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DETECTION AND CHARACTERISATION OF *PLUM POX VIRUS* (PPV) ISOLATES FROM EASTERN SLOVAKIA REVEALED THE PRESENCE OF THREE MAIN VIRAL STRAINS.

Július Rozák, Lukáš Predajňa, Zdenka Gálová, Miroslav Glasa

ABSTRACT

Plum pox virus (PPV), the agent responsible for Sharka disease, is the most important viral pathogen of stone fruit trees world-wide, having an endemic status in Slovakia. To increase knowledge of PPV diversity in Slovakia, a set of 11 isolates, originated from the eastern part of the country, was characterised. The isolates were chip-budded from their original *Prunus* hosts to the susceptible GF305 indicators, exhibiting the symptoms of variable severity. A genomic region encompassing the partial NIb and the hypervariable 5´terminal region of the CP gene was amplified from all 11 isolates in RT-PCR and directly sequenced. The phylogenetic analysis revealed the grouping of the 11 Slovak isolates into 3 distinct clusters, representing the PPV-M (2 isolates), D (7 isolates) and Rec strains (2 isolates). The strain affiliation of isolates was further confirmed by strain-specific RT-PCR, using which the presence of additional mixed infection by minor PPV variants was detected in 2 samples. The results further contribute to the understanding of PPV diversity in Slovakia and confirm the specificity and sensitivity of molecular approaches used for the virus strain determination.

Keywords: *Prunus*; virus; RT-PCR; strain; diversity

INTRODUCTION

Plum pox virus (PPV), belonging to the genus *Potyvirus*, is the causal agent of Sharka, the economically most important viral disease of *Prunus* crops (plum, apricot, peach) as well as wild and ornamental species (**Cambra et al., 2006**). Nowadays, PPV was detected in all continents apart from Australia (**García and Cambra 2007**). Depending on the *Prunus* host and its susceptibility, the PPV-infected trees may exhibit a range of symptoms on leaves (spots, rings and mosaics, vein clearings, chlorosis and/or leaf distortions). Symptoms on plum fruits consist generally of shallow ring or arabesque depressions, sometimes with brown or reddish necrotic flesh and gumming. Infected apricots develop depressed rings on fruits, which may be severely deformed. Symptoms in peach are often less conspicuous and may represent pale rings or diffuse bands on the skin of the fruits. Typically, infected fruits of susceptible *Prunus* genotypes fall prematurely and have considerably decreased organoleptic quality. The symptoms may vary considerably with the age, the temperature and nutrient status of the plant (**Garcia et al., 2013**).

PPV is efficiently transmitted by vegetative propagation of infected material and by aphids in the nonpersistent manner (**Labonne et al., 1995; Garcia et al., 2013**).

The PPV genome consists of a single-stranded positive sense RNA molecule of about 10,000 nucleotides, coding a single open reading frame (ORF) flanked by shorts untranslated regions. The single large polyprotein hydrolyses itself after translation into at least 11 proteins (**Lopez-Moya et al., 2000**).

On the basis of molecular differences, 8 PPV strains are now recognised (referred to as M, D, EA, C, Rec, W, T, CR in the order of their discovery). PPV-M, D and Rec strains are prevalent in Europe and they are considered as major strains (**Glasa and Candresse, 2008; Šubr and Glasa, 2013; Garcia et al., 2013**). RNA viruses are characterised by high mutation rates due to the lack of proofreading activity associated with their viral RNA dependent RNA polymerases (**Desbiez et al., 2011**). Besides the mutations, recombination was found to play an important role in the evolutionary history of PPV (**Glasa et al., 2004; Garcia et al., 2013**). In case of PPV, the genetic diversity might be increased by the fact that *Prunus* trees remain in the field for more years or decades, enabling a development of the heterogeneous viral populations (**Jridi et al., 2006; Predajňa et al., 2012**) or even a mixing of different strains in a single host (**Candresse et al., 1998**).

Understanding the diversity of plant viruses is the essential step to design efficient management strategies. The Sharka disease has been first described on the territory of Slovakia in the 1950´s (**Králiková, 1964**). The first attempts to characterise the diversity of the PPV isolates spread in Slovakia were based on their symptomatology on the woody and herbaceous indicator plants (**Paulechová, 1981, Glasa et al., 1997**), mobility of their capsid proteins (**Šubr and Glasa, 1999**) and the restriction analysis of the short genome fragment encompassing the capsid protein (CP) gene (**Glasa et al., 1998**). Later, the partial and complete genome characterisation of a number of PPV isolates has permitted to obtain a comprehensive view on their molecular variability on the territory of Slovakia (**Glasa et al., 2004; Predajňa, 2013**). In this work, the picture of PPV diversity was complemented by the biological and partial molecular characterisation of

11 isolates originated from the different *Prunus* species in the Eastern Slovakia.

MATERIAL AND METHODOLOGY

Viral isolates. A set of 11 PPV isolates was collected from the main cultivated and wild *Prunus* hosts from orchards, gardens or wild landscape in Eastern Slovakia in 2012 (Table 1). The isolates were maintained in the chip-budded *Prunus persica* GF305 plants under glasshouse conditions. The symptoms were evaluated visually during two consecutive seasons. In all GF305 plants, the presence of PPV, *Prunus necrotic ringspot virus* (PNRSV) and *Prunus dwarf virus* (PDV) was periodically checked by multiplex reverse-transcriptase polymerase chain reaction (RT -PCR) using the protocol developed by **Jarošová et al. (2008)**.

Strain-specific RT-PCR. Total RNAs were extracted from infected leaves of GF305 using the NucleoSpin RNA Plant kit (Macherey-Nagel). A two-step reverse transcriptase polymerase chain reaction (RT-PCR) protocol was systematically used. The first-strand cDNA was synthesized by reverse transcription of total RNA using random hexamer primers and the *Avian myeloblastosis virus* (AMV) reverse transcriptase (both from Promega Corp.) and subsequently used in the three strain-specific PCRs using the combination of primers mM5 (5'-GCTACAAAGAACTGCTGAGAG-3', forward)/mM3 (5'-CATTTCCATAAACTCCAAAAGAC-3', reverse) for PPV-M detection, mD5 (5'-TATGTCACATAAAG GCGTTCTC-3', forward)/mD3 (5'-GACGTCCCTGTCT CTGTTTG-3', reverse) for PPV-D detection, and mD5/mM3 for PPV-Rec detection **(Šubr et al., 2004)**. PCR was performed using the EmeraldAmp MAX PCR Master Mix (Takara, Bio Inc.) under the following conditions for all combinations: denaturation 98 °C 3 min, 35 cycles of 94 °C 30 sec, 60 °C 20 sec, and 72 °C 30 sec and final elongation 72 °C 5 min.

Partial sequencing and sequence analyses. A 746-bp fragment spanning the 3´-terminal part of the nuclear inclusion b gene (NIb) and the 5´-terminal part of the CP gene (nt 8316–9061, based on the complete sequence of the BOR-3 isolate, AY028309) was amplified using the TaKaRa Ex Taq polymerase (TaKaRa, Bio Inc.) and the primer pair NCuniFor 5′-GAGGCAATTTGTGC TTCAATGG-3´ (sense) and NCuniR 5´-CGCTTAACT CCTTCATACCAAG-3´(antisense) **(Predajňa et al., 2012)** under the following cycling conditions: initial denaturation at 94 °C for 5 min; 25 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min; final extension step at 72 °C for 10 min. The RT-PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corp.) and directly sequenced on both strands using the same PCR primers, using an automated DNA sequencer (ABI 3130xl Genetic Analyser; Applied Biosystems). Sequence analyses were performed using the Molecular Evolutionary Genetics Analysis software (MEGA v.5; **Tamura et al., 2011**). The obtained nucleotide sequences have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/) under Acc. Nos. KF840163-KF840173.

Table 1 List of PPV isolates analysed in this study and their characteristics.

Isolate	Locality (district)	Natural host	Symptomatology		Strain	Accession number
			natural host	GF305	Strain	Accession number
01	Vranov nad Topľov	plum (*P. domestica*), cv. Bystrická	intensive leaf mosaics and rings	+*	D	KF840172
02	Sedliská (Vranov nad Topľou)	plum, cv. Čačanská lepotica	symptomless	++**	D	KF840173
03	Trebišov	peach (*P. persica*), cv. Sunhaven	light rings on the bark of annual shoots	++	M	KF840165
04	Sedliská (Vranov nad Topľou)	plum, cv. Čačanská najbolja	symptomless	+	M	KF840166
05	Sedliská (Vranov nad Topľou)	apricot (*P. armeniaca*), unknown	symptomless	++	D	KF840168
07	Moldava nad Bodvou	plum, cv. President	symptomless	++	D	KF840169
08	Trebišov	blackthorn (*P. spinosa*)	symptomless	++	D	KF840170
09	Trebišov	plum, cv. Bystrická	intensive leaf mosaics and rings	+	Rec	KF840164
10	Trebišov	blackthorn	symptomless	+	Rec	KF840163
11	Vranov nad Topľou	plum, cv. Top five	intensive leaf mosaics and rings	+	D	KF840167
12	Moldava nad Bodvou	myrobalan (*P. cerasifera*), wild type	symptomless	+	D	KF840171

*mild leaf symptoms

**intensive leaf symptoms, mosaics, vein clearings, distorsions

RESULTS AND DISCUSSION

Indexing of GF305 demonstrated a high biological variability of PPV isolates.

The previous studies have revealed an endemic occurrence of PPV in Slovakia (**Glasa et al., 2004; Šubr and Glasa, 2013; Predajňa, 2013**). To widen the knowledge on the variability of PPV in the Eastern Slovakia, 11 isolates were sampled from various *Prunus* hosts showing a different response to natural PPV infection (Table 1).

To evaluate the biological properties, the isolates were transmitted by chip-budding into the widely used peach GF305 indicator. Infection of GF305 was expressed by intensive leaf symptoms, mosaics, vein clearings in case of isolates 02, 03, 05, 07 and 08, contrary to mild symptoms caused by isolates 01, 04, 09, 10, 11 and 12. Only PPV has been repeatedly detected in the infected GF305 (simultaneous RT-PCR tests for the presence of other common fruit tree viruses, PNRSV and PDV, remained negative), thus the observed symptoms are likely to be caused by PPV itself. It is noteworthy for several isolates, that severity of their symptoms scored on the natural hosts did not corresponded to the symptom severity observed on the susceptible GF305 indicator plants (Table 1). It was reported that the use of GF305 for indexing of PPV-Rec isolates can be compromised by the fact, that PPV-Rec isolates usually do not express the symptoms on this indicator, or the symptoms are only mild and/or time-limited (**Glasa et al., 2002, 2004**). Similar situation was observed in case of PPV-Rec isolates 09 and 10 analysed in this study. The observation of a range of symptoms within the isolates of the same strain, e.g. symptomatologically mild PPV-D isolates (01, 11, 12) vs. severe isolates (02, 05, 07, 08) further support the data about the existence of a high intra-strain divergence, limiting the possibility to draw clear-cut strain biological properties (**Glasa et al., 2010**).

Partial sequence analysis assigned the isolates into three main PPV strains.

An informative genomic region encompassing the partial NIb and the hypervariable 5´-terminal region of the CP gene was amplified from all 11 PPV isolates. Uncloned PCR products were sequenced directly to obtain the consensus (master) sequence representative of the PPV population present in the infected sample. After the primer sequences were removed, we have used a portion with a length of 717 nucleotides for further analyses. Comparisons of all obtained sequences showed their complete colinearity. The amino acid conserved Asp-Ala-Gly (DAG) block, which is essential for potyvirus aphid transmission (**Garcia et al., 2013**), was conserved in all deduced Slovak CP aminoacid sequences (Fig. 1).

The phylogenetic analysis clearly showed the grouping of the 11 Slovak isolates into 3 distinct clusters, representing the PPV-M, D and Rec strains (Fig. 2). On the contrary, the presence of other PPV strains was not detected. Especially, strains PPV-W and CR, recently identified in the eastern part of Europe (**Glasa et al., 2011; Mavrodieva et al., 2013; Glasa et al., 2013**) could have a potential emergence potential.

The intra-strain pairwise analysis of nucleotide sequences revealed that the 7 sequenced Slovak PPV-D isolates shared 96.0 - 99.3% nucleotide identity (the most distant being 01 and 08 isolates), the identity between 2 PPV-M isolates reached 99.6% and 2 PPV-Rec isolates were 100% identical. Similar results were obtained by the analysis of a more broad range of Slovak PPV isolates (**Predajňa, 2013**), indicating that the PPV-D strain has accumulated the highest molecular divergence, which can be indicative of its more ancient introduction in Slovakia as compared to other PPV strains. Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that isolates 09 and 10 are the most closely related to the previously characterised PPV-Rec isolates from Slovakia (AY028309) and Hungary (AJ566344, AJ566345).

PPV-M isolates from this study (03, 04) showed the highest identity with Serbian (AJ2433957) and Slovak isolate (AY324837), indicating much lower level of divergence within these strains.

Strain-specific RT-PCR typing confirmed the sequence data, but revealed an additional mixed infection.

A substantial molecular distinctiveness between PPV-D and M isolates in the NIb-CP region of the PPV genome enabled to develop a RT-PCR tool, specifically detecting both virus strains.

Moreover, this region spans the recombination hotspot identified in the natural recombinant PPV isolates (forming the strain PPV-Rec, **Glasa et al., 2004**), allowing also its specific detection (**Šubr et al., 2004**).

Using this method, we could specifically detect the isolates of all three strains (Fig. 3) in the leaves of infected GF305, fully confirming the results previously obtained from the sequence analysis. In addition, the presence of mixed infection was detected in two samples (no. 05 and 10). In both cases, PPV-M has been detected in mixed infection with PPV-D and Rec, respectively (Fig. 3). Although not quantified, the intensity of amplified products indicates that in both samples, the detected PPV-M represent only a minor viral population. This is supported also by the fact, that in both cases, only dominant PPV-D (sample no. 05) or PPV-Rec sequences (sample no. 10) have been revealed by the direct sequencing of the PCR products. This result shows that a single *Prunus* host can be infected by two or more PPV strains, as previously found in the field conditions (**Candresse et al., 1998**).

However, it has been observed that competitiveness between different strain isolates in a single tree might lead over time to a displacement of less aggressive ones leaving the tree infected by a single strain isolate (**Predajňa et al., 2012**).

