h; (11) determination of the presence of lactic acid bacteria in samples of chunk meat and filling (ISO 15241), Man - Ragosa Sharpe (MRS), incubated at 30°C for 48-72 h Merck, Darmstad, Germany (12) presence and total count of *Listeria monocytogenes* (SRPS ISO 11290-1, 2), ALOA, Merck.

Statistical analysis
Statistical analysis was carried out using STATISTICA 9.1 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS

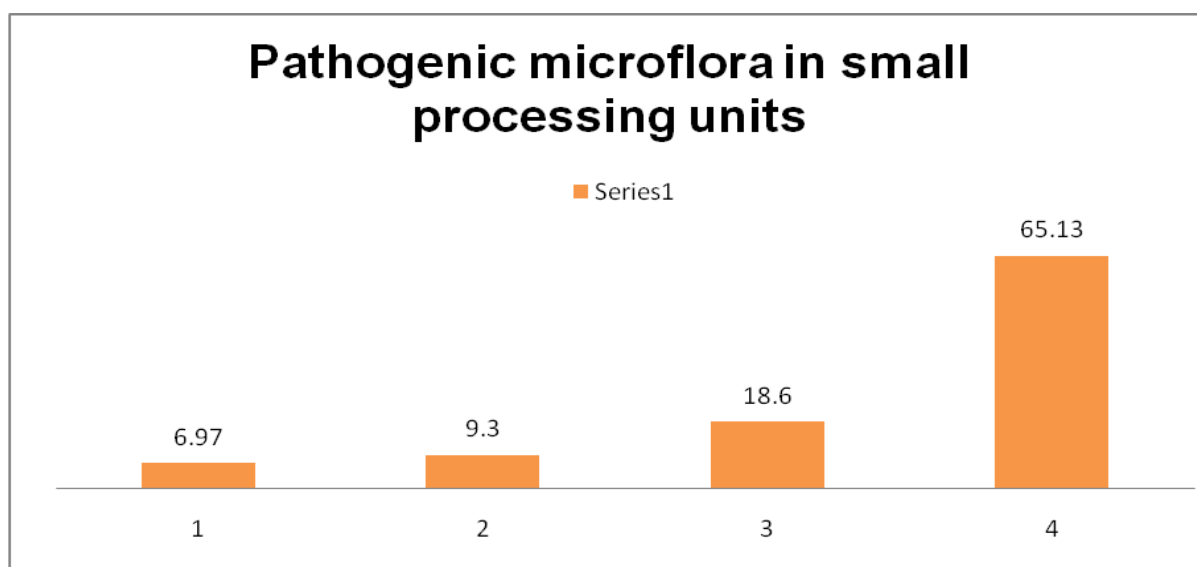Results are presented in Figure 1 and Table 1.

Figure 1. *Percentage of pathogenic microflora in small processing units (1- Listeria monocytogenes; 2-Staphylococcus aureus; 3- E. coli; 4-Nonpathogenic)*

Table 1. *Microbological contamination of batters and sausages after a drying process (MS±Sd, $log_{10}CFU/cm^2$)*

Sample	Total viable count	Micrococcaceae	Enterococcus spp.	Lactic Acid Bacteria	Enterobacteriaceae	L. monocytogenes
Batter A	7.03±0.05	4.23±0.42	3.24±0.24	ND	3.89±0.02	ND
Batter B	7.05±0.07	4.51±0.45	3.19±0.17	ND	4.19±0.12	ND
Sausage A1	4.19±0.22	2.75±0.35	2.07±0.15	5.3±0.15	ND	ND
Sausage A2	4.28±0.23	3.4±0.22	2.16±0.09	6.4±0.22	ND	ND
Sausage B1	4.5±0.05	2.64±0.1	2.25±0.25	5.7±0.15	ND	ND
Sausage B2	4.31±0.02	2.62±019	2.25±0.17	6.2±0.30	ND	ND

ND – not detected

DISCUSSION

Many authors support the belief that the microorganisms present in traditional sausages are derived from the raw materials or from the manufacturing (Talon et al., 2007). This microbiota is usually referred to as "house flora". If the microbiota isolated from traditional sausages is well described, the resident microbiota in the environment of the processing unit is still poorly known. The presence of aerobic bacteria, enterobacteria, enterococci and *L. monocytogenes* in fillings A and B surely resulted from cross contamination either with workings surfaces or after the meat mincing and mixing with spices, that is a consequence of the specific manual mixing preparation technique on the wooden table for cca. 15-30 min (Ikonić et al., 2010). Generally, *L. monocytogenes* and *S. aureus* were detected in 6.97 and 9.30%, respectively, while *E. coli* was enumerated in 18.60%. Sausage samples at the end of the storage period (270. day) were safe with presence of bacteria populations from the working environment, such as: aerobic bacteria, *Micrococcaceae, Lactic acid bacteria* and *Enterococcus* (Table 1). *L. monocytogenes* was sometimes present in environmental swabs and not detected by the end of the drying process. The results are in accordance with the results obtained by Lebert et al. (2007), Janković et al. (2013), Lakićević et al. (2014).

Several critical points were identified such as the drain, saws, workers' hands, mincing and stuffing machines. The current study revealed that the majority of the sampling sites (control point) tested were (2 to 6 log cfu/cm²) contaminated by spoilage flora (*Enterobacteriaceae*) with knives and saws, mincing machines (*Listeria monocytogenes*), workers' hands (*Staph. aureus, E. coli*), which surely indicates to an inappropriate slaughtering process, and to a low

level of personal hygiene. Detection of *Listeria monocytogenes* can be considered as a useful indicator of deterioration in hygiene or process conditions during food production. Unclean, insufficiently or inadequately cleaned pieces of equipment have often been identified as a source of pathogens. The results are unique and crucial for the improvement of hygiene control systems in traditional meat processing units.

CONCLUSION

Traditional dry sausages rely on natural contamination by environmental flora. This contamination occurs during slaughtering and increases during manufacturing. Each processing unit has a specific house flora, composed of useful micro-organisms for the fermentation and flavour of sausages, but also spoilage and pathogenic flora.

The results, during the production of the *Petrovská klobása* in the traditional manner, showed that processing units were colonised at various levels by spoilage and technological microflora with excessive contamination levels. Sporadic contamination by pathogenic microflora was recorded. *L. monocytogenes* and *S. aureus* were detected in 6.97 and 9.30% of the samples, respectively, while *E. coli* was enumerated in 18.60% of the samples. The variability of the contamination emphasized the different cleaning, disinfecting and manufacturing practices routinely followed by these (A and B households) small-scale processing units. The technological flora (staphylococci and lactic acid bacteria) were both in the environment and in products. Enterococci are present all along the manufacturing period. Also, the *Petrovská klobása*, at the end of storage period after 270th day, did not present sanitary risk as no pathogens were found.

ACKNOWLEDGEMENTS

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FOOD SAFETY EVALUATION OF THE PASTEURISING-COOKING THERMAL PROCESSES

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ABSTRACT

Concerning thermal processes, different thermal schedule establishing principles are used in the industry. These are mostly based on earlier experience, but nowadays the scientific principles have appeared mainly from the side of authority. Since the companies produce larger and larger amount of products and deliver them to longer and longer distances, there is a need to re-evaluate these principles in the food safety point of view. Therefore, in this paper, the thermal parameters from heat penetration curves were calculated and a survey about the initial and boundary conditions of the applied industrial thermal processes was made. Using this data, simulated calculations of the thermal processes stopped by the different principles were carried out. It was concluded that the earlier principles have to be corrected to fulfill the 12D bacterial destruction and that further investigation is needed for the determination of *Streptococci* population.
Keywords: *thermal process evaluation, pasteurizing-cooking, meat products, food safety*

INTRODUCTION

The thermal processing is the last possibility in providing the safety of food product in most cases. In the field of pasteurising-cooking thermal processes, we have to face some difficulties because several prescriptions are used in the thermal process evaluation such as so many mm in diameter, so many minutes for the processing time, time for reaching a target core temperature, enzyme inactivation equivalent time (USDA 1984, ZSARNÓCZAY and KÖRMENDY 1992, 1993, 1995, 1996, 1997) and 7-12D bacterial destruction equivalent pasteurising time (USDA 1998, EU 2005). In the case of the latter one, there is no agreement in the bacterial destruction parameters (decimal reduction time, z value). Furthermore, these cooking processes can be carried out in different units providing different heat transfer systems (pasteurising bath, hot smoking and cooking chambers, autoclaves) at different parameter constellations.
The aim of this study was to compare these different prescriptions for the thermal process in the food safety point of view of.

MATERIAL AND METHODS

The product was considered as infinite cylinder with diameters of 25, 40, 65, 80 and 100 mm because the length:diameter ratio reaches 4-5 in the practice. The surface heat transfer coefficient was assumed as 200W/m^{2}Kfor both holding and cooling time, while the thermal diffusivity was 1.3·10^{-7} m^2/s on the base of 200 heat penetration curves evaluated by infinite series solution of unsteady state heat transfer equation of Fourier and least squares method. According to our industrial experience, the initial temperature was 15°C, the ambient temperature range was 72-80°C, the cooling water temperature was 15°C and the target core temperature range was 69-72°C. The temperatures were calculated by the explicit finite difference method. The equivalent pasteurising time was calculated by the additive method with D and z values taken from the literature (Eszes, 2010).

RESULTS AND DISCUSSION

The min/mm thermal schedule principle was valid only for 40 mm product diameter (Figure 1). For other diameters we need to use a correction factor between 0.8-1.6 for reaching the 12D bacterial destruction. Using these correction factors is especially advisable in cases where the applied thermal parameters shift the process towards speeding up e.g. higher ambient temperature, since the equivalent pasteurising time calculation will have short summation period of time. The correction factors showed a linear relationship with the diameter. The small deviances from the total linearity can be due to the conditions of steaming off.
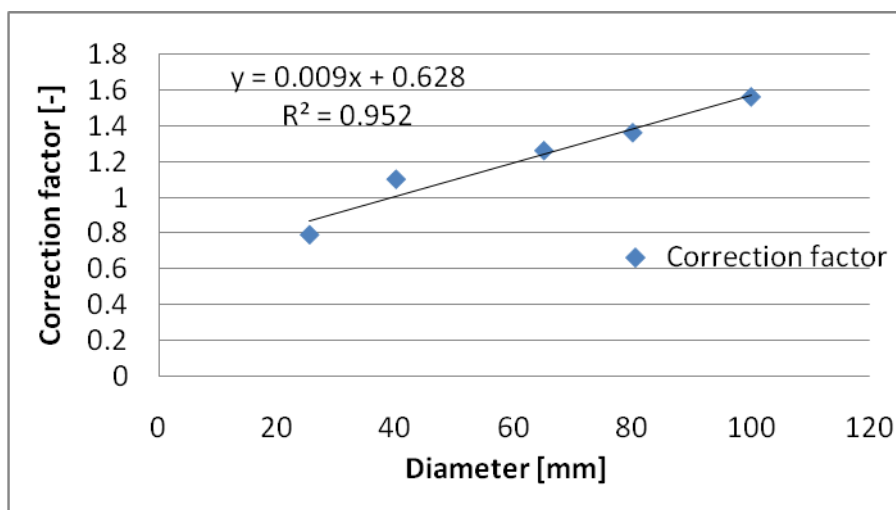

Figure1. Correction factor for the min/mm thermal process schedule principle for 12 D destruction of D-Streptococci (D=2.95, z=10°C)

The differences can be seen as well when the thermal process schedule stopping condition of reaching a target core temperature was applied. The industrial practice of using the temperature of 69°C could only be successfully applied for 12D *Streptococci* destruction in large diameter products. For the diameter less than 90 mm the target core temperature at steam off has to be increased. In case of smaller diameter, there is a need to apply only 78-80°C ambient temperature for reasonable process time and to avoid product overcooking. The small deviances from the total linearity can be due to the steaming off conditions (Figure 2).

In case of the investigation of the bacterial destruction we found very large differences between the *Streptococci* variants destruction (Figure 3). We could separate a very heat resistant and a less resistant group. The more resistant group showed a small destruction which measure only 1-2D units. It was surprising that the *L. viridescens* destruction is minimal if we apply the thermal process conditions used nowadays. Because we have no information about food safety problems or public health claims associated with this meat product group, it can be assumed that the number of them must be very low or even that these bacteria could not be involved in the raw material. The Figure 3 shows only 10-D destruction because the parameters were applied as they are used in the industry. If we use the correction decribed above, the less resistant group of *Streptococci* can be safely destroyed.
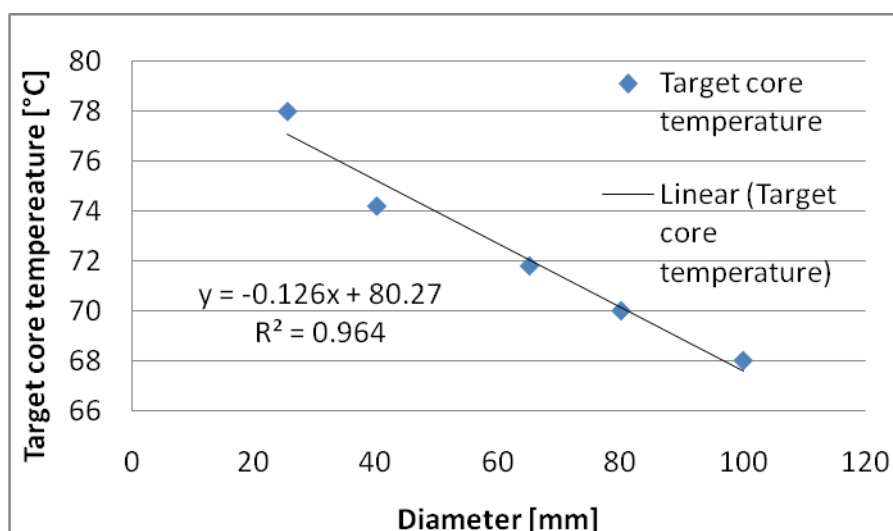
Figure2. Target core temperature for 12D destruction of D-Streptococci (D=2.95, z=10°C)
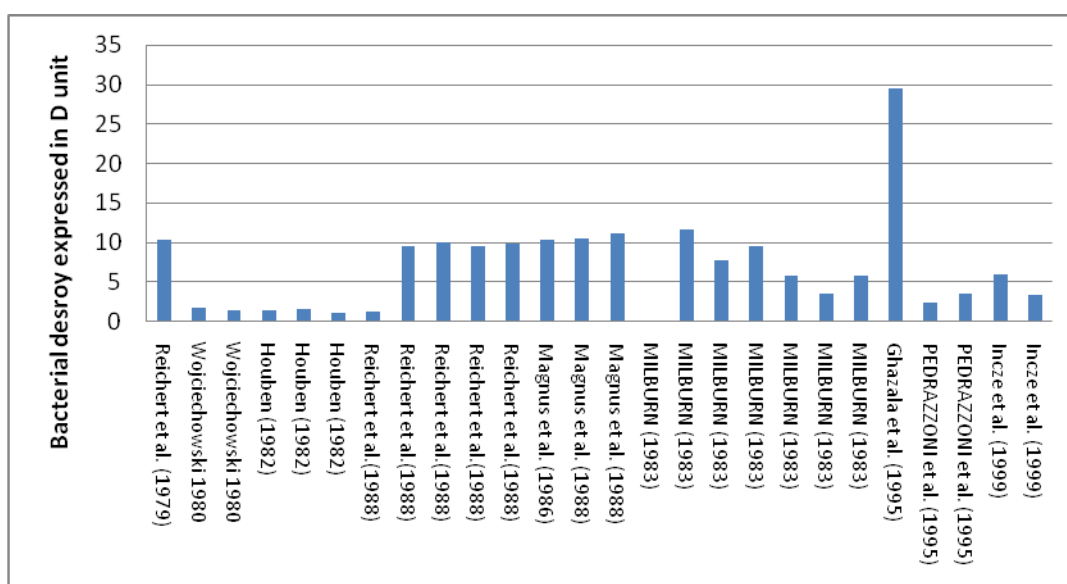

Figure3. Bacterial destruction according to the minimal and maximal D and z values presented by different authors (Eszes, 2010)

CONCLUSIONS

If we consider the bacterial destruction according to the legislations, we have to re-evaluate the previously determined thermal process establishing principles.