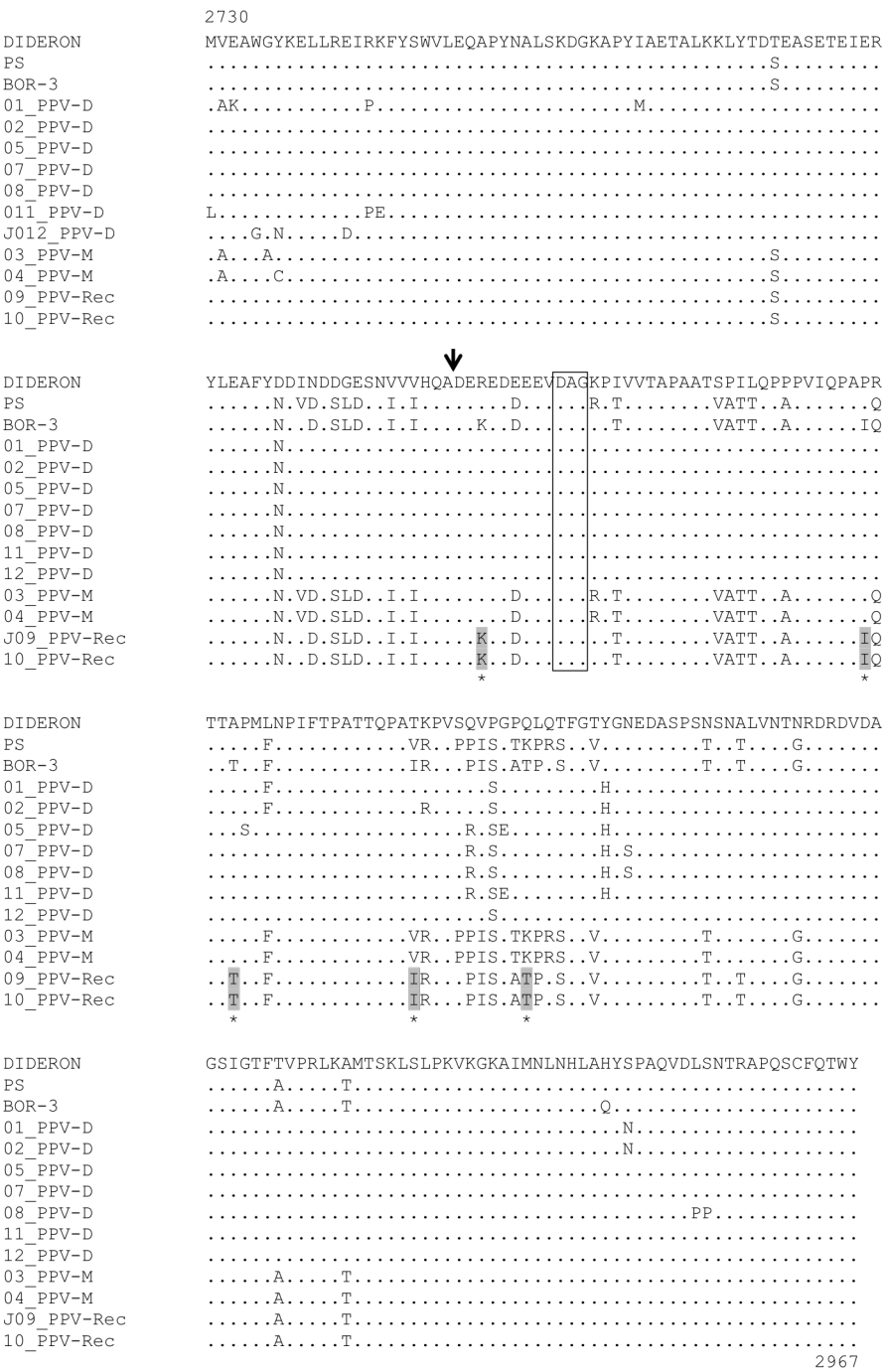

Figure 1 Multiple alignment of the deduced amino acid sequences of the C-terminal part of NIb and the N-terminal part of the capsid protein of PPV isolates analysed in this study (amino acid positions 2730-2967, numbered according to the BOR-3 polyprotein). The isolates PS (PPV-M, GenBank Acc. no. AJ243957), Dideron (PPV-D, X16415) and BOR-3 (PPV-Rec, AY028309) were included to the analysis. Identical amino acids to those of the Dideron isolate, used as the reference, are indicated by dots. The DAG motif associated with aphid transmission is boxed. The cleavage site between NIb and CP are indicated by an arrow. Specific amino acid positions in the CP N-terminus of PPV-Rec isolates (**Glasa et al., 2002**) are marked by asterisks and grey-shaded.

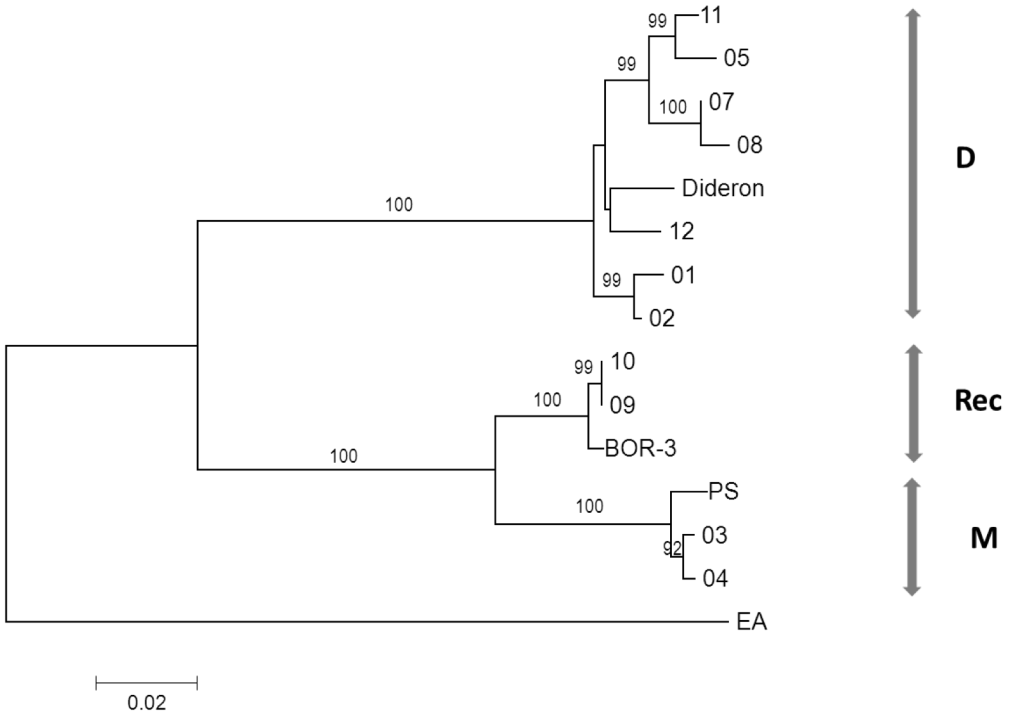

Figure 2 Phylogenetic tree of characterised PPV isolates and the representatives of the main PPV strains, generated from the NIb-CP sequences (nt 8332-9048, numbered according to the BOR-3 genome). Strain affiliation of the isolates is marked in the right margin. The divergent El Amar isolate (DQ431465) was used as an outgroup. The scale bar indicates a genetic distance of 0.02. Bootstrap values >70 (1000 bootstrap resamplings) are indicated as percentages on the branches. The tree was constructed by the neighbour-joining method, using the p-distance model.

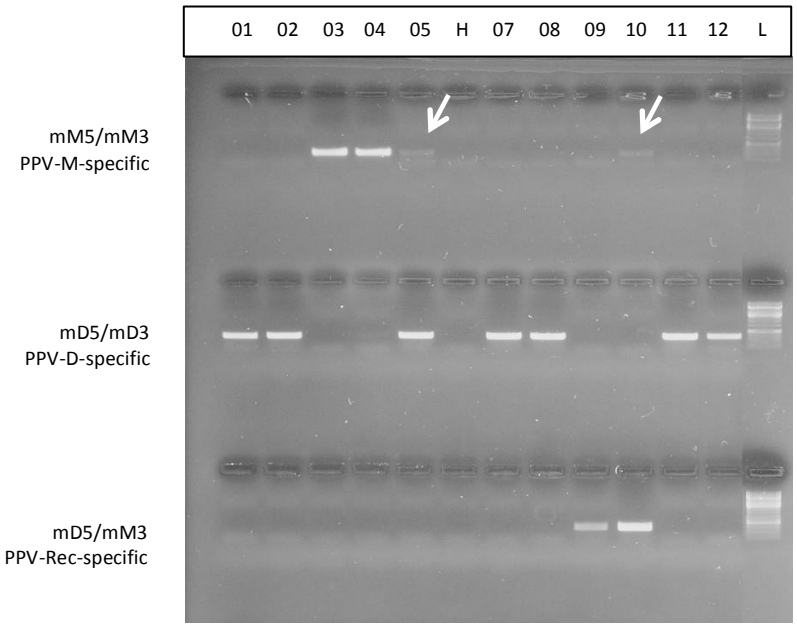

Figure 3 Agarose gel electrophoretic analysis of the strain-specific detection of PPV-M, D and Rec sequences in the NIb-CP genomic region according to **Subr et al. (2004).** Combination of primers and their specificity is indicated on the left. L = GeneRuler 1-kb DNA Ladder (Fermentas); H = healthy control; lanes 01 - 12 indicate the analysed Slovak isolates. Arrows highlight the presence of PPV-M in the samples 05 and 10.

CONCLUSION

The presence of PPV isolates belonging to the PPV-D, M and Rec strains was confirmed in Eastern Slovakia by their partial sequence analysis targeting the NIb-CP genomic region. The application of strain-specific RT-PCR has approuved the strain affiliation of isolates, and in addition, has permitted the detection of minor PPV variants in 2 samples, revealing the mixed PPV infection in these cases. The results further confirm the high incidence of PPV in the *Prunus* orchards and complement the global picture of virus diversity in Slovakia.

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EVALUATION OF MODEL WHEAT/HEMP COMPOSITES

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ABSTRACT

Model cereal blends were prepared from commercial wheat fine flour and 5 samples of hemp flour (HF), including fine (2 of conventional form, 1 of organic form) and wholemeal type (2 of conventional form). Wheat flour was substituted in 4 levels (5, 10, 15, 20%). HF addition has increased protein content independently on tested hemp flour form or type. Partial model cereal blends could be distinguished according to protein quality (Zeleny test values), especially between fine and wholemeal HF type. Both flour types affected also amylolytic activity, for which a relationship between hemp addition and determined level of Falling Number was confirmed for all five model cereal blends. Solvent retention capacity profiles (SRC) of partial models were influenced by both HF form and type, as well as by its addition level. Between both mentioned groups of quality features, significant correlation were proved - relationships among protein content/quality and lactic acid SRC were verifiable on $p < 0.01$ (-0.58, 0.91, respectively). By performed ANOVA, a possibility to distinguish the HF form used in model cereal blend according to the lactic acid SRC and the water SRC was demonstrated. Comparing partial cereal models containing fine and wholemeal hemp type, HF addition level demonstrated its impact on the sodium carbonate SRC and the water acid SRC.

Keywords: wheat-hemp composite flour; protein content and quality; SRC profile; correlation analysis

INTRODUCTION

Within cereal branch, an innovation goal presents non-traditional cereals, legumes and pseudocereals food usage, with accent on nutritional benefit of bakery products. To bring out new products of acceptable price on a market, commercial cereals premixes based on wheat flour of definite technological parameters are used in advantage; this way allows a keeping of produce process effectiveness at the same time. Among such basic characteristics belong protein content and quality according to the Zeleny test and prediction of starch polysaccharides behaviour when heated to 100 °C as the Falling Number. More precise description of cereal blends behaviour is enabled by the Solvent Retention Capacity Profile (SRC) determination, within which partial results correspond to hydration capability of flour components forming dough net structures, damaged starch rate together with pentosans content and quality.

The SRC method, registered as AACC 56-11 (**AACC Approved Method, 2000**; **Gaines, 2000**), represents a modern analytical procedure of quality prediction, both for milling products and wheat flour blends with non-traditional components. The test could be performed in a short time by usage of sample amount in grams. Its principle is based on gravimetric evaluation of absorbed amounts of distilled water, and water solutions of sucrose, sodium carbonate and lactic acid (50%, 5% and 5% w/w, respectively; signed as WASRC, SUSRC, SCSRC, LASRC).

A review on the SRC application in the cereal field was published in the Cereal Chemistry (**Kweon et al., 2011**). In recent literature, effect of agro technical factors as genotype, harvest year and planting locality are discussed (**Guttieri and Souza, 2003**), or wheat flour quality assessment (**Xiao et al., 2006**; **Duyvejonck et al. 2011**). Further scope was found out for triticale or rye quality description (**Oliete et al., 2010**), and also for wheat flour enrichment by ten types of commercial fibre of different origin (e.g. wheat, oat, apple or bamboo ones; **Rosell et al., 2009**). Within the own research results of Cereal laboratory of ITC Prague, the SRC method was validated for qualitative measurement of milling inter-products (**Hrušková et al., 2010**) or of composites containing wheat, rye, barley, oat or corn wholemeal (**Hrušková et al., 2011**).

Hemp was an important plant for its fibre and oil. Nowadays *Cannabis sativa* is the mostly planted specie due to its low content of phytochemical drug component THC (δ-9-tetrahydrocannabinol). Hempseed contains 20 - 25% protein, 20 - 30% carbohydrates, 25 - 35% oil and 10 - 15% insoluble fibre and a rich array of minerals. Hemp protein is mainly edestin, globular protein type similar to albumin found in eggs or blood. Oil is composed mostly by unsaturated fatty acids and therefore is considered beneficial for human nutrition (**Callaway 2004**). With respect to affordable references, both behaviour of cereal wheat-hemp model blend and its evaluation by means of the SRC testing was not published yet.

Aim of the presented study is to explore model cereal blends on base of wheat and hemp flours, including different commonly available food forms (conventional, organic i.e. “bio”). Statistical pattern used should reveal out relationships between single quality features and also influence of diverse recipe composition of 20 partial models.

MATERIAL AND METHODOLOGY

Preparation of model cereal blends

Based on commercial wheat flour produced in year 2010 (signed as M), model cereal blends were prepared by using five hemp flour samples (forms) singed as K1 - K5. In detail, two diverse conventional K1 and K2 samples were provided by Czech company, while organic K3 item was bought on local market; all named samples are of fine granulation. Furthermore, samples K4 and K5 are laboratory prepared ones, from dehulled and hulled hemp seeds, respectively, thus both have a wholemeal character. Model cereal blend were mixed in ratios 95:5, 90:10, 85:15 and 80:20 (w/w) of wheat and hemp flour, respectively, and were signed according to included hemp flour form and content (e.g. K1.5, or K5.20).

Cereal mixtures quality was evaluated according to **ČSN ISO 1871** (protein content according to Kjeldahl's method; abbreviation PRO), **ČSN ISO 5529** (protein quality according to Zeleny's sedimentation; ZT) and **ČSN ISO 3039** (amylolytic activity estimation as the Falling Number; FN). The analytical features were measured in duplicate, correspondingly to the mentioned Czech norms.

To gain the SRC profiles, the AACC norm No. 56-11 was followed, i.e. standard sample of 5g was used and centrifuged by using the Eppendorf 5072 apparatus (Eppendorf AG, Germany). The method accuracy was determined in terms of the test repeatability, allowing single measurements of tested model cereal blends. Calculated relative standard deviations were 0.342%, 0.727%, 0.667% and 0.476% absolutely for the WASRC, SUSRC, SCSRC and LASRC, respectively.

Statistical analysis

Represented by 20 items, cereal blends model with hemp flour was statistically described by both linear and non-linear correlation analysis, covering all observed quality features. Analysis of variance (ANOVA) serves for assessment of partial models composition, i.e. to compare the influences of hemp flour form (HF; K1 - K5) or hemp flour type (HT; fine vs. wholemeal) and hemp component addition level (AL) in pairs (HF vs. AL, HT vs. AL). The factors impact was quantified by variance components analysis (*F*-test), considering HF or HT as fixed effects and AL as a random one. The statistics mentioned were calculated using the Statistica 7.1 software (Statsoft, USA).

RESULTS AND DISCUSSION

Technological properties of model cereal blends

Basic component - wheat flour M - is characterised by higher PRO (12.5%) with standard quality (ZT 41 mL). Estimated amylolytic activity as FN equal to 310 s corresponds to that harvest year average and in terms of flour bakery usage, it is close to technological optimum.

Related to all five hemp flour forms, PRO has approx. linearly increased up to about one-quarter in relation to wheat flour standard M. The least influence was recognized during K5 fortification, while for cereal blends containing K1 and K2 on one side and for ones with K3 and K4 on the other, approx. 4% and 7% increments were found, respectively (Figure 1). According to ANOVA results, that parameter was not able to distinguish partial models with different hemp flour types, despite a revealed soft interaction of observed factors.

Reversal to content, protein quality has been significantly dwindled in all wheat-hemp flour blends in a range 7%-38% (Table 1). A negative influence was milder at fortification by commercial fine hemp K1 sample. Conversely to that, verifiable loss in protein quality was registered for wholemeal hemp flour K4 or K5 hemp forms (maximal decrease of ZT about 37%, about 68% and 66% for blends involving 20% of non-traditional flour, respectively). In this regard, cereal models containing conventional fine hemp flour could be partially distinguished from the wholemeal ones. ANOVA results also proved softly stronger impact of AL compared to one of HF factor.

Hemp component affected the SRC profile of model cereal blends, both by used form and by added amount. The broadest change was recorded for LASRC, a diminishing from 182.5% to less than one half has occurred (Table 2, variability a-d for both effects studied). Vice versa, the lowest impact of variation in cereal blend composition was noticed for WASRC. As is documented by whisker plot, arithmetic mean covering the K3 blends was similar to standard value (89.8 % vs. 90.9 % for basic sample M; Figure 2a). There is obvious dependence of the SRC profile of each blend on both fine and wholemeal hemp flour type.

Among tested cereal models with selected hemp flour concentrations, comparable trends were identified for pairs WASRC-SCSRC and SUSRC-LASRC (Figure 2b). In the former case, both SRC's level of samples enhanced by 5% and 20% of hemp flour differed minimally (about 2% and 6%), representing approx. 87% and 81% of the standard M value, respectively. Within the second couple, determined decrease was more significant, considering averages levels 83% and 58% of standard M. Such exploration of cereal blend composition brings also knowledge about the largest data scatter for LASRC parameter, likewise to case of tested hemp flour comparison. Owing to that, the parameter should be identified as identification sign of each cereal blend item.

Statistical analysis of model cereal blends

Trends observed within correlation matrixes resulted from linear or non-linear approaches were similar (data not shown), therefore only linear relationships are discussed (Table 3).

To depict quality by alternative way, the SRC profile application possibilities are nowadays studied extensively. Global properties of partial items of the model cereal blend were characterised by the procedure similarly, demonstrating possible alterations between the single SRC. The tightest correlation was found in pair WASRC and SCSRC (r = 0.96; $p < 0.01$; Table 3).

For the four single SRC, the best relationships correspondence was revealed to ZT parameter (all 6 links provable), and the fittest to LASRC ($r = 0.91$, $p < 0.01$). Also PRO was connected to LASRC, but correlations provability is weaker ($r = -0.58$, $p < 0.01$). Summarised, the LASRC has a potential to distinguish the tested partial models containing fine and wholemeal form of hemp component. Within the set of wheat, rye and triticale

testing, similar findings published **Oliete et al., 2010** for relationships of PRO to WASRC, SUSRC, and SCSRC (r = -0.64; -0.64 and -0.69; p <*0.05*). In a pair PRO-LASRC, the Pearson's coefficient reached approx. a half level (r = 0.35). Within the set of wheat flour composites containing wheat, rye, barley, oat or corn wholemeal, verifiable links between quality features and the SRC profiles were published in our previous study (**Hrušková et al., 2011**). The strongest relationship was determined between ZT and LASRC (r = 0.93, p < *0.01*).

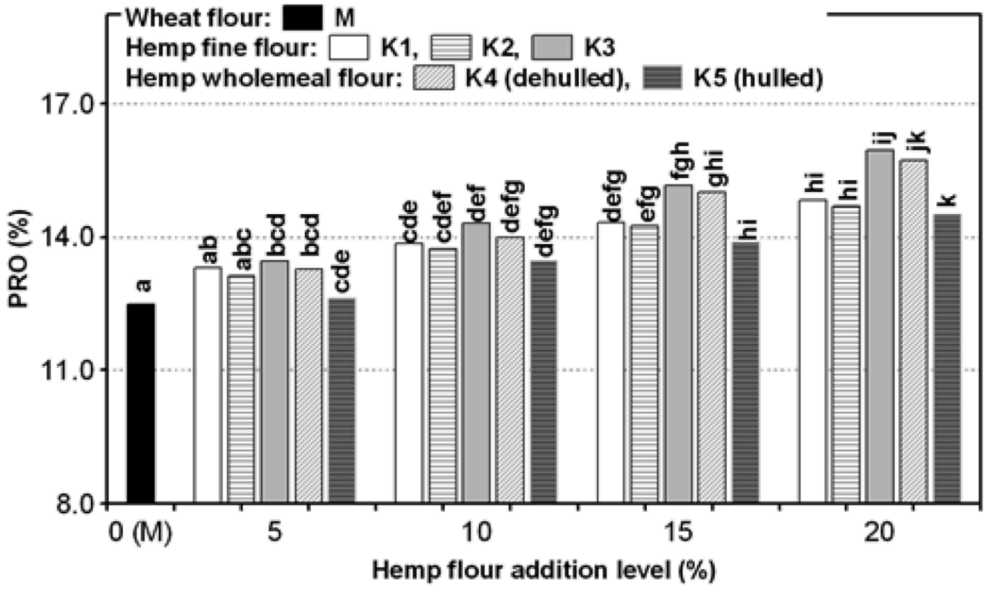

Figure 1 hemp form and addition level influences on protein content (PRO) in cereal blend models.

Table 1 Hemp form and addition level effects on analytical properties of model cereal blends.

Flour type	Sample	ZT (mL)			FN (s)		
		Range	Variability		Range	Variability	
			HF	AL		HF	AL
Wheat flour	M	41	d	E	310	ab	B
Fine hemp flour	K1.5 - K1.20	38 - 26	b-c	A-D	308 - 297	a-ab	A-C
	K2.5 - K2.20	36 - 20			309 - 292		
	K3.5 - K3.20	34 - 16			315 - 278		
Wholemeal hemp flour	K4.5 - K4.20	19 - 13	a-b		333 - 278	ab-b	
	K5.5 - K5.20	34 - 14			333 - 286		

M - commercial fine wheat flour; Hemp forms: K1, K2, K3 - commercial hemp flour of fine type;
K4, K5 - dehulled and hulled hemp flour of wholemeal type, respectively.
ZT - Zeleny sedimentation test, FN - Falling Number.
ANOVA factors: HF - hemp form, AL - addition level.
a-d, A-E: group means for HF and AL, respectively, signed by the same letter are not statistically different at p <*0.05*.

Table 2a) Hemp form and addition level effects on the SRC profile of model cereal blends.

Flour type	Sample	WASRC (%)			SUSRC (%)		
		Range	Variability		Range	Variability	
			HF	AL		HF	AL
Wheat flour	M	90.9	b	B	112.1	a	A
Fine hemp flour	K1.5 - K1.20	86.5 - 86.5	b	A-AB	109.4 - 102.3	a	A
	K2.5 - K2.20	86.3 - 86.4			100.9 - 106.7		
	K3.5 - K3.20	86.9 - 87.9			93.0 - 99.8		
Wholemeal hemp flour	K4.5 - K4.20	70.3 - 61.7	a		102.3 - 64.1	a	
	K5.5 - K5.20	69.5 - 68.3			102.5 - 74.3		

Table 2b) Hemp form and addition level effects on the SRC profile of model cereal blends.

Flour type	Sample	SCSRC (%)			LASRC (%)		
		Range	Variability		Range	Variability	
			HF	AL		HF	AL
Wheat flour	M	117.1	b	B	182.5	d	D
Fine hemp flour	K1.5 - K1.20	108.8 - 106.7	b	A	153.7 - 112.1	b-c	A-C
	K2.5 - K2.20	107.5 - 108.2			141.2 - 109.9		
	K3.5 - K3.20	106.2 - 106.9			137.1 - 102.7		
Wholemeal hemp flour	K4.5 - K4.20	88.2 - 70.5	a		108.0 - 78.3	a	
	K5.5 - K5.20	88.4 - 76.1			120.6 - 77.2		

M - commercial fine wheat flour; Hemp forms: K1, K2, K3 - commercial hemp flour of fine type; K4, K5 - dehulled and hulled hemp flour of wholemeal type, respectively.
WA-, SU-, SC-, LASRC: water, sucrose, sodium carbonate and lactic acid solvent retention capacity, respectively.
ANOVA factors: HF - hemp form, AL - addition level.
a-d, A-D: group means for HF and AL, respectively, signed by the same letter are not statistically different on $p < 0.05$.

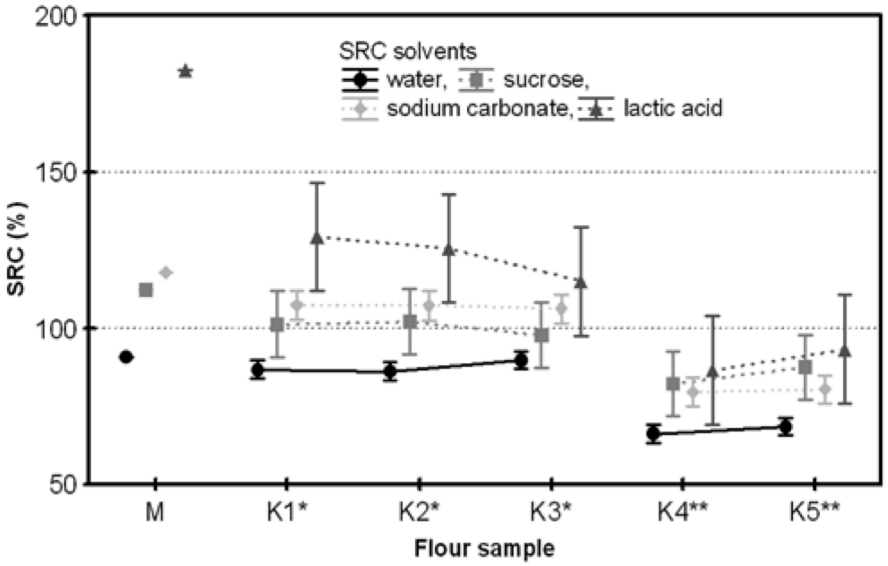

Figure 2a Effect of fine(*) and wholemeal (**) hemp flour on wheat flour M solvent retention capacity (SRC) profile.

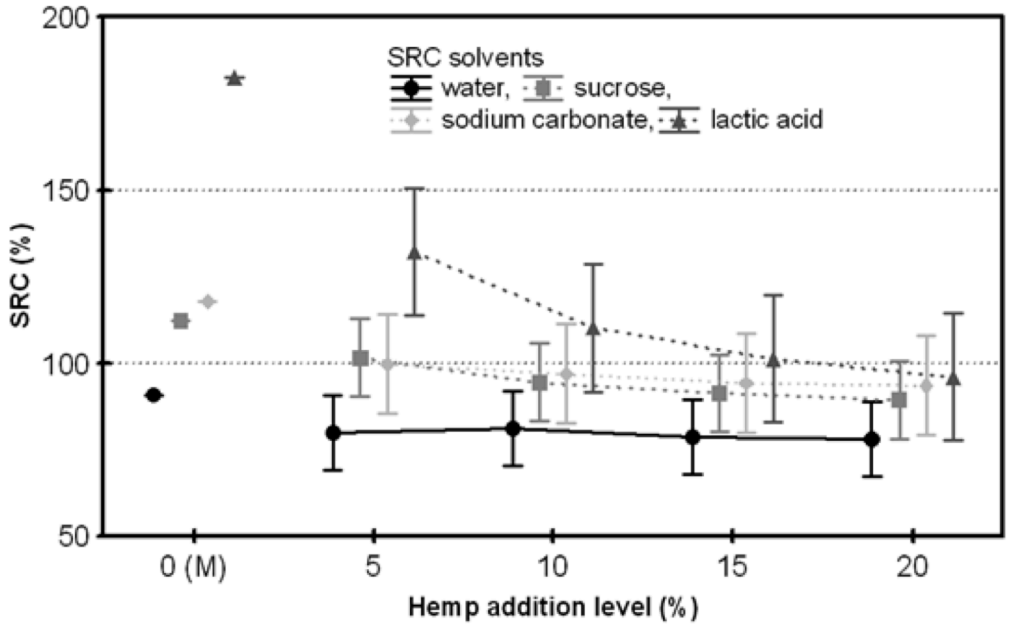

Figure 2b Effect of hemp addition level on wheat flour M solvent retention capacity (SRC) profile.

Table 3 Significant linear relationships between analytical features and SRC profiles of tested model blends

Feature	PRO	ZT	FN	WASRC	SUSRC	SCSRC	LASRC
PRO	1						
ZT	-0.73**	1					
FN	-0.79**	0.44**	1				
WASRC	*ns*	0.56**	*ns*	1			
SUSRC	-0.44**	0.66**	*ns*	0.74**	1		
SCSRC	*ns*	0.66**	*ns*	0.96**	0.84**	1	
LASRC	-0.58**	0.91**	*ns*	0.71**	0.78**	0.82**	1

PRO - protein content, ZT - Zeleny sedimentation test, FN - Falling Number.
WA-, SU-, SC-, LASRC: water, sucrose, sodium carbonate and lactic acid solvent retention capacity, respectively.
*, ** - relationships provable on $p < 0.05$ and *0.01,* respectively; *ns* - non-significant.

Table 4 a) Comparison of factors hemp form and hemp components addition level impact on analytical features and SRC profiles of model cereal blends.

a) Hemp form: samples K1 - K5

Feature	Factor	
	HF	AL
PRO	16***	79***
ZT	51***	64***
FN	3*	33***
WASRC	73***	1
SUSRC	4*	2
SCSRC	67***	3
LASRC	91***	79***

Table 4 b) Comparison of factors hemp form and hemp components addition level impact on analytical features and SRC profiles of model cereal blends.

b) Hemp flour type: fine (K1 - K3) vs. wholemeal samples (K4, K5)

Feature	Factor		
	HT	AL	HT x AL
PRO	1	12***	0
ZT	33***	16***	0
FN	14***	59***	9***
WASRC	246***	1	1
SUSRC	47***	9*	11***
SCSRC	1448***	23***	18***
LASRC	97***	23***	0

PRO - protein content, ZT - Zeleny sedimentation test, FN - Falling Number.