The companies have to investigate the composition of *Streptococci* population because if they would have larger number of these bacteria in the raw material, then they could suffer from severe underprocessing. In this case, new thermal schedules should be established. On contrary, if they assume false larger *Streptococci* count, strong over-processing can be caused by the severe thermal processing influencing the detrimental effect.

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TECHNOLOGICAL PROPERTIES OF SPELT ACCORDING TO DIFFERENT PRODUCTION SYSTEMS

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ABSTRACT

The object of the investigation was to analyse the postharvest quality of spelt (*Triticum spelta* L.) cv. 'Ebners Rotkorn' harvested in June 2012 at the University Agricultural Centre of the University of Maribor inSlovenia. Grain samples were taken from the long-term field trial established in 2007 to study the effects of different production systems (conventional (KON), integrated (INT), organic (ORG), biodynamic (BD) and control (K)) on crop productivity and food quality. The samples were analysed at the Institute for Food Technology in Novi Sad, Serbia with the purpose of evaluating the quality parameters of grain, flour and dough. The analyses were performed with classical devices (Brabender - farinograph, extensograph, amilograph and Chopin - alveograph) and Mixolab (Chopin) - a new system which measures the torque associated with dough during mixing and heating.
The results indicate that the production system significantly affected only 8 (absolute weight, the proportion of grain within the grain size of 2.0 mm, water absorption, time and torque in phase C2 and torque in phase C5, energy and dough deformation energy) out of the 48 analysed parameters. The correlation analysis between Mixolab and classically assessed parameters showed statistically significant and strong to very strong correlations (r > 0.50 in r > 0.75) between the parameters of torque C1 and wet gluten; between the stability of the dough and the tenacity parameter of resistance to extension (P),deformation energy (W) and wet gluten; between torque parameters in C2 with a curve configuration ratio (P/L) and dough extensibility (L).The water absorption is correlated with the protein content, wet gluten, gluten in grains and sedimentation index. The discriminant analysis showed a segregation of different production systems.
ORG and BD production systems do not result in poorer spelt quality when compared to KON and INT.
Keywords: *spelt, production system, quality, rheology, mixolab*

INTRODUCTION

Spelt wheat (*Triticum spelta* L.) is an old wheat species, which production has been greatly reduced during the 20th century. However, interest in this hulled low input wheat generally resistant to biotic and abiotic stresses has recently increased and spelt is being recognised as one of the most appropriate cereals for organic production (Bavec and Bavec, 2006). Since the most common form of consuming spelt is bread, information on bread making properties have appeared in the literature within this context (Ranhotra *et al.,* 1995; Jorgensen *et al.,* 1997; Bojňanská and Frančáková, 2002; Cubadda and Marconi 2002, Filipčev *et al., 2014*).
Spelt and common wheat have a lot in common but also possess many significant distinctions. Regarding dough quality and bread-making ability, the properties of spelt have been described as poor compared to common wheat (Jorgensen *et al.,* 1997; Pruska-Kedzior *et al.,* 2008).
There were no indications of any comparative research conducted on the quality of spelt in relation to its manner of production in the existing literature, however research conducted on the quality of wheat from organic farming differs in many aspects from that of wheat from conventional farming (Carcea *et al.,* 2006, Hildermann *et al.,* 2009, Ceseviciene *et al.,* 2012).

The aim of the present study is to evaluate the technological quality of spelt taken from a long-term field experiment where different production systems were compared.

MATERIAL AND METHODS

Spelt (*Triticum spelta* L.) cv. "Ebners Rotkorn' was grown in 2013 in a long-term field trial at the University Agricultural Centre of the University of Maribor in Pivola near Hoče (46°28′N, 15°38′E, 282 m a.s.l.) in Slovenia. The annual mean air temperature of the area is 10.7 °C, and the average annual rainfall in the area is around 1000 mm. The trial was established on a dystric cambisol (average pH value 5.5 in 0.1 KCl solution, soil soluble P of 0.278 g/kg and soil soluble K of 0.255 g/kg in top soil layer). In 2007, twenty plots (2.4×7 m) were randomly assigned to five production systems in four replications per system when the trial started. The farming systems mostly differed in plant protection and fertilization strategies. The following farming systems were involved: conventional farming (CON) according to the Slovene agriculture act and good agricultural practice, integrated farming (INT) according to Slovene standards for integrated farming, organic farming (ORG) according to the European Commission Regulation on Organic Farming, and biodynamic farming (BD) according to Demeter International Production Standards and European Commission Regulation on Organic Farming (Bavec *et al.,* 2010). In the control treatment (C) no fertilization or plant protection was used. Spelt grains harvested on individual plots were mixed into two samples. The first sample was a mixture of replications one and two, and the second sample of replications three and four.

The samples were analysed at the Institute for Food Technology in Novi Sad, Serbia with the purpose of evaluating the quality parameters of grain, flour and dough. Each sample was analysed in two replications. Standard procedures were used for the determination of moisture content, besatz of wheat, test weight (Shopper chondrometer), 1000-kernel weight, protein content (ICC stan-dard No. 105-2), Zeleny sedimentation value (ICC Standard No.116/1), falling number (ICC Standard No. 107/1), wet gluten and gluten index (ISO 5531), Brabender: farinograph (ICC methods No. 115-1), extensograph (ICC-Standard no. 114/1), amylograph (ICC-Standard no. 126/1), and Chopin: mixolab (Chopin+ protocol, ICC No. 173), alveograph, ICC methods No. 121).

Obtained data were subjected to an analysis of variance (ANOVA) using Statgraphics Centurion XV (Statgraphics®, 2005) to ascertain significant differences between treatments ($P < 0.05$). Differences between means were revealed by Duncan's multiple range test ($α = 0.05$). The results are presented as the mean of replications ± standard error of mean (SEM). The Pearson correlation coefficients were estimated among parameters assessed by Mixolab and classical devices (Brebender, Chopin aleveograph). Discriminant analysis was applied to obtained data in order to segregate production systems by using SPSS (IMB Corp., 2012)

RESULTS AND DISCUSSION

Due to the limiting length of the article, only the most important and quality parameters affected by the production system are presented (Table 1). The spelt grown on low-input farming (ORG and BD) treated plots exhibited lower 1000-kernel weight, the water absorption of the spelt flour taken from control plots was lower than the ones from other production systems, while energy and dough deformation energy were higher in CON production. Other quality parameters were not influenced by the production system; the content of protein (14.1 to 14.7%), falling number (298 to 342s), and sedimentation value (32.4 to 34.3 ml) are within the range reported by other authors (Ranhotra *et al*., 1995; Jorgensen *et al*., 1997; Bojňanská and Frančáková, 2002; Cubadda and Marconi 2002; Grobelnik Mlakar *et al*., 2009). A similar range of protein content and water absorption were reported by Lacko-Bartošová and Korczyk-Szabó (2011) and Filipčev *et al.* (2013) when evaluating organically grown spelt varieties in Serbia and in Slovakia, only they found a

higher average wet gluten (from 34.3 to 44.6 % and from 37.4 to 49.8 %, respectively) and a higher dough stability (0.0 to 1.5 min, and 1.5 to 2.4 min, respectively). Dough prepared from presently examined spelt flours exhibited a poor rheological quality characterised by short stabilities (0.0 to 0.25 min), a relatively high degree of softening (110 to 117 BE), low energy and dough deformation energy, as well as the configuration ratio – the P/L value (from 0.2 to 0.3) was well below 0.6, which is considered favourable for bread making.

Table 1. The effects of production system on some quality parameters assessed by classical methods and by rheological devices (faringograph, extensograph, amylograph, alveograph)

	ANOVA[1]	C	BD	ORG	INT	CON
Protein (%)	ns	14.1±0.1	14.5±0.1	14.2±0.1	14.7±0.4	14.2±0.4
Gluten (%)	ns	26.5±0.7	28.4±0.4	26.7±0.2	28.0±1.2	27.9±1.1
Falling number (s)	ns	319.0±33.5	311.8±16.0	317.5±22.5	298.3±5.3	341.8±15.3
Sedim. value (ml)	ns	32.4±0.3	33.8±0.3	32.7±0.2	34.3±1.6	33.6±1.1
H. weight (kg/100 l)	ns	76.85±0.4	76.2±0.1	76.4±0.4	75.7±1.6	77.4±0.6
Wet gluten (%)	ns	35.7±0.5	38.4±0.8	37.5±2.2	35.7±2.6	36.2±1.0
1000-kernel w. (g)	*	38.7±0.1[bc]	34.0±2.7 [d]	35.2±0.9 [cd]	43.8±0.3 [a]	42.1±0.8 [ab]
Absorption (%)	*	54.8±0.5 [b]	56.8±0.3 [a]	56.9±0.3[a]	55.9±0.1[a]	56.5±0.2 [a]
Stability (min)	ns	0.25±0.3	0.25±0.3	0.25±0.3	0.0±0.0	0.25±0.3
Softening (BE)	ns	115.0±0.0	117.5±7.0	115.0±5.0	110.0±0.0	112.5±2.5
Energy (cm^2)	*	16.0±2.0 [b]	18.5±10.0 [b]	13.5±2.0 [b]	26.0±1.0 [ab]	38.5±11.5 [a]
W (10^{-4}J)	*	47.5±4.5 [ab]	48.5±6.5 [ab]	38.5±13.5 [b]	56.5±0.5 [ab]	66.5±0.5 [a]
P (mm)	ns	24.5±1.5	24.5±0.5	24.5±1.5	25.5±1.5	28.0±0.0
L (mm)	ns	109.5±3.5	102.5±19.5	97.0±15.0	128.0±16.0	141.0±4.0

[1] *, ns: significant at the 0.05 probability level and non-significant, respectively
[a–d] *means followed by different letter(s) are significantly different (Duncan, α=0.05)*

A mixolab is a rheological device that determines the comprehensive qualitative profile of dough by measuring mechanical changes (torque) during an increase in temperature. Since it is a relatively new instrument, information on its utilisation are quite scarce and generally limited to wheat. As shown in Table 2, the production system does not affect the assessed parameters, except the minimum torque (C2) and the starch gelling (C5). The dough prepared from the spelt grown on the treatments BD, ORG and INT showed significantly lower C2 values (0.24 and 0.25 Nm) and thus more pronounced protein reduction when subjected to mechanical and thermal constraints than the dough produced from spelt grown on C and CON (0.28 Nm). Despite differences in minimum torque, parameters protein weakening (difference between torques C1 and C2) and protein breakdown (α - the slope of the curve between the end of the 30°C period and C2) are not influenced by the production system. Spelt grown on C plots exhibits the highest starch gelling and the highest degree of starch retrogradation (difference between torques C5 and C4).

The results obtained from classical rheological devices and the mixolab parameters reflect a poor technological quality which is characteristic for spelt: in comparison to wheat it produces soft, sticky, less stable and elastic dough. In accordance with this, Banu *et al.* (2011) and Dhaka *et al.* (2012) who screened different wheat flours and varieties, reported higher torques C1 (range 1.04 to 1.20), C2 (0.3 to 0.51) and lower α (in average -0.02) indicating a better quality of wheat protein, longer dough development time (in average 4:45) and stability (in average 8:46 and 6:02, respectively) which further indicates a stronger dough, lower maximum viscosity peak (C3 in average 2.21 and 1.90, respectively), higher starch gelatinisation (β in average 0.463) and higher retrogradation (1.28 and 1.3 Nm/min, respectively). Despite the lower technological quality in comparison to wheat, spelt is certainly suitable for bread making, but it requires some modification in the technological process (Filipčev *et al.* 2013).

Correlations were determined between mixolab and other assessed quality parameters to indicate the suitability of mixolab for profiling spelt quality. As is evident in Table 3, mixolab water absorption shows a very strong correlation (r > 0.75) with the protein content, gluten and sedimentation value and a strong correlation (r > 0.50) with wet gluten. The parameter stability shows a very strong correlation (r > 0.75) with alveographic P and W values, and a

strong correlation (r > 0.50) with wet gluten. Reports from literature, elaborating on classical and mixolab parameters obtained on wheat, also indicate that beside other correlations, there are two strong negative C3-connected correlations: the correlation with the sedimentation value and wet gluten, and the correlation between C4 torque and the sedimentation value (Banu *et al.*, 2011; Dhaka *et al.*, 2012).

Table 2. Effects of production system on some parameters assessed by mixolab

Parameters	C	BD	ORG	INT	CON	Average
C1 (Nm)[ns]	1.09±0.02	1.07±0.02	1.08±0.02	1.09±0.01	1.12±0.02	1.09
C2 (Nm)**	0.28±0.01[a]	0.24±0.01[b]	0.25±0.01[b]	0.25±0.01[b]	0.28±0.01[a]	0.26
C3 (Nm)[ns]	1.84±0.06	1.70±0.04	1.82±0.03	1.74±0.04	1.80±0.02	1.78
C4 (Nm)[ns]	1.79±1.04	1.60±0.09	1.74±0.31	1.68±0.05	1.70±0.03	1.70
C5 (Nm)**	2.95±0.12[a]	2.40±0.15[b]	2.63±0.06[b]	2.55±0.14[b]	2.59±0.08[b]	2.62
Absorption (%)[ns]	52.9±0.17	54.1±0.47	52.9±0.10	53.6±0.46	53.4±0.81	53.4
Amplitude (Nm)[ns]	0.06±0.00	0.05±0.00	0.06±0.00	0.07±0.02	0.09±0.02	0.07
Stability (min)[ns]	3.74±0.16	3.49±0.06	3.28±0.24	3.76±0.14	4.47±0.47	3.75
Develop. time (min:s)[ns]	3:05	2:49	2:43	2:24	1:48	2:34
α (Nm/min)[ns]	-0.064	-0.065	-0.060	-0.051	-0.065	-0.061
β (Nm/min)[ns]	0.128	0.128	0.075	0.050	0.217333	0.1196
γ (Nm/min)[ns]	-0.042	-0.017	-0.071	-0.085	-0.014	-0.046
C1-C2 (Nm)[ns]	0.81	0.83	0.83	0.84	0.83	0.83
C5-C4 (Nm)*	1.16[a]	0.80[b]	0.89[b]	0.86[b]	0.89[b]	0.92[b]

**, ns: significant at the 0.01 probability level and non-significant, respectively
[a–b] means followed by different letter are significantly different (Duncan, α=0.05)

Table 3. Pearson's correlation coefficients between mixolab and some other quality parameters evaluated

Parameter	Protein content	Wet gluten	Gluten	Sedime nt. value	Falling numbe r	P	L	P/L	W
Stability	ns	-0.64**	ns	ns	ns	0.80**	ns	ns	0.82**
Absorption	0.88***	0.64*	0.84**	0.79**	ns	ns	ns	ns	ns
C1	ns	-0.65*	ns	ns	ns	ns	ns	ns	ns
C2	ns	ns	ns	ns	ns	ns	0.65**	-0.73**	ns

***, **, ns: significant at the 0.001, 0.01 probability levels and non-significant, respectively

A discriminant analysis was carried out in order to make a distinction between the five production systems with respect to their quality based on the 48 parameters evaluated (Figure 1). The first discriminant function accounted for 99% of the total spatial variance and clearly separates production systems, especially C and BD, from each other. Further analysis revealed that the highest contribution to the discriminant function can be ascribed to the parameters maximum viscosity, resistance to extension, mixolab stability and dough extensibility.

CONCLUSIONS

As a result of the current and foreseeable expansion of organic and other low-input ways of farming, information about the influence of production systems on the quality of parameters became more and more important. The present study comparatively evaluates parameters of spelt's technological quality as a response to different production systems. The obtained results revealed that production systems do not influence the quality and thus show that low-input farming (organic and biodynamic production) does not cause a poorer bread making quality of spelt than more intensive systems of production (conventional, integrated).
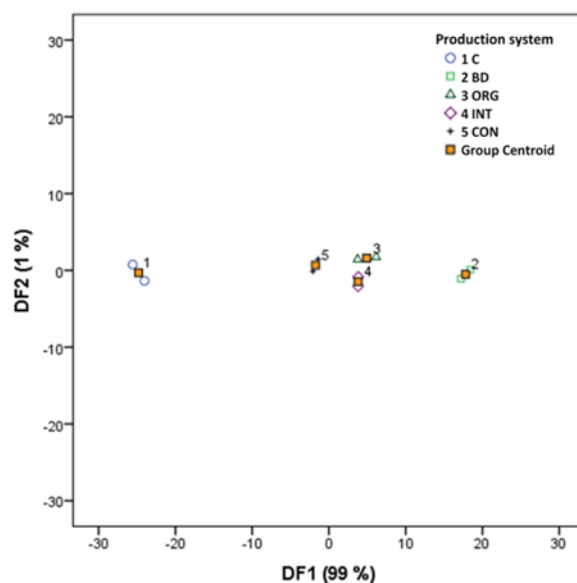
Figure 1. Plot of discriminant functions presenting the results of discriminant analysis including all the studied quality parameters in five production systems

ACKNOWLEDGEMENTS

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ANALYSIS OF THE IMPACT OF THE AMOUNT OF SPONTANEOUSLY FERMENTED SPELT DOUGH, BAKER'S YEAST AND ASCORBIC ACID ON SOME CHARACTERISTICS OF SPELT SOURDOUGH BREAD

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ABSTRACT

In the present work, a specific type of bakery sourdough made form organically grown spelt wheat was applied. It is known that the addition of sourdough provides the possibility to reduce the amount of yeast. It has been found that ascorbic acid is an important ingredient in spelt breads. The study was aimed to determine the effects of the amount of spontaneously fermented spelt dough, baker's yeast and ascorbic acid on volume and textural properties of spelt bread (volume and crumb texture). Bread volume and bread resilience increased with addition of spelt sourdough in the range from 20% to 40% flour basis. With the quadratic increase in the amount of sourdough there was a negative effect on the bread volume, positive on crumb firmness and insignificant effect on bread resilience. The optimization indicated that a formulation of spelt bread with 40% of the spelt sourdough, 2% of bakery yeast and 0.11 g/kg of ascorbic acid satisfied the specified criteria set forth in the optimization process.
Keywords: *spelt sourdough, spelt bread, Box–Behnken response surface design*

INTRODUCTION

Spelt wheat is an ancient subspecies of common wheat which is suitable for cultivation by organic methods as it does not require high-level nitrogen fertilization or heavy chemical protection (Filipčev et al., 2013a,b). In contrast to the advantages regarding some agricultural and nutritional features, the dough quality and breadmaking ability of spelt has been described as poor compared to common wheat. Although having higher gluten quantities, the quality of spelt gluten is inferior to that of modern wheat giving soft, less elastic, sticky and less dough stability (Schober at al., 2006; Pruska-Kedzior et al., 2006). Di Cagno et al., 2003 reported that, in some cases, using the combination of sourdough and some additives could reduce the risk of dough weakening and the loss of gas retention properties. On the basis of this fact can be combined sourdough and ascorbic acid in order to improve the quality of dough and bread from spelt flour. In practice, most frequently are applied sourdough concentrates which consist of spontaneous sourdough supplemented with additional organic acid (lactic and/or acetic acid). The microflora of such dough depends on the microflora of applied raw materials and the prevailing hygienic conditions, as well as the technological parameters of the applied fermentation process. The sourdough fermentations are performed as single- or multistage processes. Successful utilization of wheat sourdough demands skilled personnel and careful control over the process to obtain high quality products and is thus a much more demanding way to improve product quality in comparison to baking additives. However, the effectiveness of wheat sourdough in improving flavor, texture and nutritional quality is unique, and the utilization of sourdough has gained popularity in recent times (Kulp, 2003). Thus, control of the acidity level of sourdough and subsequent breads is a premise for the improvement of bread quality. In this study, a response surface design was used to estimate the individual and interactive effects of the level of implementation of sourdough, baker's yeast and ascorbic acid on important spelt bread parameters such as: volume, hardness and resilience.

MATERIAL AND METHODS

Spelt Flour
Spelt flour was supplied from ecological agricultural farm Jevtić, Bačko Gradište, Serbia. The spelt flour was characterized by the following parameters: moisture content 11.8% and ash content 1.70% d.b. Baker's yeast (BY) was purchased from "Fermin" Senta, Serbia. The ascorbic acid was procured from BASF, Germany.

Preparation of spontaneously fermented spelt sourdough
Spontaneously fermented dough (SFD) was prepared by mixing 150 grams of wholemeal spelt flour and 100 grams of deionized water (at room temperature) for 1 min without the addition of any microbial culture. The fermentation process was initiated by the native flora present in the flour. The formed batter was poured into the vessel, covered, and placed in an incubator at 30°C. The fermentation process was begun when dough was put in the incubator. It was left to stand in a warm place for 24 hour. Then a multiple-renewal with flour and water was commited (Table 1). The process of renewal creates conditions under which desirable microorganisms (lactic acid bacteria) suppress undesirable microorganisms (e.g. *Coli bacteria*).

Table 1. Preview of three stage process of the spontaneous fermentation spelt sourdough

Phases	Ingredients	Conditions
Starter	100 g wheat flour + 100 ml deionized water (26-28°C)	Fermentation 24 h at temperature 26-28°C
First level	The process of recovering: Wheat flour and deionized water are added to the starter in relation 1:1	Fermentation 24 h at temperature 26-28°C
Second level	Further recovery: add flour and lukewarm deionized water in a ratio of 1:1	Fermentation 24 h at temperature 32-35°C
Third level	Further recovery: add flour and lukewarm deionized water in a ratio of 1:1 and 20% flour of malt on the total sourdough amount	Fermentation 16 h at temperature 28-30°C

Bread making
Two samples of spelt bread were prepared: spelt bread without sourdough (as control) and spelt bread with spontaneously fermented spelt sourdough (abbreviated spelt sourdough bread). The ingredients for control spelt bread included: 400 g wholemeal spelt flour, 8 g baker's yeast, 6 g salt and 220 ml tap water. Spelt sourdough bread making involved mixing 400 g wholemeal spelt flour, 6 g salt, 210 ml tap water and amount of baker's yeast, spontaneously fermented spelt dough and ascorbic acid according to the experimental design (Table 2). Both samples were prepared under the same conditions: the kneading of dough for 5 min, dough fermentation was carried out at 30±1 °C for 2 h and baking was carried out at 200±3 °C, for 0.5 h.

Determination of pH and total titratable acidity (TTA)
Procedure for pH determination is the following: 15 g of breadcrumbs and 100 ml of distilled water were placed in a clean dry vial, which was sealed and stirred for 10 min. After that the pH was recorded using a Cyberscan 10 pH-meter (Eutech Instruments Pte Ltd., Singapore). The total titratable acidity (TTA) was determined in accordance with Neumann's method (Serbian Regulations 1988; Kaluđerski and Filipović, 1998).

Bread analysis
After cooling, the loaves were weighed and volume was measured by the rapeseed displacement method using a funnel and accessories (Kaluđerski and Filipović, 1998). Bread crumb firmness was determined according to the standard AACCI 74-10A method (AACCI, 2009) using a 36 mm diameter probe. Crumb resilience was determined as a percentage of

recovery of sample's height in relation to its initial height after maximal compression at 60% strain during 2 seconds followed by a recovery period of 15 s.

Experimental design
A Box-Behnken design was chosen to study the effects of three factors: spontaneously fermented spelt dough (SFD) addition level: 200-400 g/kg of flour; baker's yeast (BY) addition level: 10-20 g/kg of flour and ascorbic acid (AA) addition level: 0.1-0.2 g/kg of flour on change chemical and physical properties of spelt sourdough bread. The runs were performed in the random order presented by the program (Software Design-Expert® 8.0.7.1. Stat-Ease Inc, UK) to provide protection against the effects of lurking variables (Table 2).

Table 2. Composition of various runs of Box–Behnken design

Run	SFD addition level, %	BY addition level, %	AA addition level, mg/kg
1	20	1.0	150
2	20	2.0	150
3	40	1.0	150
4	30	1.5	150
5	40	2.0	150
6	40	1.5	200
7	20	1.5	100
8	30	1.0	100
9	20	1.5	200
10	30	2.0	200
11	30	1.0	200
12	30	1.5	150
13	30	2.0	100
14	40	1.5	100
15	30	1.5	150

RESULTS AND DISCUSSION

Acidity of spontaneously fermented spelt sourdough and spelt sourdough bread
Sourdough fermentation is based on lactic acid and alcoholic fermentation depending on the microflora of raw materials and fermentation conditions. Typical pH and TTA values of acidic wheat sourdough are 3.6 and 8.13, respectively (Brümmer and Lorenz, 1991). The degree of acidification of spelt sourdough determined through pH and total titratable acidity (TTA) is shown in Table 3. Initial pH and TTA values for SFD were 5.48 and 0.9, respectively. After 3-level fermentation (Table 1), pH value in SFD was dropped to 4.6 and TTA value was increased from 0.9 to 7.8. At the initial stage, the indigenous flora of the wholemeal spelt flour was inactive and was activated by addition of water; activated lactic acid bacteria caused a drop in pH levels at later stages of the fermentation process.

Table 3. Acidity of spontaneously fermented spelt sourdough before and after fermentation

Parameters	Spontaneously fermented spelt sourdough (SFD)	
TTA	Before fermentation	0.9
	After fermentation	7.8
pH	Before fermentation	5.48
	After fermentation	3.14

Characteristics of spelt dough and bread
Incorporation of spontaneously fermented spelt sourdough in spelt bread dough increased its acidification degree and decreased the pH values (Table 4).

Table 4. Acidity of spelt bread doughs made with or without spontaneously fermented spelt sourdough

Parameter	Spelt bread dough without SFD	Spelt bread dough with SFD
TTA value	2.40	4.96
pH value	5.56	4.90

The incorporation of sourdough in the mixing of bread dough influences, besides pH and TTA, its rheological behaviour and bread quality (Corsetti et al., 1998; Clarke et al., 2002; 2003). Effects of spontaneously fermented spelt dough in combination with ascorbic acid and baker's yeast on bread quality have been observed through this paper. Their impact on spelt bread volume (BV), bread firmness (BF) and bread resilience (BR) are shown in Table 5.

Table 5. Bread volume (BV), bread firmness (BF) and bread resilience (BR) for the control spelt bread and for the spelt sourdough bread according to experimental design

Run	Responses		
	BV, ml	BF, g	BR, %
1	1005	85.8	87.54
2	1010	848	87.93
3	1125	780	89.00
4	1117	840	88.30
5	1135	764	89.50
6	1120	820	89.40
7	990	861	87.70
8	1110	850	86.30
9	980	864	87.30
10	1115	840	88.80
11	1110	852	86.20
12	1117	84.2	88.30
13	1115	834	88.00
14	1140	750	90.00
15	1116	841	88.23
Control	850	1130	76.46

Table 6 shows the coded values of the regression coefficients (*b*) for the independent variables (spontaneously fermented spelt dough (SD), baker's yeast (BY) and ascorbic acid (AA)), the F-test and the determination coefficient (R^2). For these responses, the F_{test} was higher than $F_{tabular}$, and the models presented high values of determination coefficient ($R^2 > 0.60$; Table 6). The fitted models for BV, BR and BF were suitable to describe the experimental data. Results indicated that spontaneously fermented spelt sourdough was the variable that showed the greatest influence on BV, BR and BF in the investigated range. For BV response, the magnitude of *b* value (Table 6) indicates the maximum positive effect of spontaneously fermented spelt sourdough ($b_1 = 66.87$), while the applied doses of yeast and ascorbic acid did not significantly influence on BV observed in the range. Positive influence spontaneously fermented spelt dough is consistent with the fact that is a key to improved bread volume in the type and level of acidification sourdough. Also, some previous studies have confirmed that the application of a sourdough increases bread volume and shelf-life (Corsetti et al., 1988, 2000; Crowley et al., 2002). It is generally assumed that the utilization of sourdough improves gas retention (Clarke et al. 2002). Besides, production of CO_2 from heterofermentative lactic acid bacteria influences the fermentation process of the final bread dough. However, the quadratic coefficient of SFD showed a negative effect on BV. This means that with a too high intake of spontaneously fermented spelt dough can occur higher degree of acidification that causes the inhibition of baker's yeast (Salovaara and Valjakka, 1987).