Comparing influence of the observed factors, i.e. HF (K1-K5), HT (fine K1-K3 vs. wholemeal K4, K5), and AL (ratio in certain model blend) by the *F*-test, discrimination of partial cereal models was testified with different statistical reliability levels. In a case of the HF and the AL effects exploration, the latter factor seriously impacted protein and starch properties of model blends (e.g. PRO *F*-values 79 vs. 16, respectively; $p < 0.001$, Table 4a).

Almost levelled influence of the factors was found for the ZT parameter - calculated *F*-values were 51 and 64, respectively ($p < 0.001$). On the other hand, the SRC profiles could be distinguished according to the HF ($F = 73$ and 67, respectively; $p < 0.001$). Likewise to ZT characteristic, the LASRC presents its own capability to describe a diversity among cereal blend models, both in terms of the HF or the AL factor ($F = 91$ and 79, $p < 0.001$) (Table 4a). In contrast, the softest effect of hemp flour form was identified for the SUSRC, as could be noticed in whisker plots (Figures 2a, 2b).

Taking account of the HT factor (together with the AL one), protein properties (PRO and ZT) importance in model blends distinguishing was lessened (Table 4b). Reversely to that, *F*-value levels related to polysaccharides behaviour (i.e. FN) were magnified at least twice ($F = 14$ a 59, $p < 0.001$). Therefore it could be assumed a prediction of model cereal blends composition by indirect determination of amylolytic activity of present starch.

Investigated factors (type and added amount) influenced the SRC profiles in a different way compared to the previous evaluation. According to *F*-test, hemp component type could be traced in most precise way by the SCSRC and with somewhat higher statistical error by the WASRC ($F = 1448$ and 246, respectively; $p < 0.001$) (Table 4b). And finally, properties prediction of models with hemp wholemeal flour could be built on the SCSRC and LASRC (a larger data extent, Table 2).

CONCLUSION

Model cereal blends were prepared on base of commercial fine wheat flour and five samples of hemp components, differing in their type - fine or wholemeal one (3 and 2 samples, respectively). Hemp components partially replaced wheat flour, chosen ratios ranged between 5% and 20%. From a viewpoint of chemical components, added amounts significantly increased protein content, independently on tested hemp form. Partial model blends were distinguishable according to protein quality (Zeleny's sedimentation values), especially between fine and wholemeal hemp component type. Estimation of amylolytic activity as the Falling Number also signified differences between mentioned hemp types, counting all 20 tested cereal mixtures. Considering consecutively hemp form, hemp type and added amount as data variability factors, the SRC profiles of studied wheat-hemp model blends were verifiably affected by all three mentioned influences. Between features belonging into analytical-quality group on the one and into the SRC profile on the other side, correlation analysis confirmed presumed as well as revealed new statistically important relationships in correspondence with results of other researchers. For example, a link between protein content or their quality and lactic acid SRC could be consider as tight even as very tight on $p < 0.01$). A possibility to distinguish model cereal blends was signified by performed ANOVA test. Pair comparison of hemp form vs. added amount effects shown that capability for the lactic acid and the water SRC's. For factors' hemp type and added amount influence, such importance had the sodium carbonate and the water SRC's.

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GENETIC DIVERSITY AND RELATEDNESS AMONG SEVEN RED DEER (*CERVUS ELAPHUS*) POPULATIONS

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ABSTRACT

Deer (*Cervidae*) recently belongs to the most important species. The aim of presenting study was evaluation of genetic diversity and relationship within and among seven red deer populations from different origins - Czech Republic, Hungary, hybrids Hungary x New Zealand, Lithuania, New Zealand, Poland and Slovak Republic. This study was conducted to determine the levels of genetic variability and relationships among deer populations from a total of 637 animals originating from seven countries Czech Republic (50), Hungary (35), Hungary x New Zealand hybrids (67), Lithuania (26), New Zealand (82), Poland (347) and Slovak Republic (30). We used the hair bulbs as a source of DNA. In total, 213 alleles were observed from the 10 loci surveyed. The number of alleles per locus ranged from 11 (IOBT965) to 35 (T156, RT13). Genetic diversity and relatedness among red deer populations has been performed on a total of 637 animals. A panel of 10 microsatellite markers used in deer were optimized. On the basis of this panel of microsatellites we were investigated genetic variability and relationships by using statistical and graphical programmes. We evaluated how close populations are to each other and their genetic admixture. Molecular genetic data combined with evaluation in statistical programmes could lead to a complex view of populations.

Keywords: genetic variability; relatedness; microsatellite loci; red deer (*Cervus Elaphus*)

INTRODUCTION

This study was conducted to determine the levels of genetic variability and relationships among seven red deer populations.

Deer (*Cervidae*) belong to the most important species, which are used as a farm animal as well as hunting wild animal. For long-term conservation and development purposes, it therefore appears compulsory to manage wildlife to maintain both species survival and within species genetic diversity **(Franklin, 1980)**.

Since the 1980s genetic markers have been used, based on examination of DNA variability, for identification of the populations and individuals within it. In recent years, the importance of microsatellites for population genetics, linkage mapping, and parentage studies has increased significantly **(Poetsch et al., 2001)**. To date, about 200 microsatellite loci have been found in cervids by transferring microsatellite PCR primers derived in bovine and ovine to cervids, as well as a few loci derived directly from the deer microsatellite library **(Xu Yan-chun et al., 2001)**. The usefulness of microsatellite markers for the estimation of genetic diversity and relationships among individual populations has been documented in numerous studies **(Buchanan et al., 1994; Saitbekova et al., 1999; Schmid et al., 1999, Xu Yan-chun et al. 2001)**.

Two classes of markers are used: the first, termed type I loci, are widely conserved across species but are frequently invariable within species, the second, termed type II loci, are usually polymorphic within a species, but tend to be less widely conserved (e.g., microsatellites) **(Slate et al., 2002)**. Furthermore, primers for microsatellites isolated in other ruminants frequently amplify their deer orthologs **(Slate et al., 1998)**.

Recent developments in molecular biology and statistics have opened up the possibility of identifying and using genomic variation. It is interesting to know the genetic similarity between individuals and populations. Several different software programmes, which use molecular genetic data, have been developed for evaluating relationships among populations.

The objectives of this study were to quantify and compare levels of genetic variability and relatedness among seven red deer populations representing different uses and population sizes. The populations were analysed using by 10 microsatellite markers and genotypes of each population were used for statistical evaluation of relationships between populations.

MATERIAL AND METHODOLOGY

Samples were collected from a total of 637 red deer originating from Czech Republic (50), Hungary (35), Hungary x New Zealand hybrids (67), Lithuania (26), New Zealand (82), Poland (347) and Slovak Republic (30). We used the hair bulbs as a source of DNA.

Genomic DNA we obtained as a whole-cell lysate from 10 hair roots using PCR buffer with Tween 20. 10 microsatellite markers were runed in two optimized multiplex PCR reactions. The 8 microsatellite markers of mix 1 (Table 1) were amplified in modified multiplex-polymerase chain reaction according to **Ernst et al. (2008) (Maršálková et al., 2010)** using fluorescently labelled primers. Additional 2 microsatellites markers were amplified in the second multiplex (Table 1). PCR

Tab. 1 List of primers (name, fluorescent dye, sequences)

Primers list of master mix one:

BM888	*VIC* -	ACTAGGAGGCCATATAGGAGGC AGCTCAAAACGAGGGACAGGG	**(Talbot et al., 1996)**
OarFCB5	*6FAM* -	AAGTTAATTTTCTGGCTGGAAAACCCCAG ACCTGACCCTTACTCTCTTCACTC	**(Buchanan et al., 1994)**
RM188	*VIC* -	GCACTATTGGGCTGGTGATT GGTTCACAAAGAGCTGGAC	**(Barendse et al., 1994)**
RT1	*VIC* -	CATATGGCTAACTACCTAGCTTGCC GAGTCCCAAAGATTTCAGCCCTAC	**(Wilson et al., 1997)**
RT13	*NED* -	GCCCAGTGTTAGGAAAGAAGA CATCCCAGAACAGGAGTGAG	**(Wilson, et al., 1997)**
T26	*6FAM* -	TGCCATAGTTTTTCCTACCTTC GAAGTTCCAATAGACACGCTC	**(Jones et al., 2002)**
T156	*6FAM* -	ATGAATACCCAGTCTTGTCTG TCTTCCTGACCTGTGTCTTG	**(Jones et al., 2002)**
T501	*PET* -	CTCCTCATTATTACCCTGTGA ACATGCTTTGACCAAGACCC	**(Jones et al., 2002)**

Primers list of master mix two:

IOBT965	*6FAM* -	GGGGTTGTGGGTAAGCGGAGTT GATCTAGCGCCAGACAGACGTGTCAT	**(Kuehn et al., 2003)**
BM1818	*VIC* -	AGTGCTTTCAAGGTCCATGC AGCTGGGAATATAACCAAAGG	**(Cosse et al., 2007)**

was carried out in 10 µl volumes consisting of 1 µl of whole-cell lysate containing DNA; 1.2x Go Taq® Hot Buffer (Promega, Madison USA); 1.8 mM $MgCl_2$ (Promega, Madison USA); 0.34 mM dNTP (Applied Biosystems); concentrations of the individual primers varied from 80 to 400 nM; 0.5 U of GoTaq® Hot

Start Polymerase (Promega, Madison USA). Samples were initially heated to 95 °C for 5 minutes and then subjected to 30 cycles of PCR amplification at 95 °C for 30 s, 59 °C for 90 s, 72 °C for 90 s, and followed by a final extension at 72 °C for 60s (PTC-150 Minicycler™, MJ Research). Fragments of interest were genotyped by ABI Prism® 310 Genetic Analyzer (Applied Biosystems) and genotypes of individuals were evaluated by GeneMapper® software.

From the output of the statistical software program Tanagra was identified significant difference ($p < 0.001$) between the sample A and the sample F for shear strange textural parameter (Table 2). Therefore, we can state that storage at 12 °C two days significantly affecting the strength of chicken breast. Another significant difference ($p < 0.05$) for strength was found between the sample A and the sample B. In the case of work in shear (Table 3), we have identified a significant difference ($p < 0.001$) between the sample A and the sample F.

Genetic diversity of specific populations has been assessed on the base of allele number (NA), genotype number, gene diversity (GD), heterozygosity (Ho) and Hardy-Weinberg equilibrium (HWE). These values have been calculated from genotypes using the POWERMARKER 3.23 programme **(Liu et al., 2003)**. Distance measures were estimated by the method of **Nei et al. (1983)**. The neighbour-joining tree topology was obtained with the PHYLIP-3.69 **(Felsenstein, 1993)** software using the Cavalli-Sforza distance and the neighbour-joining tree construction was carried out with the Dendroscope software **(Huson et al., 2007)**.

Results and discussion

This study was conducted to determine the levels of genetic variability and relationships among deer populations from a total of 637 animals originating from seven countries. In total, 213 alleles were observed from the 10 loci surveyed. The number of alleles per locus ranged from 11 (IOBT965) to 35 (T156, RT13). Selected microsatellite markers are more polymorphic than the marker used in **Frantz et al. (2008)**, number of alleles ranged from 2 to 14, and are more polymorphic than microsatellite loci used for the analysis of paternity in **Walling et al. (2010)**, where the number of alleles ranged from 6 to 15. The number of alleles of two bovine microsatellite loci (BM888, RM188), which we used in our study, is greater than in **Slate et al. (1998)**.

He per locus overall population was between 0.716 (RT1) and 0.917 (RT13) (Table 2) and, on average, **0.831** ±0.019. No locus had a significant ($P < 0.05$) deviation from HWE. Ho per locus ranged from 0.638 (RT13) to 0.854 (BM888) and mean number of Ho was **0.738 ±0.017**, which is comparable with the mean number of Ho per locus in **Perez-Espona et al. (2008)** and **Walling et al. (2010)** and is greater than mean number of Ho in the population of Java Rusa Deer in **Webley et al. (2004)**. Values of He and Ho are relatively high and values of He are greater than Ho for all markers. He and Ho of markers are greater than expected and observed heterozygosity of markers in the Bavarian red deer population used in **Kuehn et al. (2003)**.

Tab. 2 Genetic diversity per alleles

Marker	GenotypeNo	SampleSize	AlleleNo	He	Ho
OarFCB5	74	637	16	0.845	0.794
T156	118	637	35	0.874	0.751
BM888	137	637	31	0.907	**0.854**
RT1	82	637	20	**0.716**	0.673
RT13	141	637	35	**0.917**	**0.638**
T501	55	637	15	0.817	0.740
T26	67	637	18	0.872	0.791
RM188	57	637	16	0.759	0.701
IOBT965	35	637	11	0.738	0.693
BM1818	63	637	16	0.853	0.745
Mean	82.9	637	21.3	**0.830**	**0.738**

P <0.05 in all observed loci
He- expected heterozygosity
Ho- observed heterozygosity

Tab. 3 Genetic diversity per populations

Population	GenotypeNo	SampleSize	AlleleNo	He	Ho
CR	19.3	50	11	0.774	0.768
HU	17.4	35	9.2	0.788	0.680
HUxNZ	22.4	67	10.2	0.781	0.788
LTV	16	26	9.7	0.793	0.746
NZ	22	82	9.7	0.748	0.720
PL	59.8	347	18.4	0.815	0.737
SR	14.6	30	8.3	0.777	0.703

He- expected heterozygosity
Ho- observed heterozygosity

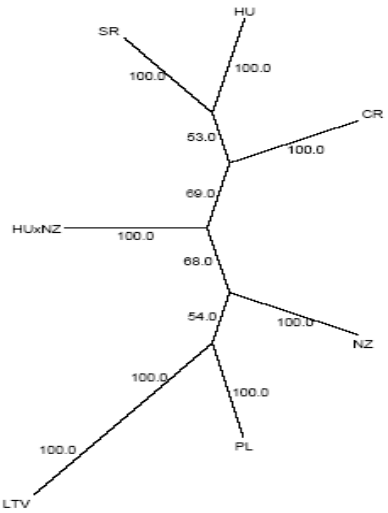

Fig. 1 Genetic distances among seven red deer populations

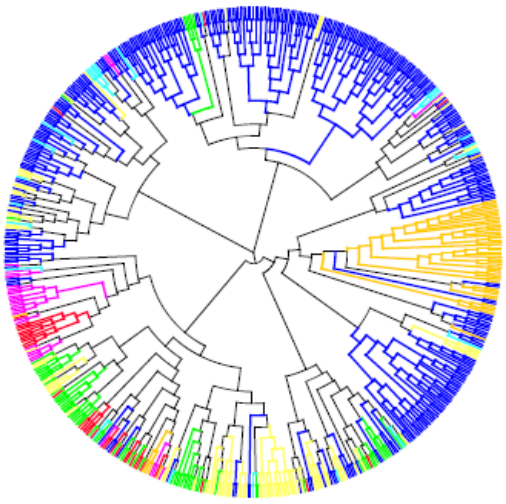

Fig. 2 Cluster diagram of all evaluated red deer individuals from different populations.

A total of 11 alleles combined in 19.3 genotypes were observed in population from CR, 10.2 alleles combined in 22.4 genotypes in hybrids HUxNZ, 9.7 alleles combined in 16 genotypes in LTV population, and 9.7 alleles combined in 22 genotypes in NZ population. The largest number of alleles (18.4) combined with the largest number of

genotypes (59.8) were found in a population from PL. The Hungarian population (9.2 alleles, 17.4 genotypes) and population from Slovak Republic (8.3 alleles, 14.6 genotypes) have the smallest number of alleles that forms the smallest number of genotypes (Table 3). Allele number in HU and SR population is higher than mean number of alleles in all populations in **Maudet et al. (2009)**.