Table 6. Significant coded values of regression coefficient of investigated models responses

Coefficient	Bread volume (BV)	Bread resilience (BR)	Bread firmness (BF)
b_0	1117	841	88
$b_{1\ SD}$	66.87**	0.93**	-39.62**
$b_{2\ BY}$	3.12**	0.65*	-6.75
$b_{3\ AA}$	3.75	0.04	-10.12
b_{12}	1.25	-	-1.50
b_{13}	2.50*	-	-16.75*
b_{23}	-	-	1
b_{11}	-51.62**	-	+24.37*
b_{22}	3.37	-	-4.12
b_{33}	-7.87	-	7.12
R^2	0.99	0.62	0.97
F_{test}	54.42	5.43	14.30
$F_{tabular}$	6.00	3.71	6.00

**$P \leq 0.01$; *$P \leq 0.05$;

Besides, an excessive dosage of sourdough causes a greater drop of pH values what causes a decreased maximal extension and decreased dough stability (Galal, et al., 1978; Tanaka et al., 1967; Tsen, 1966). The consequence of this is reduced of bread volume and increased crumb firmness. In order to improve the volume of bread made with the addition of sourdough, it must be combined with other raw materials that have a significant effect on the hardening of gluten network and create better properties for gas holding (Korakli et al. 2001). Thus ascorbic acid was used in combination with the spontaneously fermented spelt dough. Ascorbic acid alone in the applied dose had no significant influence on the increase in bread volume, but in combination with spontaneously fermented spelt dough significantly ($P \leq 0.05$) increased the volume of spelt sourdough bread and significantly ($P \leq 0.05$) decreased bread firmness. Baker's yeast, in the applied dose, significantly ($p \leq 0.01$) affected the rise in bread volume and bread resilience. This effect was expected due to the high correlation between bread volume and crumb resilience (Angioloni and Collar. 2009). Furthermore, it is necessary to optimize the amount of spelt sourdough, baker's yeast and ascorbic acid in the spelt sourdough bread. The optimisation using the desirability function was performed on maximum bread volume (BV) and bread resilience (BR) and minimum bread firmness (BF). The criteria used for optimization along with the predicted and actual response values are presented in Table 7. The insignificant difference between the measured values for the bread volume (1135 ml), bread resilience (87.66%) and bread firmness (747.65 g) and the predicted ones (1140 ml, 89.77% and 747.00 g, respectively) confirms the adequacy of the developed model. The quality of spelt bread with sourdough using RSM optimized doses of SFD (30% on flour basis), BY (1.8% on the bases flour) and AA (110 mg/kg flour) was higher than that of the spelt bread without sourdough (the control sample). Bread volume was approximately 1.3-fold higher, bread resilience 1.7-fold higher and bread firmness was 1.17-fold lower than in the spelt bread without sourdough.

Table 7. Criteria for optimization and solution along with the predicted and observed response values

Factors	Goal	Lower Limit	Upper Limit	Importance	Solution	
SFD, %	In range	20	40	3	30	
BY, %	In range	1	2	3	1.8	
AA, mg/kg	In range	100	200	3	110	
Responses					**Predictive values**	**Realized values**
BV, ml	maximize	980	1140	5	1140	1135
BR, %	maximize	86.2	864	4	89.77	87.66
BF, g	minimize	750	90	4	747.00	747.65

CONCLUSIONS

Spontaneously fermented spelt sourdough in combination with a lower share of baker's yeast and ascorbic acid can be used to improve the quality of spelt sourdough bread. Incorporation of 30% spontaneously fermented spelt sourdough with 1.8% baker's yeast and 110 mg/kg ascorbic acid affected positively the overall quality of breads, yielding bread with desirable volume, crumb elasticity and firmness.

ACKNOWLEDGEMENTS

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INFLUENCE OF *CORDYCEPS SINESIS* EXTRACT ON FERMENTATION PROCESS

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ABSTRACT

Cordyceps species, are very valued in traditional Chinese medicine. Their extracts show significant therapeutic activities, as it is well known that these medical fungi have anti-oxidant, immunomodulatory, anti-inflammatory, anti-tumor and many more beneficial effects on human body. Extracts of *Cordyceps* sp. also increase strength and endurance and they are commonly used for the replenishment of health. They have influence on cell metabolism, but mechanisms of action in cells still remain unknown. The purpose of this article is to examine the influence of *Cordyceps* extract on growth of yeast's cells and fermentation process. Three different species of yeast were used as models to examine influence of *Cordyceps* extract on cells metabolism, especiallly CO_2 production. Two concentrations of water extract of *Cordyceps sinesis* were added to yeast cell suspensions. Yeast cells (*Saccharomyces cerevisiae*, *S. cerevisiae K1* and *S. pastorianus* - as representatives of baker's, wine and brewer's yeasts), were cultivated in malt broth. Suspensions of cells were added to malt broth with sugar concentration equal to the industrial wort. Maximal and minimal concentrations of *Cordyceps* extract were added to malt broth and fermentation was performed in incubators for several days. After defined time of fermentation, samples were measured and decrement of their masses was evaluated. It was shown that maximal concentration of *Cordyceps* extract has greater effect on decrease in mass, than minimal concentration and also extracts have greater influence on brewer's and wine yeasts than on baker's yeast. This experiment opened a new field of research and further analysis need to be performed to examine possible influence of the *Cordyceps* extract on yeast fermentation in order to get shorter period of fermentation and more healthy product.
Keywords: *Cordyceps sinesis*, *fermentation, extract, yeast.*

INTRODUCTION

Cordyceps is one of the most precious mushrooms used in Chinese traditional medicine. There are more than 680 species under the genus *Cordyceps*, but the most famous is *Cordyceps sinesis. Cordyceps sinesis* is entomopathogenic fungi found in the soil of a prairie at an altitude of 3500 to 5000 meters above the sea level, on the Tibetan Plateau of Asia and Himalayas (1-4). It is known as „winter worm summer grass", because of its appearance during different seasons, and usually called caterpillar fungus because it grows from the head of caterpillar (4,5). With or without dead larva, fruiting body have been traditionally used as therapeutic agents and as a health food in Asian countries for several centuries (5).This exotic parasitic complex of fungus and caterpillar is very rare in nature and expensive at the local Asian market, but both natural and cultured *Cordyceps* products demonstrate health benefits (2). *Cordyceps* is commonly used for the replenishment of health, preventing aging and improving physical performance (2). Many studies showed that *Cordyceps* possesses pharmacological actions in modulating the immune system and has also an antioxidant and antitumor activity (1,2).

Strong anti-oxidation activity of mushroom is confirmed in literature and this activity is mostly due to the presence of polysaccharides, which are major contributors and active components. They have scavenging effect on free radicals, but the anti-oxidation activity could also be due to other components. Important bioactive compound called cordycepin is also produced by *Cordyceps*. Cordycepin (3'-deoxyadenosine) is nucleoside analogue and

has biological and pharmacological activities such immunological stimulating, anti-cancer, anti-virus and anti-infection activates (2,6).

Researchers suggest that supplementation with fermentation product of *Cordyceps sinensis* effects of energy metabolism, improves exercise performance especially in healthy older subjects and might contribute wellness (7,8). Also is shown that polysaccharides from *Cordyceps* influence the swimming capacity of mice. A group of mice supplemented with Cordyceps extract have extended swimming endurance time. They revealed that polysaccharides from Cordyceps have significant anti-fatigue effect. The effect of the extract on the convalescence from exhaustion can be related to the resistance to stress-induced exercise and strengthen immune system. Other authors examined similar effect on rats and prove that swimming endurance was significantly prolonged with a lessening of fatigue (4, 9, 10).

Clinical researches proved that intake of Cordyceps increase both cellular ATP level and oxygen utilization (11). In one experiment mice treated with *Cordyceps Sinesis* survived up three times longer than untreated ones in low oxygen environments. *Cordyceps* has ability to utilize oxygen more efficiently. The stimulation of ATP generation is paralleled by an enhancement in mitochondrial electron transport. At the same time it has immunopotentiating effect (12,13). In human organism, the use of Cordyceps increases blood supply and also improves respiratory function. This findings inspired Slovenian scientifics to research influence of C*ordyceps* extract on yeast cells as a model organism. In their experiments it is shown that enrichment of yeast suspension with *Cordyceps* extract leads to more release of the CO_2 (9-11,13-16).

Inspired by their results and according to their experiment we have done similar experiment and examined influence of *Cordyceps* on yeast's cells with attempt to get efficient fermentation. For that purpose three types of yeasts were used and examined influence of the *Cordyceps* in two different concentrations.

MATERIAL AND METHODS

Three different types of yeast were used: *Saccharomyces cereviseae* (baker yeast) – commercially available on the market; *Sacharomyces cereviseae* K1 (liofylised yeast) as wine yeast and *Saccharomyces pastorianus* (beer yeast) - operating yeast from local brewery. All yeasts were first isolated as pure cultures. When pure yeast cultures were obtained, yeasts were transferred to malt broth enriched with glucose and cultivated for 24 hours at 28°C. Malt broth was made from yeast extract, peptone, malt extract and glucose in concentration similar to worth in brewing industry.

Pure Cordyceps Super^{TM} powder was kindly donated by Aloha Medicinals Inc from America. *Cordyceps* was dissolved in sterilized distilled water in two different concentrations - maximal 50 mg/ml and minimal 50 µg/ml. It was noticed that dissolvation of *Cordyceps* in distilled water wasn't complete at this maximal concentration.

After 24 hours of cultivation, inoculums of yeast in amount of 2 ml were transferred to Erlenmeyer flasks with 100 ml of malt broth. For every type of yeast triplicates were done with corresponding control. In every yeast sample was added 1 ml of *Cordyceps* solution, while 1 ml of distilled water was added to controls. Every Erlenmeyer flask was closed with cotton stopper. Samples were further incubated at 28°C in an incubator and the fermentation took place.

After defined time of fermentation, at the beginning every 2 hours, and then until the end of fermentation once a day, samples were measured and decrement of their masses were evaluated. Alcohol, original extract, real extract, degree of fermentation was determined (Alcolyzer Beer ME Analyzing system, Antoon Paar Gmbh.)

RESULTS AND DISCUSSION

Samples enriched with minimal concentration of *Cordyceps* showed higher decrement of mass after 24 hours. The difference between samples enriched with *Cordyceps* and samples without *Cordyceps* remains at samples with beer yeast till the end of fermentation, while samples of wine and bakery yeasts tends to equalize with controls.
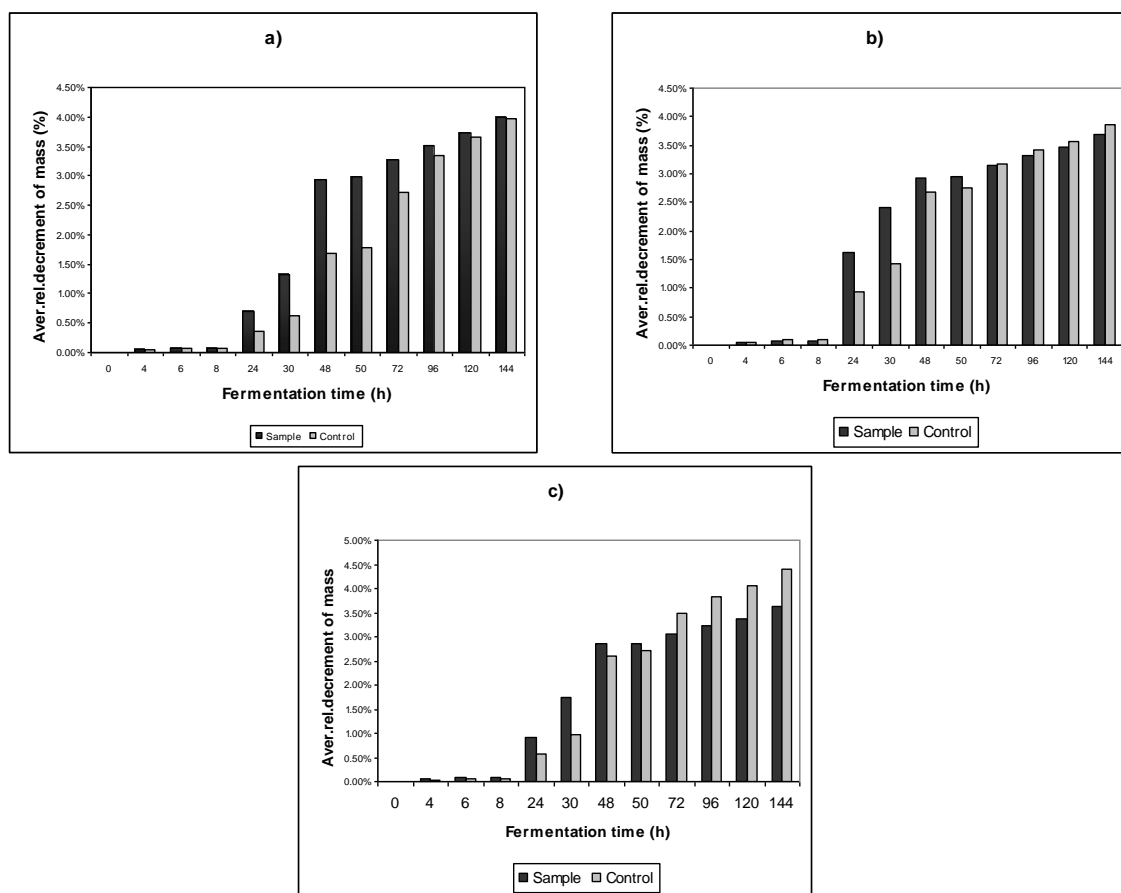

Figure 1. Average relative decrement in mass depending on time of fermentation for samples enriched with solution of Cordyceps extract in concentration 50 µg/ml; a) beer yeast; b) wine yeast; c) baker yeast

At the other side, maximal concentration of *Cordyceps* has higher effect on decrease in mass. The difference between enriched samples and controls is also visible after 24 hours of fermentation. This difference was higher than with smaller concentration, but remains shorter time, just till the 3th day of fermentation. After third day, the decrement was almost equal for all samples, with a little increment of the controls. This difference in decrement between samples enriched with *Cordyceps* and samples without *Cordycpes* is due to the increment of CO_2 production which confirms enhancement in metabolic activity because of the increment of ATP level (9). Parameters of obtained beer are shown in Table 1.
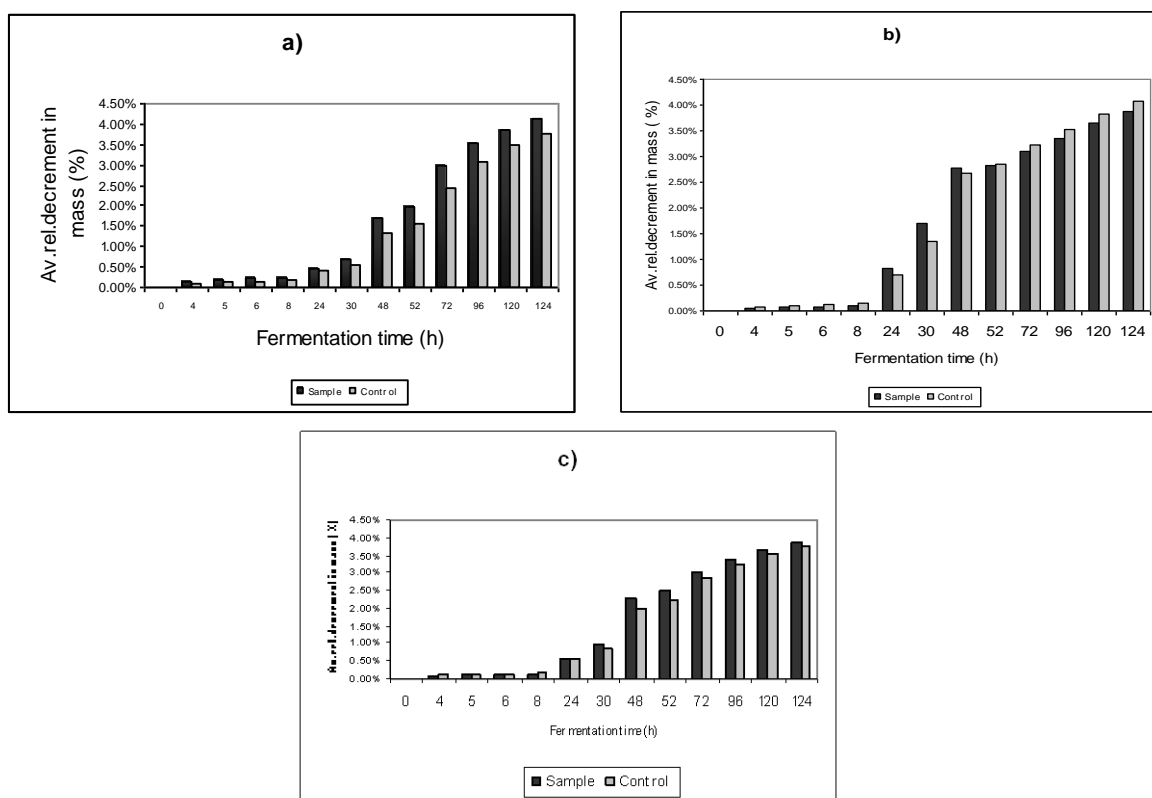
a)

b)

c)

Figure 2: Average relative decrement in mass depending on the time of fermentation for samples enriched with solution of Cordyceps in concentration 50 µg/ml; a) beer yeast; b) wine yeast; c) baker yeast

Table 1. Parameters of obtained beer with Cordyceps in concentration 50 µg/ml

Sample	A	B	C	D	E	F
Plato %	13,13	16,03	13,14	14,31	13,39	14,14
Alcohol % v/v	6,60	7,65	6,25	6,99	6,53	7,13
Er % m/m	3,11	3,60	3,67	3,78	3,49	3,37
Ea % m/m	0,74	0,89	1,43	1,30	1,15	0,83
RDF %	77,58	77,59	73,43	75,02	75,26	77,52
ADF %	94,37	94,08	89,11	90,92	91,38	94,13

A-sample with beer yeast and *Cordyceps*, B – sample with beer yeast, C- sample with wine yeast and *Cordyceps,* D- sample with wine yeast, E- sample with baker yeast and *Cordyceps,* D- sample with baker yeast; Er – real extract in beer, Er – apparent extract in beer. RDF -real degree of fermentation, ADF-apparent degree of fermentation.

CONCLUSIONS

In this study is presented a new attempt to accelerate yeast fermentation. It is shown that *Cordyceps* has effect on mass decrement especially on beer yeast which opened a new field of research. Knowing that *Cordyceps* has such positive impact on health, its dual role in obtaining product in shorter time and with improved healthier characteristics can be very interesting. Further analysis need to be carried to examine possible influence of *Cordyceps* on yeast fermentation in order to get shorter period of fermentation and healthier products.

ACKNOWLEDGEMENTS

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EFFECT OF HEAT TREATMENT ON ANTIOXIDANT PROPERTIES OF ROSE HIP (*ROSA CANINA* L.) PRODUCTS

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ABSTRACT

The fruits of rose hip (*Rosa canina* L.) are mainly used for the production of marmalade and in dried form for making herbal teas. Rose hips are rich source of natural antioxidants such as ascorbic acid, carotenoids and phenolic compounds.

The aim of our study was to estimate the influence of elevated temperatures used during the production of different rose hip products on their antioxidant capacity. The total phenolic and flavonoids content as well as antioxidant activity were evaluated by three contemporary and compatible methods. The rose hip fruits were drying in laboratory dehydrator, whereas marmalade was produced by traditional method. The values obtained for the fresh fruits were used as a control.

In the dried fruits the losses of 31.84%, 22.93%, 20.69%, on a dry basis for total flavonoids concentration, total phenolic content and antioxidant activity, respectively, has been obtained, in relation to the values of the fresh sample. During the production of marmalade the complete loss (100%) of total flavonoids concentration and antioxidant activity has been performed, while 99.69% on a dry basis were lost for total phenolic compounds, calculated in relation with control fresh sample.

Based on these results, it can be concluded that long-term effect of elevated temperatures and aeration during production of marmalade by traditional method very significantly influenced the losses of antioxidant properties.

Keywords: *dried rose hip*, *rose hip marmalade, antioxidant activity, phenolic compounds, flavonoids*

INTRODUCTION

The fruits of wild rose (*Rosa canina* L.), known as rose hip, have been the subject of numerous scientific studies because they possess biological active compounds with potential positive effects on human health. Rose hip contains vitamins B_1, B_2, C, E, carotenoids, minerals, amino acids, organic acids (Szentmihalyi *et al.,* 2002; Ercisli, 2007), as well as phenolic compounds that exhibit antioxidant, anticarcinogenic and antimutagenic properties (Kilicgun and Altiner, 2010). In addition, rose hips fruit contain about 30% of seeds rich in rose hip oil (Szentmihalyi *et al.,* 2002) used mainly in pharmaceutical and cosmetic industry due to compounds which have been found to have therapeutic effects in the treatment of dermatitis and other skin changes (Zlatanov, 1999; Szentmihalyi *et al.,* 2002; Ozcan, 2002; Concha *et al.*, 2006). In food industry rose hips are mainly used for the production of jam, jelly, syrup, beverages and marmalade whereas in dried form for the production of herbal teas often blended with hibiscus. A great number of plant materials contain biological active substances such as phenolics and vitamin C, exhibiting antioxidant properties (Ramamoorthy *et al.,* 2007; Rajić *et al.,* 2012; Rekha *et al.,* 2012). A measure of total antioxidant capacity of foods enables better understanding of their functional properties (Prakash *et al.,* 2000).

The aim of this study was to investigate the effect of elevated temperature applied during the manufacture of various rose hip products on their antioxidant capacity compared to antioxidant capacity of fresh, unprocessed fruits.

MATERIAL AND METHODS

Preparation of rose hip products. The fruits of rose hip (*Rosa canina* L.) used in this experiment were harvested in the locality of Lazarevac (Kolubara district, West Serbia).

Fresh rose hip (whole fruits with seeds) was used as a control (sample A). Rose hip dried by convective drying in a laboratory dehydrator Stöckli with controled heater of 600 W, maintaining the set temperature of air, as described by Paunović *et al.* (2014) was used as sample B. Rosehip marmalade was produced by traditional method (sample C), previously described by Paunović *et al.* (2014).

Total dry matter. The total dry matter was determined by using standard gravimetric method (AOAC).

Determination of total phenolics. The amounts of total phenolics (TPC) in samples were determined according to the Folin-Ciocalteu method described by Singleton and Rossi (1965). Briefly, 0.5 mL of diluted samples were mixed with 2.5 mL of 10-fold diluted Folin-Ciocalteu's phenol reagent and allowed to react for 5 minutes. Two milliliters of sodium carbonate solution (75 g/L) was added to the mixture and then shaken. After 2 h of reaction at room temperature, the absorbance at 760 nm was measured. The calibration curve was prepared with gallic acid solution, and the results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g). Triplicate measurements were performed.

Determination of flavonoids. The total flavonoid concentration (TFC) was determined using a method developed by Zhishen *et al.* (1999) with some modification. Briefly, 0.5 mL of appropriately diluted sample was added to 2 mL of distilled water. At time zero, 0.15 mL of 5% $NaNO_2$ was added; at 5 min, 0.15 mL of 10% $AlCl_3$ was added; at 6 min, 1 mL of 1 M NaOH was added. Afterwards, the total volume of solution was immediately made up to 5 mL with distilled water and mixed well. The absorbance was measured at 510 nm against an appropriate blank. The calibration curve was prepared with quercetin standard solutions in ethanol, and results were expressed in milligrams of quercetin equivalents per gram of sample. Measurements were performed in triplicate.

DPPH radical-scavenging activity. DPPH radical-scavenging activity of samples was estimated following the slightly modified procedure described by Kaneda *et al.* (1995). Every diluted sample (0.2 mL) was added to the DPPH working solution (2.8 mL) (mixture of 1.86×10^{-4} mol/L DPPH in ethanol and 0.1 M acetate buffer (pH 4.3) in ratio 2:1 (v/v)). The absorbance at 525 nm was measured after the solution had been allowed to stand in the dark for 60 min. The Trolox calibration curve was plotted as a function of the percentage of inhibition of DPPH radical. The results were expressed as millimoles of Trolox equvivalents per gram of sample (mM TE/g). Triplicate measurements were performed.

RESULTS AND DISCUSSION

The total dry matter was 50.77%, 91.13% and 71.75%, for the samples A, B and C, respectively, while the content of total phenolic compounds was 45.6 mg/g (89.82 mg on a dry basis), 63.08 mg/g (69.22 mg on a dry basis) and 0.20 mg/g (0.28 mg on a dry basis) for the samples A, B and C, respectively. The loss of total phenolic compounds in relation to their content in fresh sample was 22.93% on a dry basis for the sample B and even 99.69% on a dry basis for the sample C (Table 1).

Fresh fruits were dried at moderate temperatures (60 °C and 50 °C), as a whole fruits. These temperatures have not significantly affected the loss of total phenolic compounds. The fruits were dried whole therefore plant tissue was not injured resulting in lower oxidation processes of conversion of polyphenol compounds into reactive quinones. In addition, polyphenoloxidase responsible for oxidation processes and enzymatic browning of foods has not been activated. During the production of marmalade the elevated temperature was applied (cooking temperature above 100 °C), the fruits were mashing and seeds were removed through cheesecloth. In this way, the plant tissue has been injured whereas the mixing during cooking provided intensive aeration, resulting in a significant loss of total phenolic compounds.

Table 1. Chemical composition and antioxidant activity of rose hip products

Sample	Dry matter content (%)	TPC (mg/g d.b.)	TFC (mg/g d.b.)	Antioxidant activity (mM TE/g d.b.)	The losses of TPC (% d.b.)	The losses of TFC (% d.b.)	The losses of antioxidant activity (% d.b.)
A	50.77	45.6 (89.82)	18.24 (35.93)	0.15 (0.29)	-	-	-
B	91.13	63.08 (69.22)	22.32 (24.49)	0.21 (0.23)	22.93	31.84	20.69
C	71.75	0.20 (0.29)	0	0	99.69	100	100

d.b. - on dry basis
A - control sample; B - dried by heat; C - produced by traditional method

The total flavonoids concentrations were 18.24 mg/g (35.93 mg on a dry basis) and 22.32 mg/g (24.49 mg on a dry basis) for the samples A and B, respectively, while it was 0 mg/g for the sample C. The loss of total flavonoids in relation to their content in fresh sample was 31.84% on a dry basis for sample B and even 100% for the sample C (Table 1).

The objective was to determine the influence of flavonoids which make only a part of total phenolic compounds on antioxidant capacity of rose hip products. Basis on the obtained results the effect of elevated temperature on the total flavonoids concentration was significant in relation to the effect of temperature on total phenolic substances. Considering that flavonoids are plant polyphenols, the reasons for their loss were the same as those listed above for the loss of the total phenolic compounds. During drying of rose hip fruits about one third of the total flavonoids concentration has been lost.

The completely loss (100%) of the total flavonoid concentration in relation to the fresh sample has been obtained in marmalade just because of the effect of elevated temperature, plant tissue injury and intensive aeration, suggesting that marmalade produced in laboratory conditions (or in household) possess reduced nutritional value. In order to preserve the components that affect the antioxidant capacity it is necessary to produce the marmalade at low temperature, feasible only in industrial conditions in a vacuum evaporator in the absence of air. Concerning that the Regulations on the quality of fruit and vegetables allow the use of pectin mixture, which bind water enabling reduction the time of cooking, a recommendation would be to apply the pectin preparations at home made production (Paunović *et al.,* 2014).

Antioxidant activity was 0.15 mM TE / g (0.29 mM TE on a dry basis) and 0.21 mM TE / g (0.23 mM TE on a dry basis) for the samples A and B, respectively, while it was 0 mM TE/g for the sample C. The loss of antioxidant activity in comparison to the antioxidant activity of the fresh sample was 20.69% on a dry basis for sample B and even 100% for sample C (Table 1).