He per population was between 0.748 ±0.033 (New Zealand) and 0.815 ±0.022 (Poland). Ho per population ranged from 0.680 ±0.032 (Hungary) to 0.788 ±0.026 (hybrids HUxNZ) (Table 3), which is slightly lower than lowest and greatest value of observed heterozygosity in **Pérez-Espona et al. (2008)**. The values of He and Ho are relatively high in all populations and therefore populations appear to be sufficiently heterogeneous. The values of He are greater than Ho also in all populations. For correct output, it is necessary to analyse at least 30 individuals, otherwise the results cannot provide relevant insight into the test group.

As we seen in Figure 1, Hungary and Slovak Republic populations are the closest of all. CR population is on the same main branch with SR and HU population and create first group. The LTV population is genetically farthest from the SR and HU populations. The PL and NZ population with LTV population create another group that is genetically distant from the first group. HUxNZ hybrids have approximately the same distance from these two groups of populations.

Figure 2 is more detailed view and represents the mixing of individuals from populations. It is cluster analysis where each branch represents an individual. Data source for constructing cluster diagrams is genetic distance. Matrix of genetic distance is calculated with the observed allele frequencies for each individual. The graphical view is divided into four main branches. Individuals of each population mostly tend to cluster together. The first main branch is created by individuals from Polish (blue) population and a few individuals from New Zealand (yellow) and Lithuanian (light blue) population. The second main branch is mainly created by individuals from Polish (blue) population. The third main branch is created by individuals from Czech and Polish population. The Polish population (blue) is mixed with individuals from New Zealand population (yellow) and hybrids HUxNZ (green). The fourth main branch is created by individuals from New Zealand population (yellow), hybrids HUxNZ (green), Hungarian (red) and Slovak population (pink).
The most mixed populations are Hungarian population and HUxNZ hybrids. The least clustered individuals are from the Lithuanian population and the most clustered are individuals from Czech and Polish population.

Based on these graphical views we can see the genetic distances among seven populations as well as distances among individuals within these populations. Molecular genetic data combined with evaluation in statistical programmes could lead to complex view of populations.

CONCLUSION

Genetic variability and relationship analyses have been performed on seven red deer populations from a total of 637 animals of different origin (Czech Republic, Hungary, Hungary x New Zealand hybrids, Lithuania, New Zealand, Poland and Slovak Republic). We can affirm that optimized panel of microsatellite markers are polymorphic and suitable for this kind of evaluation. We were able to evaluate and visualise genetic diversity and relatedness between populations on the basis of this panel of microsatellites by using statistical programmes. Neighbour-joining trees showed that individuals from each population tend to cluster together, except Lithuanian population, which have the least clustered individuals. Most of those clustered are individuals from CR. The results indicate the genetic uniformity of individual groups of animals and we can conclude that populations have enough distance from each other so they have sufficient genetic diversity.

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THE INFLUENCE OF FEEDING GMO-PEAS ON GROWTH OF ANIMAL MODELS

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ABSTRACT

Introduction of genetically modified (GM) food or feed into the commercial sale represents a very complicated process. One of the most important steps in approval process is the evaluation of all risks on the health status of people and animal models. Within our project the genetically modified peas was breeded that showed significant resistance against *Pea seed-borne mosaic virus* and *Pea enation mosaic virus*. Preclinical studies have been conducted to found out the effect of GMO peas on animals - rats of outbreeding line *Wistar*. In a total, 24 male, specific pathogen free *Wistar* rats were used in the experiment. At the beginning of the experiment, the animals were 28 days old. The three experimental groups with 8 individuals were created. The first group of rats was fed with GMO peas, the second group of rats consumed mix of pea cultivar Raman and the third group was control without pea addition (wheat and soya were used instead of pea). In the present study we focused our attention on health, growth and utility features of rats fed with GM pea. All characteristic were observed during the experiment lasting 35 days. Consumed feed was weighted daily and the weight of the animals was measured every seven days. The average values were compared within the groups. The aim of the experiment was to verify if resistant lines of pea influence the weight growth of animal models. The results of our experiment showed that even a high concentration (30% of GM pea) did not influence growth rate of rats to compare with both rats fed with pea of Raman cultivar and control group. We did not observe any health problems of animal models during the experiment.

Keywords: geneticaly modified crops; pea preclinical studies

INTRODUCTION

Crop that have been genetically modified include plants with changes in DNA structure through genetic engineering **(Morisset et al., 2008)**. Genetic modifications have been considered as modern plant breeding methods in biotechnologies and depend on spontaneous processes in nature. It does not mean creating and transfer of artificial genes. GMO crops have some specific features such as resistance to harmful conditions including pests and diseases **(Han and Jung 2013)**, low temperatures **(Sakamoto et al., 2004)**, drought **(Lawlor, 2013)** etc., or tolerance to affusion against non-selective herbicides used to kill ineligible plants **(Gryson, 2010; Ujhelyi et al., 2012)**. Genetic engineering facilitates the transfer of desired characteristics into other plants, which is not possible through conventional plant breeding **(Ahmad et al. 2012)**.

Pea (*Pisum sativum L.*) is attacked by wide range of pests and pathogens. Some of them can cause economically significant diseases and losses. The pea is sensitive to a large number of viruses transferred by pea weevil. More than 120 species of viruses that are able to infect a pea has been noticed. Only some of them occurred in such rate that they could be considered as economically significant **(Larsen et al., 2007; Pflughoft et al., 2012)**.

Recently researches detected that only two of the viruses - Pea enation mosaic virus and Pea seed-borne mosaic virus had economic effect on pea production. The Pea enation mosaic virus (PEMV) is unique within plant viruses. In fact, it is occurred in the form of two viruses in obligatory symbiosis - PEMV-1 (Enamovirus) and PEMV-2 (Umbravirus). The presence of both viruses is necessary for induction of wild type infection **(Hodge and Powell 2010)**. Pea seed-borne mosaic virus (PSMV) is a typical representant of genus Potyvirus. The disease was first described in the former Czechoslovakia by Musil **(Musil, 1966)**, and a year later in Japan **(Inouye, 1967)** and two years later it was recorded in the USA **(Stewenson and Hagedorn, 1969)**. Typical symptoms of infection with this virus are pea leaf roll leaves, shortening of internodes, degree of stunting of infected plants, further deformation of the flowers and development of small deformed pods. Transfer by seeds is a reason for its easy spreading as it has been noticed in worldwide important crops as pea, lentic or broad bean. The spreading by seeds up to 30% in sensitive pea seeds has been noticed, but there were also cases in which 90% of commercial seed was infected. Nowadays these two previously mentioned species of viruses are extended in leguminous plants worldwide **(Safarova et al., 2008)**. The viruses are naturally transferable by vectors, namely by aphids, non-persistent type such as *Mysus persicae*, *Aphis craccivora, A. fabae* **(Aapola and Mink, 1973; Kvicala and Musil, 1973)**. Nowadays four pathotypes PSbMV called P1, P2,

P3 and P4 has been described, from which in the Europe commonly occurs the pathotype P1 and last year the occurrence of pathotype P4 was confirmed **(Alconero et al., 1986, Hjulsager et al., 2002; Johansen et al., 1991)**. Resistance to standard pathotypes PSbMV is based on recessive genes *sbm-1*, *sbm-2*, *sbm-3* and *sbm-4*, which correspond to the mentioned pathotypes PSbMV **(Nicaise et al., 2003)**.

The aim of the present experiment was to study the effect of GM pea resistant to PEMV and PSMV viruses on morphological parameters and weight gain in rat models.

MATERIAL AND METHODOLOGY

The experiment was conducted at Department of animal nutrition and forage production of Agronomic faculty of Mendel University in Brno (in according with animal cruelty law No. 246/1992 Sb).

In a total, 24 male, specific pathogen free *Wistar* rats (Biotest, Konarovice, Czech Republic) were used in the experiment. At the beginning of the experiment, the animals were 28 days old and differences in body weight were in a range ±5 g. The animals were kept in an air-conditioned room with stable temperature 23 ±1 °C, light period 12 hours and humidity 60%. Photoperiod was based on pattern 12 hour/day and 12 hour/night with maximum intensity 200 lx. The monitored air content of CO_2 was - max. 0.25% and NH_3 max. 0.0025%. Food and water was provided ad libitum.

The experiment started after 8 days of quarantine period. The animals were divided into three groups, each of 8 animals. The experiment lasted 35 days. One group served as a control and these rats had not been fed with GM peas. Instead of pea the mixture of whey and soya was used. The remaining two groups were supplemented with 30% of GM pea or *Raman* pea according to the following scheme:

Composition of feed mixture is given in Table 1. Feed mixture KS1 was used as a negative control, the second KS 2 contained GMO pea and the third KS 3 contained was created by pea cultivar Raman.

The data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 10.0 (Czech Republic). Differences with p-value ≥0.05 (α = 5%) were considered significant and were determined by T-test, which was applied for means comparison.

Following parameters were monitored and calculated individually in groups of rats during the experiment: net intake of feed, conversion of feed, weight increment and health (were studied anatomical pathology and bacteriology parasitological virological indicators) status of animals.

The animals were treated and fed every day and once a week they were weighted.

The conversion of feed mixture was calculated according to the following formula:

Conversion = Feed consumption / (Final weight - Starting weight)

RESULTS AND DISCUSSION

During the last years the research and breeding activities have been focused on problems of pea viruses **(Safarova et al., 2008).** This concern was caused by frequent and repeating pea viruses occurrence **(Jeger et al., 2012; Soylu and Dervis 2011)**. It has been confirmed that only using of cultivars resistant to economically significant viruses represent effective measures against their negative influence on pea production and its seeds.

Changes in body weight of experimental animals

The changes in body weight were observed daily during the period of 35 days. The weights of the animals in three experimental groups are recorded in tables 2, 3 and 4.

The average weight values of the three groups were compared in. In the first group (rats feeded by mixture 1, negative control) was noticed the average weight 343 g at the end of experiment. In the second group (experiment utilized GMO pea as feeding mixture), the average weight reached up 331 g and in the third group (positive control) it was 348 g. There have not been noticed any statistically significant differences among compared groups. Standard deviations between groups were less than 3 %. Average values of weights at the end of experiment (after 35 days) are presented in Figure 1.

All experimental animals were in good health condition without any differences in growth and changes in behaviour.

Table 1 Composition of feed mixture in experimental groups

Feed mixture /Experimental groups	KS 1	KS 2	KS 3
Composition (%)	Negative control	GM peas	Positive control
Wheat	60.00	43.80	43.80
Peas – species *Raman*	0.00	0.00	30.00
Peas – resistant line	0.00	30.00	0.00
Maize	11.00	10.00	10.00
Pollards from soya (47.5%)	12.00	0.00	0.00
Starch	10.84	10.00	10.00
Lysine (78%)	0.46	0.00	0.00
Premix of micro and macroelements	3.00	3.00	3.00
Premix of vitamins	0.20	0.20	0.20
Sunflower oil	2.50	3.00	3.00
Total	100.00	100.00	100.00

Table 2 Increase of weight of rats fed mixture 1

Negative control	1. day	7. day	14. day	21. day	28. day	35. day
1	134	179	213	257	293	360
2	114	158	200	247	289	349
3	133	169	212	262	306	342
4	123	158	202	251	284	333
5	131	164	213	260	297	338
6	134	171	227	267	308	347
7	125	160	216	256	289	328
8	141	179	218	248	330	345
Average	129	167	213	256	300	343
Sandard deviation	8.4	8.8	8.4	7.0	14.0	9.9

Table 3 Increase of weight of rats fed mixture 2

GMO peas	1. day	7. day	14. day	21. day	28. day	35. day
1	136	168	235	277	324	358
2	124	160	214	258	306	306
3	130	172	220	270	311	338
4	120	160	211	252	292	309
5	120	164	215	262	311	352
6	121	158	202	258	289	339
7	120	154	215	271	277	321
8	120	167	215	258	297	327
Average	124	163	216	263	301	331
Standard deviation	5.8	5.9	9.3	8.3	15.1	18.9

Table 4 Increase of weight of rats fed mixture 3

Positive control	1. day	7. day	14. day	21. day	28. day	35. day
1	118	149	202	273	310	314
2	145	183	232	266	304	341
3	116	160	198	241	287	346
4	136	174	214	251	301	372
5	125	161	196	234	273	354
6	123	177	218	268	322	370
7	133	175	213	242	288	356
8	120	156	212	278	315	330
Average	127	167	211	257	300	348
standard deviation	9.9	11.9	11.9	16.6	16.2	19.6

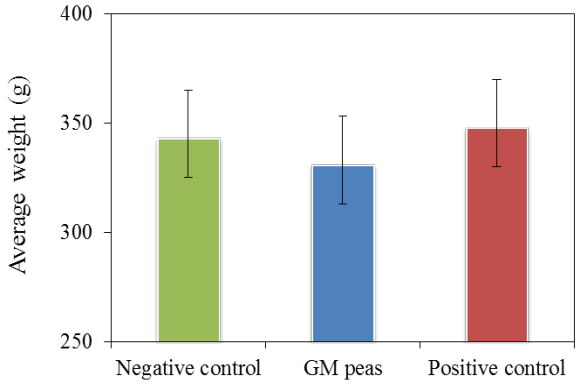
The average values of weight were compared in three groups. In the first group (rats fed mixture 1, negative control) was noticed the average weight 343 g at the end of experiment. In the second group (experiment fed GMO pea), the average weight reached up 331 g and in the third group (positive control) it was 348 g. There have not been noticed any statistically significant differences among compared groups. Standard deviations between groups were less than 3%. Average values of weights at the end of experiment (after 35 days) are presented in Figure 1.

All experimental animals were in good health condition without any differences in growth and changes in behaviour.

Study of feeding conversion

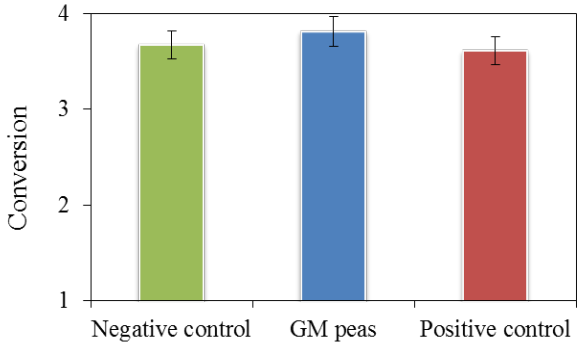
Feed conversion is defined as ratio between total increase of animal to feed consumption. It is calculated difference between weight at the begging and end of experiment. This total increase is rated by feed consumption during experimental period. All necessary values of 24 experimental animals are expressed in table 5.