Antioxidant activity of dried rose hip fruit reduced about one-fifth compared to the fresh sample, while the loss in the marmalade was total (100%). Concerning that content of vitamin C and total phenolic compounds contributes to antioxidant capacity (Ramamoorthy *et al.,* 2007; Rajić *et al.,* 2012; Rekha *et al.,* 2012), it was expected that the reduction of these components will reduced the value of antioxidant activity. In the previous study of Paunović *et al.* (2014), the content of vitamin C was 395.19 mg, 181.54 mg and 73.59 mg, on a dry basis for the samples A, B and C, respectively. Correlations between the total phenolic content, total flavonoid concentration, vitamin C content and antioxidant activity are given in Figures 1, 2 and 3.
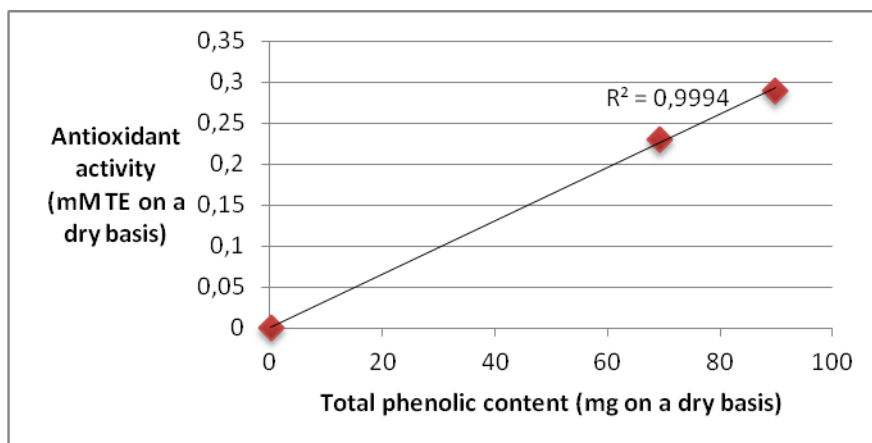
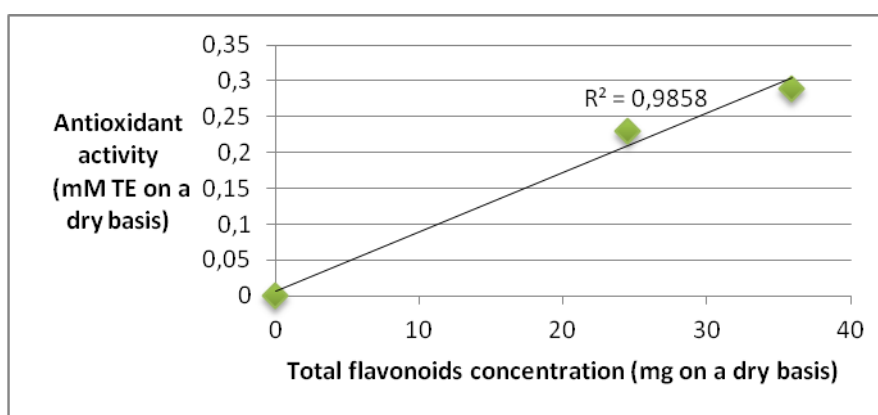*Figure 1. The correlation between the total phenolic content and antioxidant activity*

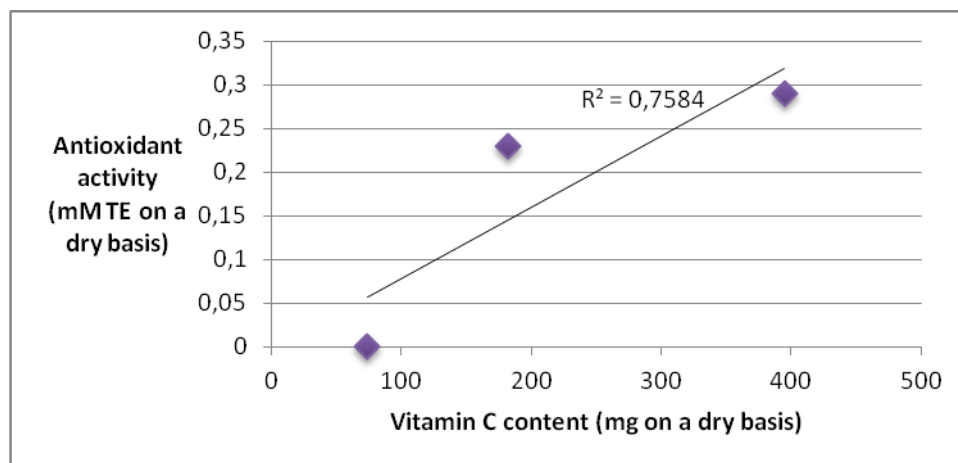*Figure 2. The correlation between the total flavonoids concentration and antioxidant activity*

Figure 3. The correlation between the vitamin C content and antioxidant activity

CONCLUSIONS

Convective drying of fruits for making herbal teas can provide a satisfactory quality. Drying at moderate temperatures (60 °C and 50 °C) provided the loss of 22.93%, 31.84% and 20.69% for the total phenolics, total flavonoids and antioxidant activity, respectively, compared to fresh fruits, calculated on a dry matter. On the other hand, in the production of marmalade the loss of tested compounds was very significant amounted to 99.69% on a dry basis for the total phenolic compounds whereas the completely loss (100%) was obtained for the total

flavonoids and antioxidant activity, compared to the fresh material. The loss of the tested components has been affected by the elevated temperatures, operating time of elevated temperatures, injuries of plant tissue during mashing and aeration.

Generally it can be concluded that antioxidant capacity of the tested samples has been primarily influenced by the total phenolic compounds, followed by vitamin C content.

ACKNOWLEDGEMENTS

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INTERACTIONS OF INDIGENOUSLY LACTIC ACID BACTERIA ISOLATED FROM VEGETAL SOURCES WITH SPOILAGE FUNGI

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ABSTRACT

Significant losses in food industry are due to spoilage fungi that contaminate all kinds of raw materials, including legumes, fruits, cereals or meats. Fungal contamination can induce several undesirable effects, including mycotoxin production, off flavors, rotting, discoloration etc. Lactic acid bacteria, generally accepted as GRAS („Generally Recognized As Safe") organisms have large impact in food industry, not only for their fermentation abilities but also for protective action against spoilage microbes. For this reason, the aim of our study was the examination of the inhibitory action of some strains of *Lactobacillus* spp. isolated from Romanian traditional foods against fungi like *Botrytis cinerea, Alternaria* spp., *Aspergillus* spp., *Monilinia fructigena, Rhizoctonia solani* and *Penicillium* spp. which contaminate fruits and legumes. Eight broad-spectrum LAB strains were selected by double layer techniques and their protective antifungal action was evaluated in an *in vitro* fruit model, using tomato, apricot and peach homogenate as culture substrate. Organic acid production was evaluated by HPLC method and revealed a good correlation with antifungal action of some LAB. Two out of the eight LAB tested demonstrated high protective effects against at least *Botrytis cinerea, Alternaria spp.* and *Monilinia fructigena*, important plant pathogens involved in fruits and legumes alteration. Moreover, the selected LAB strains were able to inhibit some *Aspergillus* spp. (*A. niger, A. flavus*) mycotoxigenic strains. In conclusion, the results demonstrate the functionality of these antifungal isolates as bio-protectants that could reduce the level of contamination by spoilage fungi.
Keywords: LAB, spoilage fungi, in vitro fruit model

INTRODUCTION

Microbial safety of foods and feeds is of great interest for industry and consumers. Molds growth create major quantitative losses (Pitt and Hocking, 1999) and qualitative depreciation (Filtenborg *et al.*, 1996), including hazardous effects such as mycotoxin contamination (Bryden, 2007). Public demand for safe, preservative free food, with extended shelf-life increased the interest for competitive biological traits. LAB are good candidates for industrial and commercial exploitation being regarded as safe microorganisms („GRAS" microorganisms), able to produce various bio-active compounds (Yang and Chang, 2010; Wang *et al.*, 2012). In the last few years LAB have been proven to inhibit various foods and feed microbial contaminants by bioactive compounds like organic acids, fatty acids, hydrogen peroxide or bacteriocins (Gerez et al., 2013), as well as they are able to control mycotoxins (Dalie et al., 2010; Tsitsigiannis et al, 2012). The inhibition of some plant pathogens by selected LAB was also communicated (Mauch et al., 2010; Trias et al., 2010; Lutz et al., 2012) proving their possible utilization as biocontrol agents.

The current study aims to demonstrate the ability of some selected LAB to inhibit both potentially mycotoxigenic fungi (*Aspergillus* spp. and *Penicillium* spp. and plant pathogens like *Alternaria* spp*., Botrytis cinerea, Monilinia* spp*.* using an *in vitro* fruit model.

MATERIAL AND METHODS

Microbial strains and growth conditions
The lactic acid bacteria used in our study were formerly isolated from plant materials and Romanian traditional fermented foods, sauerkraut and pickles (Sicuia et al., 2014). Seven out of them, designated as L22, L26, L35, L43, L49, L58 and L120 were used in this work. Three collection strains of LAB, *Lactobacillus plantarum* IC12353 (Lpl), *Lb.paracasei* CCM 1837 (Lpa) and *Lb.acidophilus* IC11692 (Lac), were used as reference strains. The bacterial stocks were maintained frozen at -20°C in De Man, Rogosa and Sharpe (MRS)(de Man et al., 1960) broth medium with 25% glycerol (v/v). Before use, the LAB strains were propagated twice in MRS broth at 36°C, than cultures were obtained in the same condition by incubating for different times, 24, 48 or 72 hours.
All molds (Table 1) were cultivated on Potato-Dextrose-Agar (PDA) medium at 27°C.

Table 1. Fungal strains used in this study

Fungal species	Number of strains	Source
Alternaria spp.	3	Tomato fruit
Aspergillus flavus	11	Grain seeds
Aspergillus niger	5	Grain seeds
Aspergillus ochraceus	4	Bread
Botrytis cinerea	2	Strawberry fruits
Botrytis cinerea	3	Raspberry fruits
Monilinia spp.	3	Apricot and pear fruits
Rhizoctonia solani DSM 63002	1	German Collection of Microorganisms and Cell cultures (DSMZ)
Penicillium spp.	3	Citrus fruits
Penicillium crustosum		Cucumber pickles

Antifungal activity assays
The LAB antifungal activity was examined by double layer technique and/or by the double cultures technique, as described by Sicuia *et al.* (2014).
LAB organic acids quantification was performed by HPLC, using HPLC System (Waters) for chromatographic separation and quantification, and Empower 2 software for the data acquisition and management.
In vitro fruit model assay
The antifungal activity of LAB was also evaluated using the *in vitro* fruit model described by Crowley *et al.* (2013) with slight modifications. As culture substrate, we used tomatoes, apricots or peaches prepared as fresh smashed fruits (20% w/v) in 2% agar, neutralized to pH 7-7.2 with sodium hydroxide. Petri plates (Ø=5cm) containing fruits homogenate were overlaid with 100µL of fresh LAB culture, and then inoculated in the center of plates with fungi. The *Alternaria* spp., *Botrytis cinerea* and *Monilinia fructigena* inoculum consisted in mycelia plugs (5mm in diameter), while conidial suspension (10 µL) was used for *Alternaria* spp., *Aspergillus* spp. and *Penicillium* spp. Plates were incubated at 30°C, and fungal growth was daily examined for seven days. The inhibition efficacy expressed by the LAB strains was calculated based on Islam *et al.* (2009) algorithm:

$$E\% = \frac{CG - RI}{CG} \times 100$$

Where: E (%) = antagonistic efficacy on fungal growth inhibition; CG = mycelia growth in the control (mm), RI = mycelia growth after antagonistic interaction (mm).
Statistical analysis
The unpaired student t test was used to determine significant differences in fungal growth (diameters of fungal growth) on fruit homogenate containing media between the control and test samples. A P value of <0.05 was considered statistically.

RESULTS AND DISCUSSION

Previous screening experiments allowed the selection of various lactic acid bacteria that exhibit broad-spectrum antifungal action against plant pathogens and potential mycotoxigenic fungi (Sicuia et al., 2014).

Eight out of these strains (seven new isolates and one collection strain - Lpl) were used in this study in order to examine their effects against selected potential food contaminants. Strains of *Alternaria* spp*, Botryrtis cinerea, Monilinia* spp., *Penicillium* spp., *Aspergillus niger, A.flavus, A.ochraceus* isolated from fruits or seeds sources were used as targets for LAB action. It was shown that at least three LAB strains were able to inhibit the growth and/or conidial maturation of *A.flavus* and *A.ochraceus* strains (Figure 1).
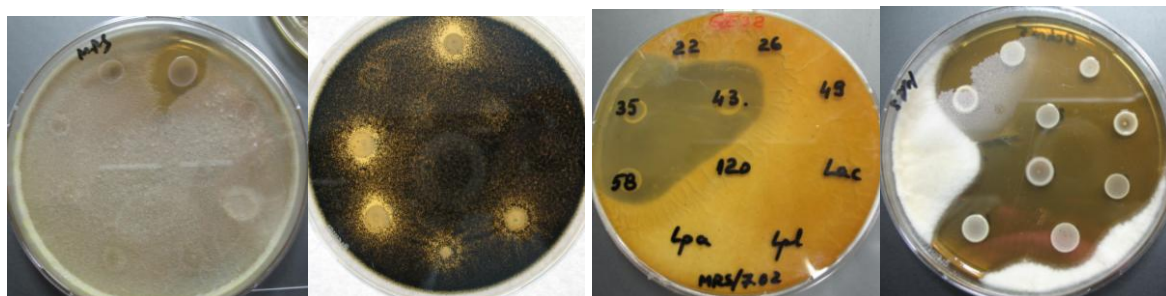
Figure 1. Fungal growth/conidial maturation inhibition of Penicillium crustosum CM, Aspergillus niger An4, A. flavus GE32, and A.ochraceus (from left to right) produced by selected LAB strains

All the selected LAB produce lactic acid but the quantities varied from 74.23 mmol/L for L43 to 308.4 mmol/L for L22 (after 24h of growth in MRS); the acetic acid was produced in reduced quantities: less amount was detected in the strains that synthesized more lactic acid (29.37 mmol/L in L43 and 7.8 mmol/L in L22). Phenyllactic acid and hydroxyphenyllactic acid were detected in some strains.

The protective effect of selected LAB as well of the reference strains against some spoilage fungi (*Alternaria* spp*, Botrytis cinerea, Monilinia* spp., *Penicillium* spp*., Rhizoctonia solani)* was examined on fruit homogenate (apricot, peaches and tomato) containing plates. Significant growth inhibition of *Alternaria* spp. on tomato containing medium was observed for L35 – 40%, L43 – 53,5% and L58 – 59,1% (Figure 2).
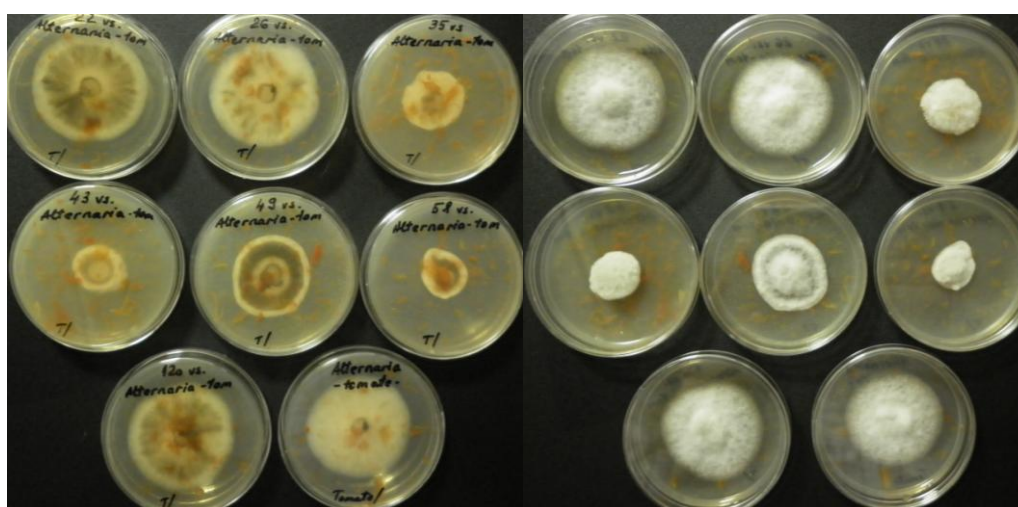

Figure 2. Growth inhibition of Alternaria spp. produced by LAB on tomato homogenate containing medium

Similar results against *Alternaria* spp. were obtained when peaches homogenate was used as growth substrate (Figure 3).
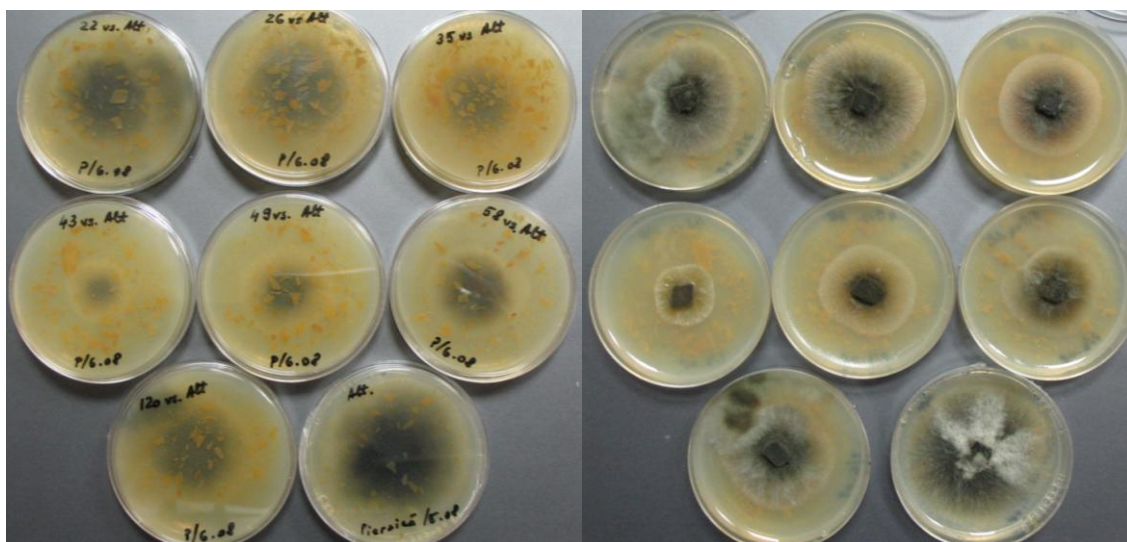

Figure 3. Growth inhibition of Alternaria spp. produced by LAB on peaches homogenate containing medium

The use of apricot homogenate as growth substrate and selected LAB rendered inhibition of *Penicillium* spp. growth (isolated from citrus), best action being observed for L120, L22 and L26 (Figure 4). No significant inhibitory effect of LAB on this medium was observed against the other fungi.
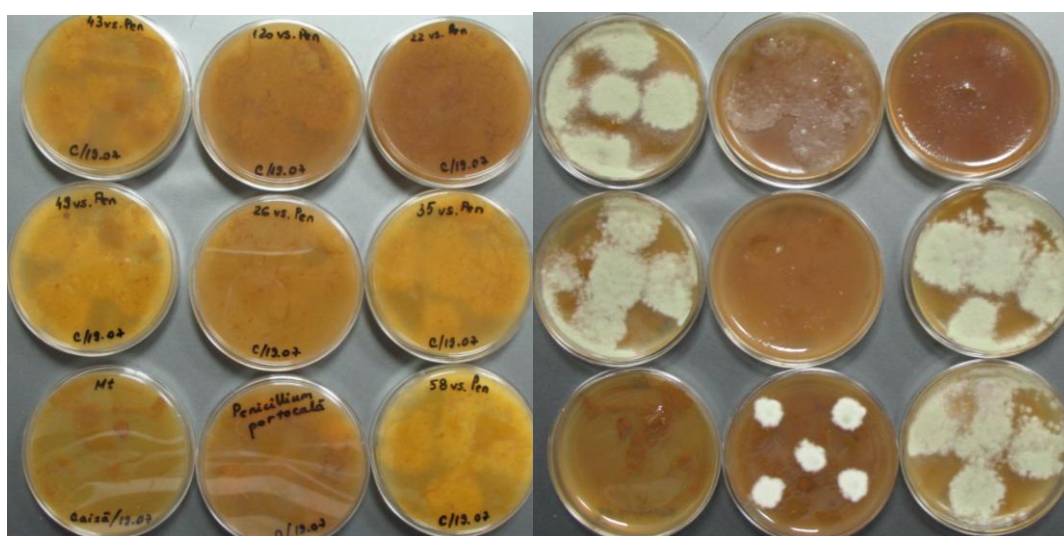
Figure 4. Penicillium spp. development inhibition produced by LAB on apricot homogenate containing medium

Regarding the development of *B.cinerea* on tomato and peaches homogenate containing media in the presence of LAB it was proven that the best inhibition was produced when peaches were used as substrate: the inhibition efficacy of all the strains, except L58, increased by 2-3 times (Figure 5). This effect could be due to the higher sugar concentration of peaches and/or to other molecules that promote the biosynthesis of antifungal bioactive compounds in LAB.
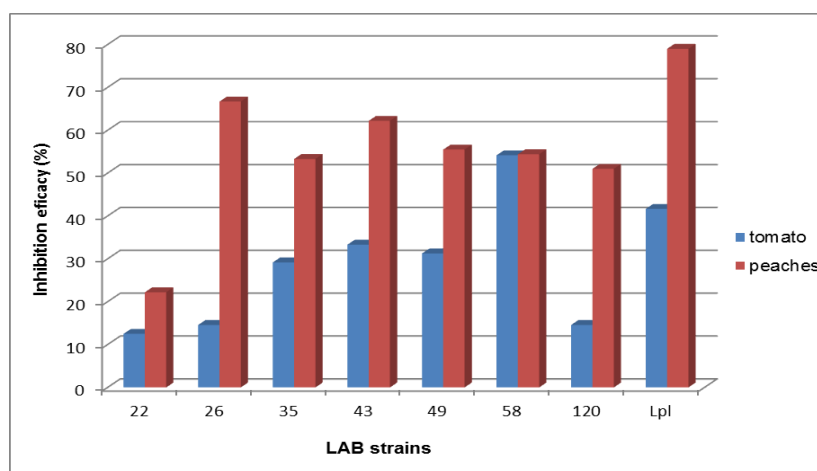
Figure 5. Comparative antifungal eficiency of LAB on tomato or peaches containing medium

The strains of *Monilinia* spp. tested in our experiments exhibited generally resistance to LAB action on apricot based medium while on peaches homogenate the rate of inhibition was slightly increased: Lpl and L58 proved to be the most active LAB strains on this plant pathogen (Figure 6).
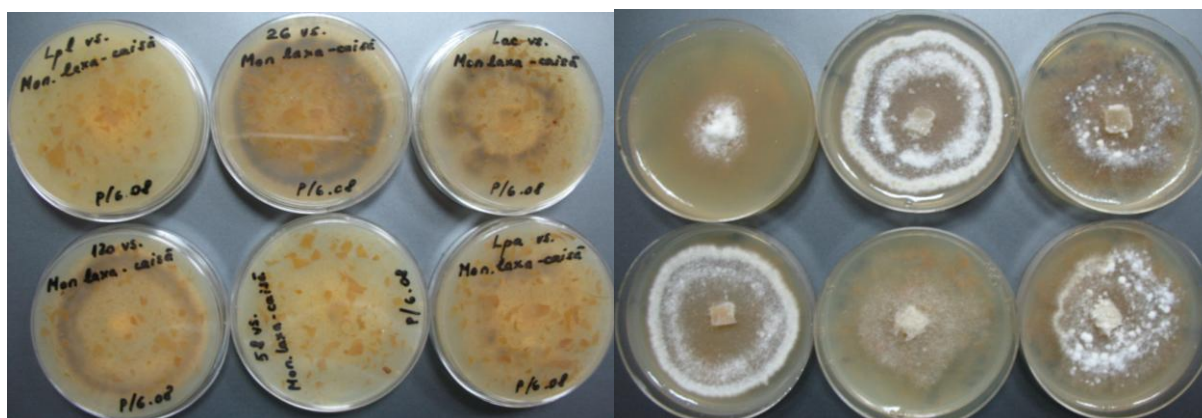

Figure 6. Growth inhibition of Monilinia spp. by selected LAB co-cultivated on peaches containing culture medium.

CONCLUSIONS

The use of LAB as biocontrol agents against plant pathogenic fungi presents both challenges and opportunities for management of plant diseases in order to obtain high quality food products.
Inhibitory action of LAB is generally detected *in vitro* by direct interaction with target fungi on specific growth media. The use of fruit homogenate based media for detection of antifungal effect of LAB could offer more natural conditions to study the mechanisms of interactions. The seven LAB selected in an extended program of screening natural bacterial isolates with antimicrobial abilities presented broad spectrum antifungal action by „classical" *in vitro* interaction. Their inhibitory action is due, mainly to organic acids production, but other mechanisms are not excluded (at least for L43 strain). On the fruit based media differences among antifungal action of LAB were detected: the inhibition of *Penicillium spp.* was clearly demonstrated on apricot containing medium by three LAB strains (L22, L26 and L120); *Alternaria* spp. and *B.cinerea* were clearly inhibited by strains L35, L43 and L58 when co-cultivation was performed on tomato or peaches based media. The use of peaches as

growth substrate increased 2-3 times the antifungal action of all bacterial strains, probably by compounds that promote the synthesis of bioactive compounds.

The results obtained in this study demonstrate the functionality of the antifungal LAB isolates as bio-protectants that could reduce the level of contamination with spoilage fungi.

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FUMONISINS IN FOOD: ORGANIC VERSUS CONVENTIONAL

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ABSTRACT

The European Union regulated the maximum concentrations of fumonisins (as the sum of FB1 and FB2) from 200 to 2000 µg/kg in maize-based products and unprocessed maize. The present study was carried out to obtain information on the presence of mycotoxins FB1 and FB2 in conventionally and organically produced food. The LC-MS/MS method was used for the detection of fumonisins in a one-step chromatographic run using an MS Agilent Triple Quad 6410B. The extraction was performed with acetonitrile/water. After the filtration the extract was cleaned up using Fumoniprep®, R-Biopharm AG. The mobile phase was a mixture of methanol and water with 0.1% formic acid, flow 0.5 ml/min in gradient program starting from 90% water to 5% water for 15 min. The LODs, defined as the lowest concentration that the analytical process can reliably differentiate from background levels, were estimated for those concentrations that provide a signal to noise ratio of 1:3. These values of the LODs are 0.25 µg/kg for the FB1 and 0.5 µg/kg for FB2. The LOQs estimated as those concentrations of analytes which yield a signal-to-noise of at least 1:10, were 1.0 µg/kg for FB1 and 2.0 µg/kg for FB2. The aim of this study was to compare the results of samples of cereals-based baby food produced from organically grown cereals as well as commercially produced raw materials. The organic farming system produces food that is less likely to be contaminated with *Fusarium* species, although no significant difference in fumonisins was found between organic and conventional production.
Keywords: *fumonisins, LC-MS/MS, organic, conventional, food*

INTRODUCTION

Grain based products are one of the main sources of mycotoxins which are harmful to both humans and animals (Kuzdralinski *et al*., 2013). The fumonisins are a group of mycotoxins produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum*. There are at least 28 different forms of fumonisins, most designated as A, B, C, and P-series. Fumonisin B_1 is the most common and economically important form, followed by B_2 and B_3 (Silva *et al*., 2009). The European Union regulated the maximum concentrations of fumonisins (as the sum of FB1 and FB2) from 200 to 2000 µg/kg in maize-based products and unprocessed maize (Regulation (EC) No 1126/2007). The organic farming in the EU is a system of agriculture and food production that combines favourable environmental and animal welfare standards and is supported by the EU law (Regulations (EC) No 834/2007 and No 889/2008). The organic production system strives for minimal disruption of the natural equilibrium while ensuring the production of high-quality food. Organic methods in farming are considered as environmentally friendly, mainly due to a fundamental principle of harmonious cooperation with nature and prohibition of chemical products such as fungicides and growth regulators. Currently conventional processing allows the use of plant protection products. In the scientific literature, the possible health risks caused by mycotoxins in organic products have been examined.
The aims of this survey were to investigate the distribution of fumonisins in maize grains from organic and conventional production systems and to calculate the relationships between FB1 and FB2 mycotoxins content in analysed samples.