Results of feeding conversion showed that there were no statistically significant differences between observed groups. Values reached up from 3.61 up to 3.81. Results of feeding conversion are given at Figure 2.



p-value ≥0.05, α = 5%

Figure 1 The average increases in weight of rats in three experimental groups



p-value ≥0.05, α = 5%

Figure 2 Feeding conversion during experimental period

Table 5 Feeding conversion during experiment period

	Starting weight	Final weight	Total growth	Feed consumption	Conversion
Negative control	1038	2746	1707	6268	3.67
GM peas	994	2651	1657	6312	3.81
Positive control	1020	2786	1766	6371	3.61

Although the genetic transformation of leguminous plants has been considered as very difficult and published protocols are reproducible with difficulties, there have been created new GM materials with declared resistance to biotic factors. From studied legumes - soybean, bean, pea, chickpea, peanut and vigna was released only soybean **(Huyghe, 1998; Wang and Brummer 2012)**. Precising and acceleration of breeding process has been possible by rapid development of information and techniques. It has been realised thank to adequate DNA markers in binding to genes of resistance, in ideal cases by direct detection of responsible genes **(Gilliland et al., 2003)**. The results of our experiment suggest that feeding GM pea to rats does not have detrimental effect on their health. There is potential possibility of GM peas utilisation and ranking GM pea into the List of approved GM crops.

CONCLUSION

The aim of presented experiment was to verify the influence of feeding of GM pea on health conditions, growth and quality parameters of rats. The results of the experiment showed that high level of GM pea in feed had no statistically significant effect on weight increase in comparison with both the group fed by pea cultivar Raman and negative control. No health complications were noticed during the experiment in model animals.

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DETERMINATION OF TEXTURAL PROPERTIES OF DIFFERENT KINDS OF KETCHUPS OF TWO DIFFERENT RATES UNDER DIFFERENT CONDITIONS OF STORAGE FOR THE DETERMINATION OF THEIR CONSUMAL QUALITY

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ABSTRACT

The aim of this work is an evaluation of structural properties of ketchups originating from five different producers at two different batches and consecutively their comparison in different storage conditions and a determination of their consumable quality. Using the objective instrumental method based on physical deformation of the sample we evaluated properties like, viscosity and consistency of ketchups on texturometer TA.XT plus (Stable Micro Systems, Godalming, United Kingdom). These tests were completed on the basis of regressive extrusion using a hyperbaric disc. The consistency of each ketchup from the first rate determined by regressive extrusion ranged from 2656.82 $g.s^{-1}$ to 5137.37 $g.s^{-1}$. The lowest value 2656.82 $g.s^{-1}$ was found in the sample A after 3 days of storage at the temperature 5 °C, the highest value 5137.37 gs^{-1} was found in the sample C after 10 days of storage at the temperature 5 °C. The value of the consistency in each of the samples didn´t change much, not even after the ketchups were stored at a room temperature for 24 hours. The consistency of each of the ketchups from the second rate determined by the regressive extrusion ranged form 2700.14 $g.s^{-1}$ to 5133.94 $g.s^{-1}$. The lowest value 2700.14 $g.s^{-1}$ was found in the sample A after 10 days of storage at the temperature 5 °C, the highest value 5133.94 $g.s^{-1}$ was found in the sample C after buying in a market and consequential cooling to 5 °C. The value of the consistency in each of the samples didn´t change much, not even after the ketchups were stored at a room temperature for 24 hours. The results obtained from the evaluation of the texture can be used to optimize the production and thereby prolonging the duration of maintaining its characteristics during the period of minimum durability.

Keywords: texture; *TA.XT plus*; ketchup; consumer

INTRODUCTION

Ketchup is a tomato puree seasoned with spices. It´s usually made of tomato puree and flavoured with salt, sugar, vinegar, oil but also onion, garlic, mushrooms, spice, aromatic herb essence and other additives improving colour, taste and consistency **(Stýčková & Teslíková, 2005)**.

Usually at least 6% of dry matter should come from fresh tomatoes or tomato puree. Glucose or glucose syrup makes 20 - 30% of the total solids. The high glucose content and high dry matter content in ketchup leads to a reduction of water activity on the level from 0.93 to 0.95. The amount of added vinegar results in 0.8% and 1.0% acetic acid in the product. Spices can be replaced with oleoresins or extracts. Often starch is added to achieve the desired consistency. However, some countries don´t allow this. In these countries the final product is referred to as a „tomato sauce“. To achieve a certain durability of a closed product benzoic acid and sorbic acid are used for preservation. Durability of a closed product is then approximately a year, whereas a durability of an open product is a few weeks or months provided it is kept at a safe temperature depending on the structure and the presence of preservatives **(Lund et al., 2000)**.

The ketchup is required to be a clear tomato colour, smooth looking, without any blots with a consistency that is neither too waterish nor too compact so that it doesn´t spurt or separate the liquid part from the solid after shaking and then opening the bottle. Also, there must be a pleasant ketchup fragrance preserved **(Ranken et al., 1996)**.

The production of ketchup uses a so-called „hot break“ process. It means that the tomatoes are very quickly warmed up to at least 77 °C but usually 90 °C. At this temperature enzymes begin to sunder which preserves a higher pectin level. It means that by destroying all the enzymes using a „hot break“ process a more viscous product is made, less susceptible to separation. There´s also a „cold break“ process in which the tomatoes are warmed up to 65 °C. The pectin is destroyed and therefore a more sparse liquid is made with a brighter colour and a fresher taste **(Pritchard & Burch, 2003)**.

In food processing, food scientists add special ingredients for the textural properties of food. Starches, pectin and rubber are added for the purpose of thickening, gel consistency or to increase viscosity. The addition of glucose affects the texture of food by improving the sensation in mouth or it may be added in a higher concentration so the food is more chewable and insubstantial because crystallization is in progress. Viscosity and consistency are interesting properties, which affect the textural as well as the visual attributes of food quality **(Vieira, 1996)**.

The effect of processing on the texture of the product can be better understood after considering the structural changes within the process of manufacturing, when the

tomatoes are cut, warmed up and homogenized and the cell and cell-wall disperse arises, which affects the textural properties of the ketchup **(Ouden, 1996)**.

Consistency and the level of serum separation largely determine the quality of the ketchup. Consistency of the ketchup depends directly on the rate of the tomato pulp used in the production, whilst the serum separation depends on the heat used. There are demonstrable differences in viscosity of the ketchups produced by „hot break“ and „cold break“ methods (**Marsh et al., 1997)**.

The term „consistency“ refers to non-newtonian fluids with dispersed particles and dissolved long-chain molecules. Usually the product flow is measured. Newtonian fluids are characteristic by their static viscosity. Their deformation is directly proportional to the pressure used, whilst on the other hand we know non-newtonian fluids, including ketchup, which we characterize as pseudoplastic fluids. Here the viscosity decreases with the pressure and the heavier we affect the simplier it deforms. It follows that ketchup is a highly viscous substance and flows slowly at a slow pressure **(Fishman et al., 1991)**.

The consistency of tomatoes and products depends on the presence of intact cells and cell fragments, pectin substances on their surface, the solubility of pectin and suspended particles in serum. Insoluble constituents present in these products may be intact, frangible or pulverised cells and cell fragments and long chains of lignin polymers, cellulose and hemicellulose and pectin constituents insoluble in water. These dispersed particles are highly hydrated and they take up considerably large content, however, they contain very few solid constituents. Consistency depends primarily on the ratio of water-insoluble substances to total solid substances **(Kertesz and Loconti, 1994)**.

Viscosity has been described as a resistance to motion when mixing or pouring ketchup. Ketchup is an excellent example of flow properties. When the ketchup bottle flips upside down, its contents will remain in its place for a while. On the other hand, if the content was mixed and shaken before flipping, it would immediately start flowing along the sides of the bottle as a relatively sparse fluid. The viscosity of the ketchup is defined as an internal friction of the motion, when it easily arises the motion (i.e. liquid materials) but only a small friction arises. In reverse, for solid materials the friction is relatively high **(Beckett, 2004)**.

Density has a great influence on the taste since denser ketchup remains in the mouth longer and releases its flavour slowly. Moreover, the longer it stays in mouth the more it heats and releases more volatile compounds. Approximately 95% of the ketchup consists of smaller molecules like water, vinegar, sugar, salt and aromatic compounds. The remaining 5% consist of larger molecules like polysaccharides, most of which are starch and fibres. These have an ability to hold a large amount of water, which results in a bigger density of a ketchup. Pectin is the examples of larger molecule, which is naturally found in fruits and vegetables, where this molecule holds together the cell walls. Pectin is released from the cell walls during the boiling and its amount depends on the degree of ripeness of the fruit or vegetable. Pectin has been found in ketchup extracted from the tomatoes, however it hasn´t been able to hold a larger amount of water. One of the solutions is the decrease of water by further boiling, however this makes the texture „thicker“ and less desirable. Another solution is the addition of larger molecules, which hold the water in the ketchup. That is why ketchups are being added different kinds of polysaccharides, which prevent from larger losses of water and therefore sustain a fitting texture of the product **(Vega et al., 2012)**.

During the storage and the transport some undesirable changes can occur. Partly because the product hasn´t been in a thermo-dynamical balance when leaving the production line and partly because of the fluctuating temperature within the storage and transport. These changes can result in the fault of the product, caused by structural changes of the product **(Kilcast, 2003)**.

Within the storage the viscosity of the product may be changed. As the ketchup homogenizes within the production process, an increase of apparent viscosity occurs up to 40% regardless of the processing temperature or homogenisation pressure **(Race 1991)**. It means that all the homogenised products are in advantage within the storage, therefore even after expiration of some time the viscosity remains unaffected.

Szczesniak (2002) explains the texture as a sensory and functional demonstration of structural and mechanical and surficial properties of food, which we perceive sensually (vision, hearing, feel, kinesthesis composed of sensation of the presence of motion and location) and which affect the nervous receptors. Complex and dynamic character of the texture is evaluated analytically by a sensory analysis.

The texture properties of ketchups have an important meaning in a number of technological operations. Understanding of the basic texture properties such as viscosity is important not only in characterising the raw material, but also in a number of technological calculations or supervision of processing devices. This results in a development of texturometry, which helps to increase the competitiveness of the products in the markets. Texturometer is one of the possibilities to evaluate the texture of foods and even though it´s an instrumental method in which the device imitates the human senses and measures the basic mechanical properties, only humans can evaluate the quality completely, by their sensory rating. However, the device measuring the attributes related to texture and quality are necessary for research and supervision of foodsstuffs **(Kilcast, 2003)**.

Texturometer *TA.XT plus* (Stable Micro Systems, Godalming, United Kingdom) is basically able to measure any physical property of the product. It´s very effective and capable of making high-speed testing. It´s also very easy to use. In connection with the software, the device is well projected, constructed and made by the company Stable Micro System for a long-term reliability and exactness.

The relationship between the instrumental measurement of the texture and sensory evaluation is still an object of examination. Instrumental methods imitate human testing methods or measure the basic mechanical properties or indirectly set optical and chemical properties. Only humans can evaluate the quality with a sensory evaluation,

however the devices measuring the attributes related to quality are necessary for research and supervision **(Abbott et al., 1997)**.

MATERIAL AND METHODOLOGY

During the work, the texture properties of ketchups originating from 5 different producers were studied. We compared 2 different production batches, bought in the market (A-E) (Tab. 1). These properties were measured by a texturometer *TA.XT plus* (Stable Micro Systems, Godalming, United Kingdom). This device was projected by the company Stable Micro System for the measurement of texturometrical properties of commodities like foodstuff, cosmetics, pharmaceutical products and different industrial materials.

This device continually records the force, distance and a time whilst simultaneously deforming the material with pressure or tensile force. Deformation of the sample placed on the base of the device is carried out by a fluctuant arm with a strain gauge to record the acting force. There are different extensions and probes attached to the arm and the base of the strain gauge. The process of measurement is recorded by the PC program *Exponent* (Stable Micro Systems) in a form of deformation curve. This sophisticated PC program allows further curve processing such as statistical evaluation of the recording (determination of the maximum, minimum and average values, standard deviation and coefficient of variation of the endpoint). Mathematical calculations (labeling the maximum and minum of the curve, calculation of the extent below the curve, determination of the maximum, minimum and average curves as well as the comparison with remaining curves, saving the recordings and their further processing. This allows us to watch the measured material within a certain time period.

The device works on the base of its setting given by its user. The handeling is defined according to the chosen mode (measurement of the force or path during the pressure or tensile force) and the conditions of the adjustable parameters (Pre Test Speed, Test Speed, Post Test Speed, Distance, Force, Time, Trigger). Utility is designed so that the user can save all the parameters and settings for the next measurement as a routine again. The usage of the device is simplified to minimum using the projects in the program in which there are parameters adjusted for each commodity.

In this case, we used the adjustment of those Exponent projects in which the ketchups were evaluated by back extrusion using the A/BE probe. This device consists of a disc made of plexiglas. The disc penetrates centrally into the ketchup in the pattern book and pushes the ketchup up and round the edge of the disc by a pressure test.

This probe is appropriate for measuring viscosity and product like soft gels, processed fruits and vegetables, ointments, sauces. There are discs of 3 different sizes. The selection of the disc depends on the measured product. In our case we´ve chosen a disc with the diameter of 45 mm. To determine the texture properties we used ketchups coming from five different producers A-E (Tab.1) and two different batches, we monitored the changes within the storage of both batches during 12 days immediately after the opening of the ketchups, after 3 days of storage at 5 °C, after 7 days of storage at 5 °C, after 10 days of storage at 5 °C and consecutively after 24 hour storage at room temperature. Furthermore, we compared 2 ketchups (soft and hot) after the expiration date with 2 ketchups (soft and hot) before the expiration date. The changes within the storage were monitored in 14 samples. 5 samples from the first rate were measured twice within every evaluation. The other 5 samples from the second rate as well. Consecutively, 2 samples before the expiration date were compared to the 2 after the expiration date, which were also measured twice. Altogether there were a hundred measurements made and consecutively evaluated.

The measurements were carried out at same times (10 A.M). We tested the ketchups in common sample books with a diameter of 45 mm. The sample book was filled app. to 75% of its height. We measured each ketchup twice within every measurement to achieve better results. The content of the sample book was cooled to a fridge temperature of 5 °C each time. There was a probe used with each test. The probe was placed centrally above the sample book which was placed of the board where it fitted exactly to avoid the movement of the sample book during the lifting of the probe. We used software *Exponent* (Stable Micro Systems) preprogramed adjustments for ketchup testing. We started the measurement using the command „run test“. From the first contact with the ketchup the probe continued to a depth of 30 mm. As soon as this depth was achieved, the probe began to lift to its original position. During the penetration of the probe into the ketchup, the program *Exponent* recorded the measurement in a form of a curve. This curve depicted all the properties we monitored such as rigidity, consistency, cohesion and viscosity. As soon as the test was carried out, all the sample readings were recorded.

The results of the measurements of ketchups´ texture properties (consistency, viscosity) we expressed graphically in *MS Excel* (Microsoft, Redmond, Washington, USA) and using the matematical and statistical analyses: arithmetic mean (x) , standard deviation (sd), coefficient of variation (c.v).

Statistical evaluation of the results were carried out in the program *Tanagara 1.4.43*. (Rakotomalala, Lyon, France). Based on the parameters arised from our results, we chose a nonparametric statistical test Kruskal-Wallis 1-Way ANOVA.

Consistency: The area in the positive part of the graph. The higher is the value, the greater is consistency of the sample. The consistency relates to non-newtonic fluids with dispersed particles and dissolved long-chain molecules. Usually the product flow is measured.