MATERIALS AND METHODS

Chemicals and reagents

FB1, 50 µg ml⁻¹ in 50:50 acetonitril: water, was purchased from Sigma Aldrich, (Germany) and FB2, 50 µg ml⁻¹ in acetonitrile was purchased from Sigma-Aldrich, (Germany). Referent material (Product No.P64/F428) purchased from R-Biopharm Rhône Ltd (Glasgow, Scotland), Methanol and Acetonitrile (for HPLC, Gradient Grade), were from J.T.Backer (United Stated). Formic acid (98/100%, laboratory reagent grade) was from Fischer Scientific (Loughborough, UK). Pure water was obtained from Purelab® ELGA water purification system (Vivendi Water Systems Ltd UK). Glass microfiber filters (GF/A) were from Whatman, Cat. No. 6880-2504 (Maidstone, UK). Econofilters regenerated cellulose (0.45µm) were from Agilent, Germany. Fumoniprep (Product Code P31) immunoaffinity columns were from R-Biopharm Rhône Ltd, (Glasgow, Scotland). Potassium chloride, potassium dihydrogen-phosphate, anhydrous disodium hydrogen-phosphate, and sodium chloride were obtained from Merck (Darmstadt, Germany).

Preparation of standard solutions and reagents

The stock standard solution for every fumonisin was prepared at 1000 µg ml⁻¹ in acetonitrile (ACN) and stored at -20 °C. The working standard solutions containing both compounds were obtained by the further dilution of stock individual solutions with methanol:water (50:50, v:v).

The phosphate buffer solution (PBS) was prepared from 0.2 g potassium chloride, 0.2 g potassium dihydrogen phosphate, 1.2 g anhydrous disodium hydrogen-phosphate, and 8.0 g sodium chloride to 990 mL deionized water, adjusted to pH 7.0 with 25% HCl, and the solution was made to 1 L.

Samples and sample procedure

Conventional samples of different kinds of cereals-based baby food were purchased in commercial shops and border inspection, during 2013. The organic food samples were collected from health food stores and the same samples prepared for export. (When needed, the samples were finely milled). Ground samples (10 g) were extracted with 100 mL acetonitrile: methanol: water mixture (25:25:50, v:v:v) by blending, using commercial blender with glass blender jars (Vicam) for 1 minute. Sampe extracts were filtered through Whatman No. 4 filter paper and centrifuged for 5 minutes at 12000 rpm. For clean-up, 10 mL of supernatant was diluted with 40 mL of phosphate buffered saline (PBS, pH 7.4), mixed well and filtrated through CA filter 0.45 µm (Whatman). All 50 mL was added to Fumoniprep (R-Biopharm) IA column attached onto a vacuum manifold. The column was washed twice with 10 mL of PBS, and FBs were eluted with 1.5 mL methanol followed with 1.5 mL of deionized water.

Instrumentation and chromatographic conditions for LC-MS/MS

LC was performed with an Agilent 1200 HPLC system equipped with a G1379B degasser, a G1312B binary pump, a G1367D autosampler and a G1316B column oven (Agilent Technologies, USA). Chromatography separation was achieved by Zorbax Ecllipse XDB C18 column (50 x 4.6 mm, 1.8 µm) (Agilent, USA) maintained at 30 °C. The analytical separation was performed using gradient program starting from 90% water to 5% water for 15 min, with methanol as mobile phase A, and as water mobile phase B, both containing 0.1% formic acid (70:30, v:v). The flow rate was maintained at 0.5 mL min⁻¹. The mass analysis was carried out with an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA) (Turner et al., 2009). The data acquisition and quantification was conducted using MassHunter Workstation software B.03.01 (Agilent Technologies 2010). The following ionization conditions were used: electrospray ionisation (ESI) positive ion mode, drying gas (nitrogen) temperature 325 °C, drying gas flow rate 5 l/min, nebulizer pressure 50 psi and capillary voltage 3000 V. The dwell time was 100 ms. External standard method was used for quantification of FBs.

RESULTS AND DISCUSSION

Optimization of the chromatographic separation for FB1 and FB2

For the quantification the one with the best signal sensitivity (Q) was preferred and for the confirmation the second transition (q) and the ratio of abundances between both ion transitions (Q/q) were used. The cone voltages were selected according to the sensitivity of the precursor ions and the collision energies were chosen to give the maximum intensity of the fragment ions obtained. Table 1 lists the mass spectrometer parameters as precursor and product ions as well as the optimized cone voltages and collision energies used. The product-ion spectra obtained on triple quadrupole instrument generally provide fragments which are of diagnostic value for structural elucidation and confirmation. FBs have a common fragmentation pathway involving sequential losses of water and two tricarballylic acid (TCA) side chains from the alkyl backbone (Sulyok *et al.*, 2006).

For the detection of FB1 the precursor ion was *m/z* 722.5, being the product of the ions selected *m/z* 352.3 and 334.4. For FB2 and FB3, different retention time was obtained but with the same transitions, the precursor ion was *m/z* 706.4, and the product ions *m/z* 318 and 336. Fumonisin B3 (FB3) was qualitatively determined in CRM, since analytical standard was not purchased.

One significant drawback in ESI/MS quantitative analysis is the matrix effect (Cun *et al.*, 2012). In our investigation the matrix effect is not important, because the samples were prepared by immunoaffinity column.

Table 1. MRM conditions for QQQ

Fumonisins	Polarity	R $_{time}$ (min)	Precursor (m/z)	Product (m/z)	CV (V)
Fumonisin B1	$[M+H]^+$	6.5	722.5 722.5	352.3 334.4	40 40
Fumonisin B2	$[M+H]^+$	7.6	706.4 706.4	336 318	35 35
Fumonisin B3	$[M+H]^+$	8.5	706.4 706.4	336 318	35 35

Method validation

The calibration curves were linear in the studied working range with correlation coefficients greater than 0.99 (FB1 y=238.16x+2438.84, R^2=0.9985; FB2 y=535.88x+2325.79, R^2=0.9974) (Fig 1). The chromatogram obtained from real sample of food is depicted in Fig 2. demonstrating good chromatographic separation and satisfactory sensitivity. The average recoveries of FB1 and FB2 by adding different spiking levels to cereal-based baby food samples are presented in Table 2. and they varied from 92-96% with a relative standard deviation from 3-6% for FB1 and from 94-97% with a relative standard deviation from 4-7% for FB2.

The precision of the method in terms of repeatability (r) (intra-day precision) and reproducibility (R) (inter-day precision) was evaluated calculating the relative standard deviation (%RSD) of five samples of reference material also analyzed in triplicate on different days (Table 3). The limit of detection was calculated by MassHunter Software, for those concentrations that provide a signal to noise ratio of 3:1. These values of the LODs are 0.25 µg/kg for the FB1 and 0.5 µg/kg for FB2. The limits of quantifications (LOQs) estimated as those concentrations of analytes which yield a signal-to-noise of at least 1:10, were 1.0 µg/kg for FB1 and 2.0 µg/kg for FB2. LOQs obtained were well below the maximum levels for FBs set by the European legislation for foodstuffs.
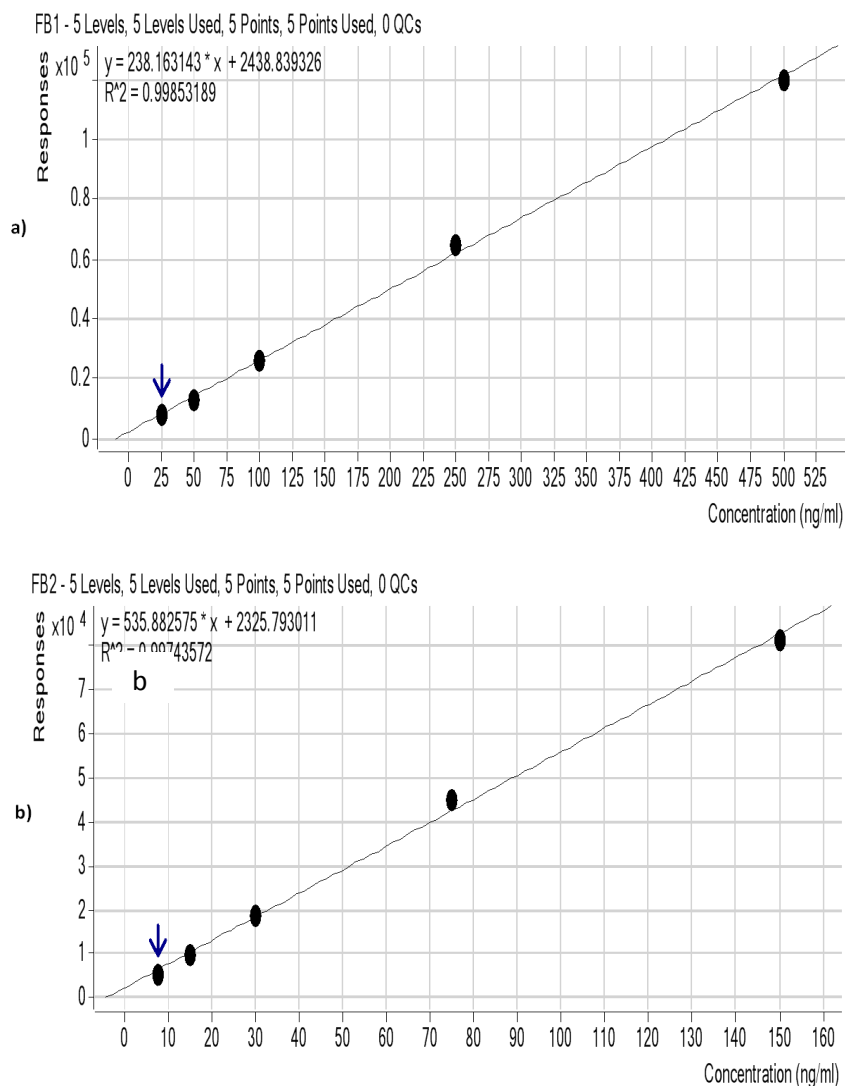
FB1 - 5 Levels, 5 Levels Used, 5 Points, 5 Points Used, 0 QCs

$y = 238.163143 * x + 2438.839326$
$R^2 = 0.99853189$

a)

FB2 - 5 Levels, 5 Levels Used, 5 Points, 5 Points Used, 0 QCs

$y = 535.882575 * x + 2325.793011$
$R^2 = 0.99743572$

b)

Figure 1. Calibration curve of FB1 (a) and FB2 (b)

Table 2. Recoveries and RSDs

Recovery					
FB1			**FB2**		
Fortification levels (µg/kg)	**%**	**RSD (%)**	**Fortification levels (µg/kg)**	**%**	**RSD (%)**
10	92	3	10	94	4
400	93	3	400	95	4
1000	96	6	1000	97	7

Table 3. Precision determinated on CRM

Compounds	**Intra - day precision (n=5)**	**Inter - day (n=3)**
	RSD (%)	**RSD (%)**
FB1	2.8	7.6
FB2	2.0	11.2

Application to real samples

The applicability of the proposed method was evaluated by analyzing 19 samples of different organic (10 samples) and conventional cereal-base baby food samples (9 samples). The FBs were detected in two organic food samples (the concentrations of FB1 were 331 and 565 µg kg⁻¹, and of FB2 they were 75 and 151 µg kg⁻¹). The concentrations of FB1 detected in four

conventional samples were 35, 85, 86 and 230 µg kg^{-1} while the concentrations of FB2 were 10, 14, 22 and 35 µg kg^{-1}.

According to the legislation in our country the MRL value for FBs (FB1+FB2) is 200 µg/kg (Off. Gazette RS 45/2010). Bearing in mind the regulations the detected values for FBs in 20% of samples from the organic production (FBs were 406 and 716 µg kg^{-1}) and in 11% of samples from the conventional production (FBs was 265 µg kg^{-1}) were above the MRL values (Off. Gazette RS 45/2010). Fig. 2 shows a chromatogram of samples of organic food from rice and maize with prebiotics.
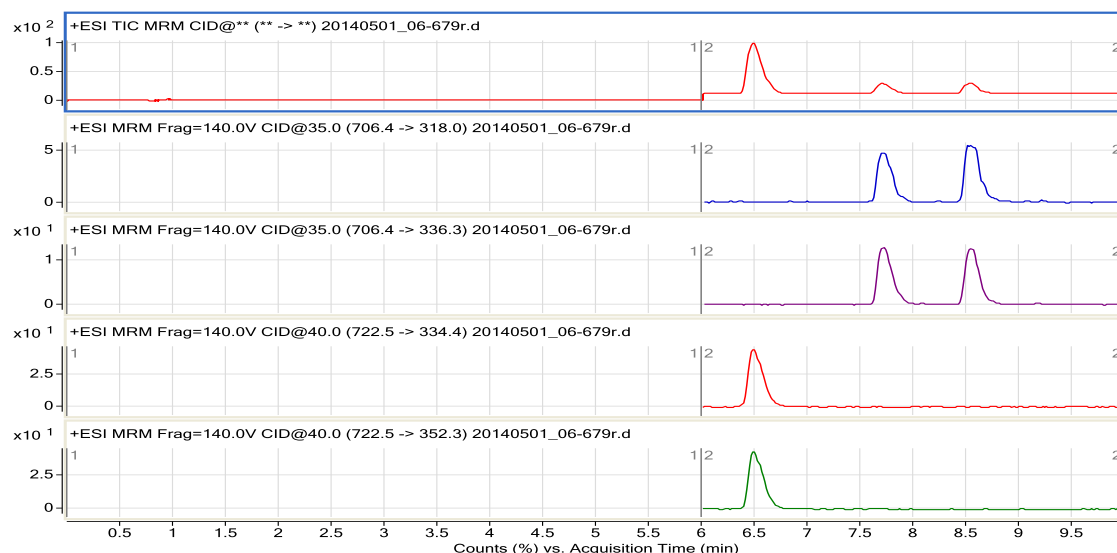
Figure 2. LC-MS/MS chromatogram of sample of organic food from rice ond maize with prebiotics

CONCLUSION

Fumonisins were analyzed in 10 samples of cereal-based baby food produced from organically grown cereals as well as commercially produced raw materials. The most dominant fungi recorded were *Fusarium verticillioides*, and *Fusarium* sp., the already well known producers of the fumonisins. The detections of FB1 in the samples from the organic production were in the range from 331 to 565 µg kg^{-1} and FB2 from 75 to 151 µg kg^{-1}. In the conventional production they were more frequent but lower i.e. FB1 and FB2 ranged from 35 to 86 µg kg^{-1} i.e. from 10 to 85 µg kg^{-1}, respectively. This study is the first to evaluate organically-grown-cereals samples in Serbia for the presence of FB1 and FB2. The organic farming system produces food that is less likely to be contaminated with *Fusarium* species, although no significant difference in fumonisins was found between the organic and conventional production. The developed method was satisfactorily applied as routine procedure to identify and quantify fumonisins in the laboratories of food quality and safety control, because of its robustness and feasibility.

ACKNOWLEDGEMENT

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