Viscosity: The area in the negative part of the graph determines the viscosity index. Viscosity is a physical property of food, defined as the flow rate of the unit of force. The higher is the value, the greater is viscosity of the sample.

Table 1 Samples of ketchup used to measure

Sample	A	B	C	D	E
Type	Fine ketchup with alternate sweetener	Spicy ketchup with chili	Sweet ketchup	Spicy hot ketchup	Fine ketchup
Composition	Tomato paste, drinking water, fermented vinegar, salt, modified corn starch, citric acid, spice mix, xanthan gum, sucralose.	Drinking water, tomato paste, isoglucose syrup, vinegar fermentation, modified starch E1422, salt, spices (0.07% chili).	Water, concentrated tomato paste, sugar, fermented spirit vinegar, modified starch, salt, condiments (pepper extract).	Tomatoes, fermented vinegar, sugar, salt, herbs, spices kajanské, herbal extracts (contains celery), spices, spice extract.	Water, tomato concentrate (28%), sugar, fermented vinegar, modified starch E1422, salt, sodium benzoate, potassium sorbate, natural flavor.

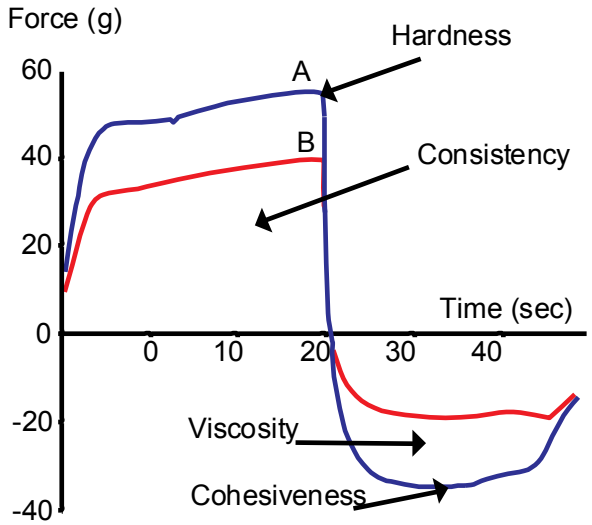

Figure 1 Curve showing the textural properties of ketchup in the program *Exponent*

RESULTS AND DISCUSSION

The consistency of each ketchup from the first batch determined by regressive extrusion ranged from 2656.82 $g.s^{-1}$ to 5137.37 $g.s^{-1}$. The lowest value 2656.82 $g.s^{-1}$ was found in the sample A after 3 days of storage at the temperature 5 °C, the highest value 5137.37 $g.s^{-1}$ was found in the sample C after 10 days of storage at the temperature 5 °C. The value of the consistency in each of the samples didn´t change much, not even after the ketchups were stored at a room temperature for 24 hours (Fig. 2). The consistency of each of the ketchups from the second batch determined by the regressive extrusion ranged form 2700.14 $g.s^{-1}$ to 5133.94 $g.s^{-1}$. The lowest value 2700.14 $g.s^{-1}$ was found in the sample A after 10 days of storage at the temperature 5 °C, the highest value 5133.94 $g.s^{-1}$ was found in the sample C after buying in a market and consequential cooling to 5 °C. The value of the consistency in each of the samples of second batch didn´t change much, not even after the ketchups were stored at a room temperature for 24 hours (Fig. 3).

Ketchup is a food with a thin consistancy so within its production there are various additives added in order to improve this property **(Sinha, 2011)**.

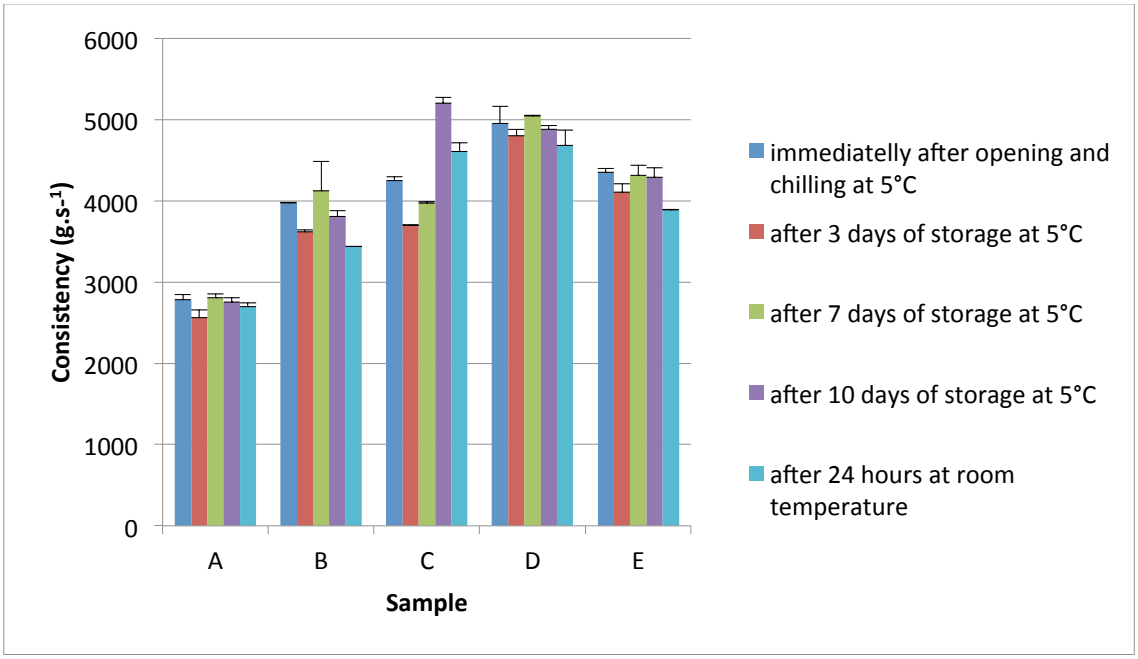

Figure 2 The average values of consistency for each of ketchup A-E of the first batch depending on storage

The graph shows the average value of the consistency of ketchups of the first batch (sample A – E), depending on storage, where fault segments with standard deviation is shown.

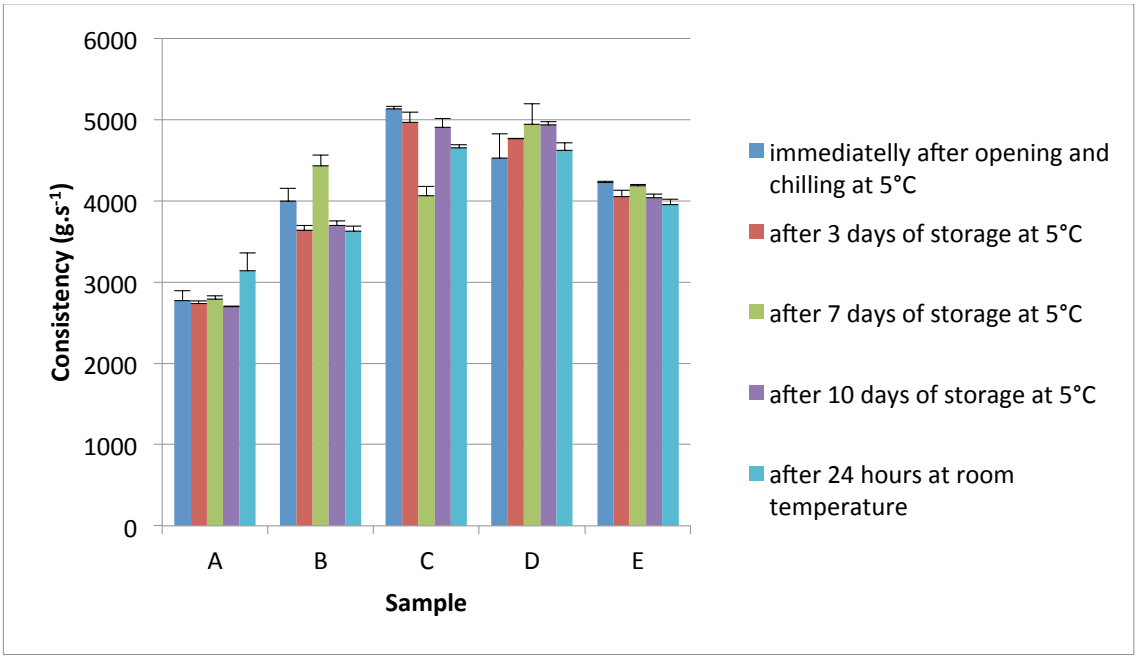

Figure 3 The average values of consistency for each of ketchup A-E of the second batch depending on storage

The graph shows the average value of the consistency of ketchups of the second batch (sample A – E), depending on storage, where fault segments with standard deviation is shown.

The consistency of ketchups depends on the presence of intact cells, cell fragments and pectin substances as well as on the solubility of these in a serum. Consequently it arises that the consistency depends on the ratio of the water insoluble substances to the number of solids **(Kertesz and Loconti, 1994)**.

Marsh et al. (1997) found out that the consistency of the ketchup depends directly on the rate of the tomato pulp used in the production, and also on whether there was any thickening used. Besides the additives used, glucose may be used as a thickening **(Marsh eta al., 1997)**.

Table 2 Statistical comparison of the consistency of the first and second batch of ketchup A-E

Storage conditions	*p* - value
Immediately after opening and chilling 5 °C	0.004223
After 3 days of storage at 5 °C	0.004223
After 7 days of storage at 5 °C	0.003077
After 10 days of storage at 5 °C	0.001545
After 24 hours at room temperature	0.001637

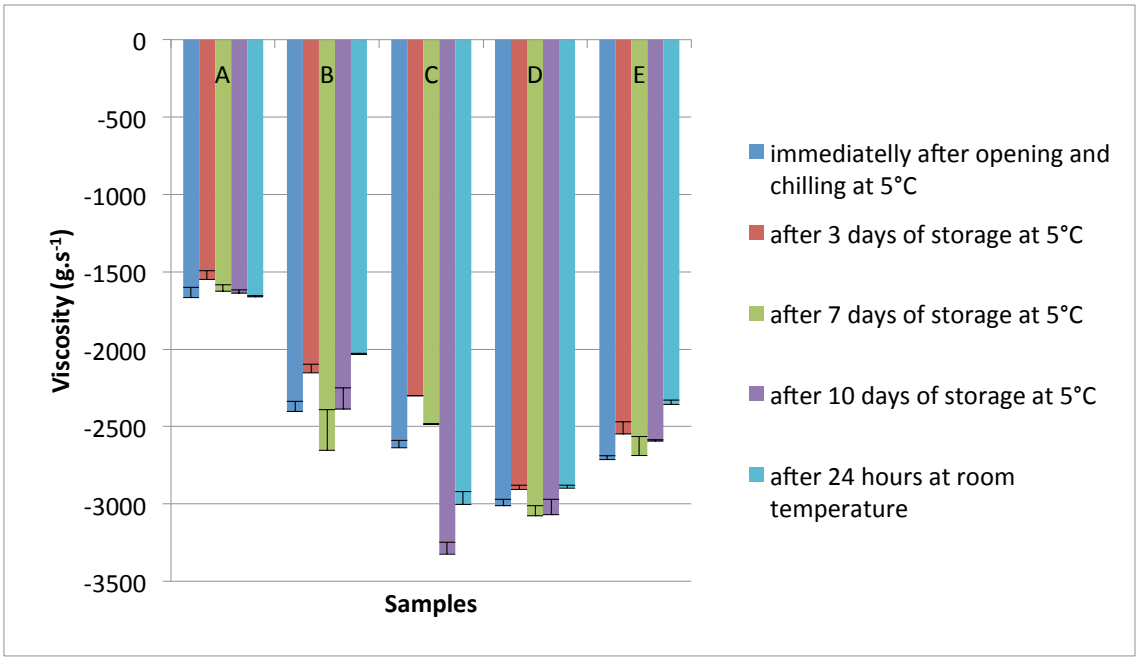

Figure 4 The average values of viscosity for each of ketchup A-E of the first batch depending on storage

The graph shows the average viscosity of ketchups of the first batch (sample A – E), depending on storage, where fault segments with standard deviation is shown.

After applying a nonparametric statistical method Kruskal-Wallis 1-Way ANOVA 1 to compare the consistency of the first and second rate of ketchups A-E, it´s safe to say that there´s a highly significant statistical difference between them at different storage conditions, where $p < 0.01$.

The viscosity of each ketchup from the first batch determined by regressive extrusion ranged from 1548.38 $g.s^{-1}$ to -3323.77 $g.s^{-1}$. The lowest value -1548.38 $g.s^{-1}$ was found in the sample A after 3 days of storage at the temperature 5 °C, the highest value -3323.77 $g.s^{-1}$ was found in the sample C after 10 days of storage at the temperature 5 °C. The value of the viscosity in each of the samples didn´t change much, not even after the ketchups were stored at a room temperature for 24 hours (Fig. 3).

The viscosity of each of the ketchups from the second batch determined by the regressive extrusion ranged form -1586.04 $g.s^{-1}$ to -3294.74 $g.s^{-1}$. The lowest value -1 586.04 $g.s^{-1}$ was found in the sample A after 10 days of storage at the temperature 5 °C, the highest value -3294.74 $g.s^{-1}$ was found in the sample C after buying in a market and consequential cooling to 5 °C. The value of the viscosity in each of the samples didn´t change much, not even after the ketchups were stored at a room temperature for 24 hours (Fig. 4).

The viscosity of ketchup is highly influenced by the approach in production. The production of ketchup uses a so-called „hot break“ process. It means that the tomatoes are very quickly warmed up to at least 77 °C but usually 90 °C. At this temperature enzymes begin to sunder which preserves a higher pectin level. It means that by destroying all the enzymes using a „hot break“ process a more viscous product is made, less susceptible to separation **(Pritchard & Burch, 2003)**.

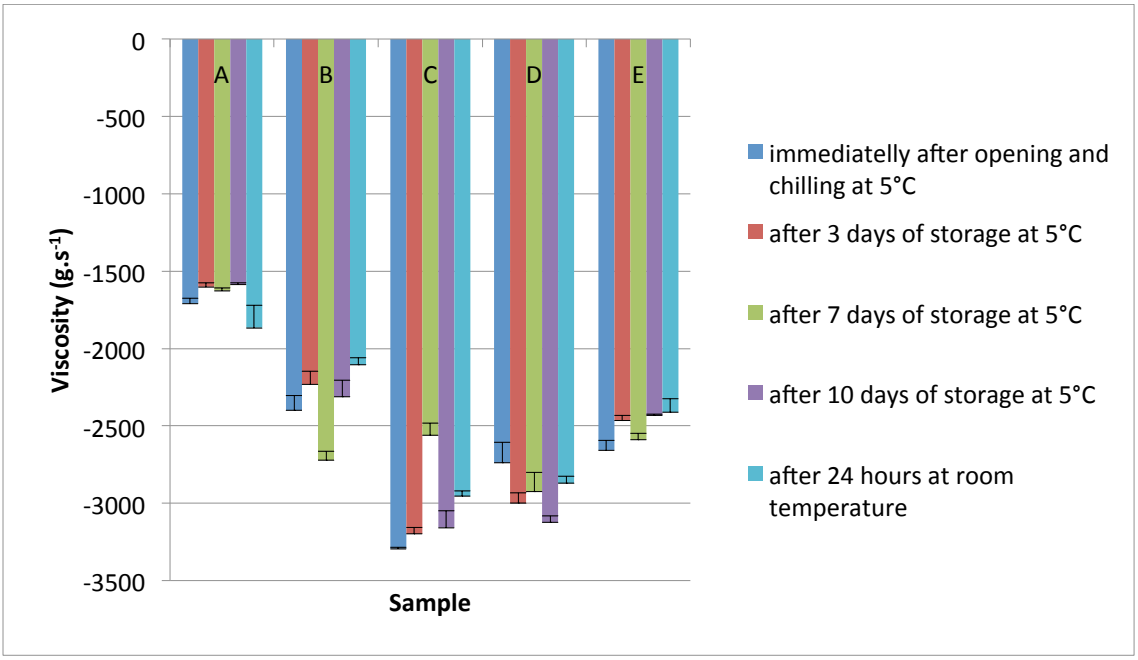

Figure 5 The average values of viscosity for each of ketchup A-E of the second batch depending on storage

The graph shows the average viscosity of ketchup of the second batch (sample A – E), depending on storage, where fault segments with standard deviation is shown.

Table 3 Statistical comparison of the viscosity of the first and second batch of ketchup A-E

Storage conditions	*p* - value
Immediately after opening and chilling 5 °C	0.003722
After 3 days of storage at 5 °C	0.005399
After 7 days of storage at 5 °C	0.005167
After 10 days of storage at 5 °C	0.001790
After 24 hours at room temperature	0.001535

The viscosity is the ketchup most important property, which is invariable, thanks to the fact that the ketchup homogenises during the production process, the viscosity of ketchup is preserved even after expiry of a period of time during storage. Ketchup that is adequately viscous gives a better taste in your mouth and it seems denser, which has the effect that the ketchup remains longer in the mouth and releases its flavour more slowly **(Race, 1991)**.

After applying a nonparametric statistical method Kruskal-Wallis 1-Way ANOVA 1 to compare the consistency of the first and second rate of ketchups A-E, it´s safe to say that there´s a highly significant statistical difference between them at different storage conditions, where $p < 0.01$. Statistical comparison of the consistency of the first and second batch of ketchup A-E is on Tab. 2. Statistical comparison of the viscosity of the first and second batch of ketchup A-E is on Tab. 3.

Ketchup A from the first and the second batch had the lowest consistency value, which could be caused by sucralose, which was used as a sweetener instead of glucose. The ketchup C from the first and the second batch had the highest consistency value. The ketchup D achieved also quite high consistency value but during its production there were no thickenings or additives used. All the other ketchups contain a modified starch E 1422, which adjusts the consistency.

CONCLUSION

The samples of ketchups A-E originating from common supermarkets. The samples were different kinds of ketchup such as soft, hot and sweet. They also had different structures and wrappings. The properties like consistency and viscosity were monitored right after bought and cooled to 5 °C. Further measurements were carried out after 3 days, 7 days and 10 days always at a temperature of 5 °C. Finally, we left the samples at a room temperature for 24 hours and consecutively measured them. From the PC programe *Exponent* we chose a methodology for measuring ketchups, we used regressive extrusion method using a probe and a hyperbaric disc. It arises from the

viscosity and consistency values of the measurements that there´s a highly statistically important difference between the first and the second rate.

Based on the results of the texturometric measurements of the properties of ketchup and their consecutive statistical evaluation, our recommendations are following:

- Choose wisely the appropriate method „hot break“ or „cold break“, which directly influences the texture properties of the product.
- Use quality raw materials while producing. These influent the qualitative properties of the ketchup highly.
- Use a right amount of additives which influent mostly the consistency but also the durability of the product
- Follow the required conditions while storage as they are presented on the wrapping of the product.

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EFFECT OF MALT MILLING FOR WORT EXTRACT CONTENT

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ABSTRACT

Beer manufacturing is one of the most ancient procedures of food manufacturing. The four (in many cases much more) ingredients, the great numbers of technological steps and variations of technological parameters (temperature, time, pressure etc.) have a major influence on both type and quality of the final product. As a result of this, studying beer brewing may offer a great deal of possibilities for numerous researches, scientific examinations, and can provide useful informations for the manufacturing companies as well. At the „Slovak University of Agriculture in Nitra" we examined an entire beer brewing process in October 2013, utilising the Ahlborn sensors, which were integrated in the instruments. Simultaneously, in Gödöllő at the Szent István University, we analysed the effect of malt milling on extract yield and the filterability of wort. We used the brewing parameters (temperature, time, volume ratios), which were experienced in the microbrewery and published in professional literature. Our results verify the conclusions drawn in the professional literature, however they point out the importance of grinding. Results performed on the yields with different grain-constitution might directly be utilised for the specialists of recently in Slovakia and Hungary spreading small-scale, handicraft, and homemade beer brewing.

Keywords: milling; malt; grain-size distribution; wort; extract

INTRODUCTION

Malting, mashing, brewing, fermentation and bottling are usually mentioned among the beer brewing operational steps. From our perspective, the mashing is a crucial area, during which the malt grist is mixed with water and slowly heated up. The purpose of this process is that the useful substances, proteins and still in soluble phase existing starch shall be dissolved to the highest possible extent and be transformed into sugar. For this process the brewery mostly utilises decoction mashing. Characteristic for this procedure is, that 1/3 portion of the mash from the mash tun is led into a brewing kettle, there it is gradually boiled and then pumped back into the mash tun to mix it up again with the rest of the mash. This step is repeated twice or three times thus reaching the final temperature of 62 - 79 °C of mashing. In smaller factories, home breweries, infusion-method is more commonly used; here the entire content of mash tun is gradually heated up to the desired temperature, applying necessary multiple pause periods. After reaching the desired mashing temperature, mash is stirred until transformation of starch into fermentable sugars terminates. This can be checked by the so-called iodine-test **(Briggs, 1998; Fix 2000; Géczi 1994; Narziss, 1981)**. For quick determining the extract produced during the mashing is carried out by using Balling-grade [°B] or Brix-grade [°Bx] measurement unit, in laboratories the extrakt and dry substance content of wort might be determined by analytical means as well **(Bamforth, 2006; Fix and Fix, 1997)**.

Methods of brewing and later on hop boiling (electrical, gas-operated, etc.) determine beer brewing from both economical and environmental aspects. Nowadays the technologies are deemed as environmental-conscious where energy-consumption is reduced, water-consumption is reduced or the amount of waste is reduced. From this perspective it is worth examining the grinding process from the beer production technological steps. The grinding is an extraordinarily energy-consuming procedure; at the same time it determines the quality of the final product. **(Korzenszky, 2007; Korzenszky and Judák, 2009)**. Grinding the malt is actually milling, executed between rollers, which is of vital importance from the aspects of chemical-biological transformations during mashing, quality content and extraction of wort, plus yield. Malt milling is very special because the grain husk and the internal core need different preparation. Milling the peel must be avoided as it plays an important role at wort separation, forming a filter-layer. In spite of this internal core needs fine milling because this contains the important ingredients from extraction point of view. Checking the malt grist in small-scale production-routine takes place by visual control, but by means of using sieves-series it is possible to acquire quantified evaluation. With the sieve-series, structure of grist can be established, distribution of grain-size is easy to depict in graphs and average grain size which is characteristic for the grist might also be determined **(Frančáková et al, 2011; Ivanišová et al., 2011; Korzenszky, 2012; Miller, n.a.; Mousia, 2004; Reilly et. al., 2004; Warpala and Pandiella, 2000)**.

Quality of beer is influenced by other processes (for example mashing, brewing, chilling, fermentation) and the technological parameters of those **(Tóth et al., 2013; Goode et al., 2005)**. During our researches we examined the wort's extract-content and filterability as a function of grist formed by different milling methods.

MATERIAL AND METHODOLOGY

In the microbrewery of Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences we examined, documented and analysed a beer brewing procedure on 5th November 2013. Quantities of basic materials, determining the technological temperatures and periods happened according to the recipe, which was based on the previous experiences. Ahlborn sensors mounted on the mash tun, brewing kettle and fermentation tank made it possible that pressure and temperature values of brewing and fermentation procedures can be continuously recorded and evaluated. Due to continuous sampling, the parameters (malt humidity, grain distribution after milling, extract content of wort, etc.), which are characteristic for brewing procedure, were also determined under laboratory circumstances after brewing in Gödöllő, at Szent István University, Faculty of Chemistry and Biochemistry.

Table 1 Sieve analysis of grain-size distribution of malt grist used in Nitra brewing

Grind	Size [mm]	%
Husk	>1.0	43.7
Coarse grits	0.5 - 1.0	30.5
Fine grits	0.25 - 0.5	15.0
Flour	<0.25	10.9

Making the beer started with milling 13 kg of malt. A little portion of malt was milled twice, for the sake of more favourable grain-size distribution. Grain-size distribution was determined by sieve-analysis, (VEB MLW Labortechnik Ilmenau Thyr2, DDR). The received data is shown in Table 1. More than ten percent of malt grist was of flour quality, 15% of it can be characterised as fine grist and 30.5% was the so-called coarse grist. The fraction larger than 1.0 mm comprised of husks and uncrushed grain particles. The malt grist can be characterised with 0.99 mm average grain size. Country of origin of the malt is Slovakia.

The planned and measured temperature-rise of mashing is shown in Figure 1. Based on the recipe, mixing the malt took place in 50 °C water. After 10 minutes rest-time we increased the temperature to 55 °C. Including heating time, we had intended to keep it at this temperature for 20 minutes, and then the next target was 64 °C. Temperature territory of 55 - 64 °C we had aimed to maintain for at least 60 minutes. Based on the recipe, the final temperature is 76 °C, at which the wort is kept until transformation of starch into fermentable sugars. It was checked by iodine-test. Due to inertness of electric heating the planned temperature-steps are not obvious based on our measured values. Temperature of wort increased continuously, after two and half hours of brewing we could start the separating of liquid wort from the residual grist (brewers' grains). This operation is the lautering or filtering.

As a result of quick evaluation performed by Balling-hydrometer the extract-content of wort is 15.2 °B, which was finally modified by the amount of mash and extract content to 9.8 °B. The introduction also pointed out that beer brewing is influenced by several parameters. Out of these factors we would like to emphasize the importance of malt milling hereafter. The aims of laboratory examinations investigated the extract-content and filterability of wort. It was prepared based on the values experienced during the hereby-presented beer-brewing and professional literature data.

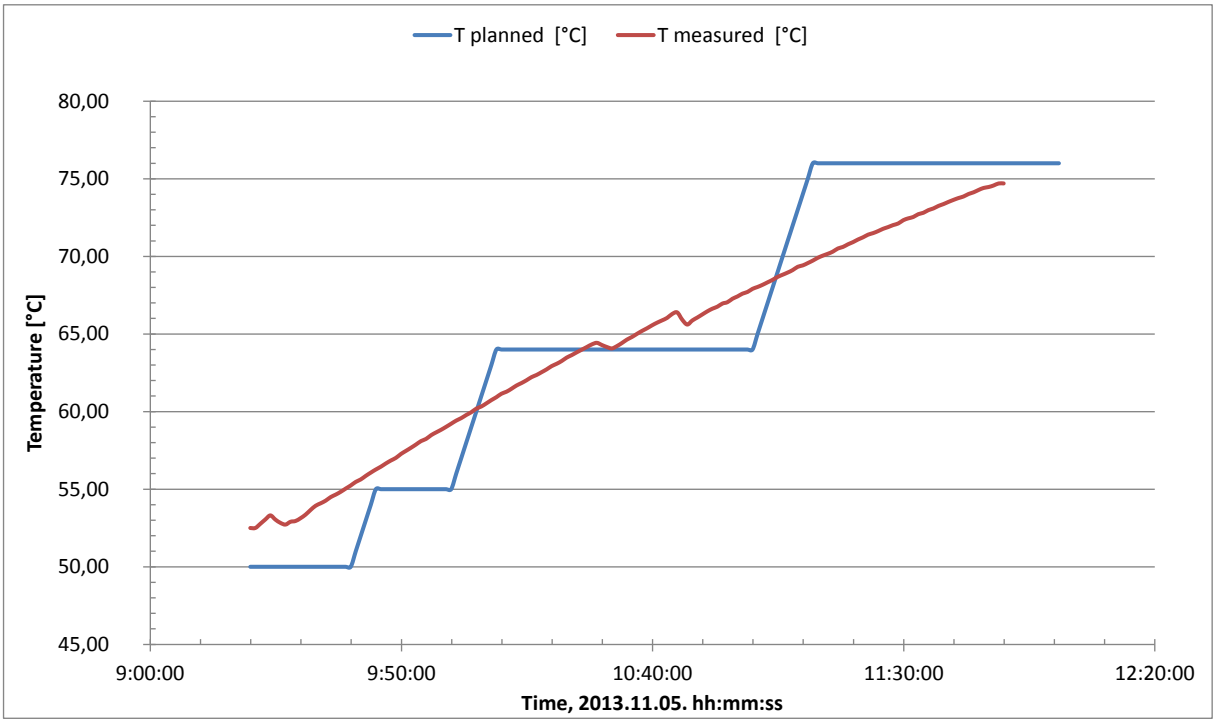

Figure 1 The planned and measured temperature-rise of mashing as a function the time

Table 2 Grain-size distribution of malt grist used in laboratory mashing, Gödöllő

Size [mm] / Methods of milling (grinding)	>1.0*	0.5 - 1.0	0.25 - 0.5	<0.25	$X_{average}$
	[%]				[mm]
A – Laboratory mill	5.9	15.9	64.4	13.8	**0.46**
B – Poppy seed grinder	37.6	38.5	17.5	6.5	**0.78**
C – Mortar	68.9	15.3	9.4	6.4	**1.39**
D – Walnut grinder	90.9	5.2	2.2	1.8	**1.94**
E – Grape press	93.0	3.9	1.8	1.3	**2.21**
F – Without milling	100	0	0	0	**>2,5**

* not husks only, but in case of D,E,F whole grains also.

Quality of milling is interpreted by the grain-size distribution. Depending on the malt-type, breweries formed an "ideal" grain-size distribution, which results in greater and greater yield, however at the same time it does not obstruct separating wort from brewers' grains. To examine this, we made 5 samples of Münch Malt type malt (w = 3.54%, MSZ 318-3:1979) with different grain-size distribution, using laboratory mill (Mlyn walcowy Typ SK, Sadkiewicz Instruments, Poland, Bydgoszcz) and household grinders (poppy-seed grinder, walnut grinder, grape press and mortar). For control purposes, we used the 6th sample of malt without milling. The characteristics of the 6 malt samples with different grain-size distribution were summarized in Table 2 as well.

Figure 2 Mashing in laboratory in water-bath thermostat

Grain-size distributions are rather different from each other; the malt used during the brewing in Nitra resembles the most to the sample prepared with poppy-seed grinder. The mashing was modelled under laboratory circumstances with using 100 g malt and 330 ml of water, in water-bath thermostat (Memmert WB 14, Germany). The "mashing" of the 6 samples took place simultaneously (Figure 2), mixing in the malt at 35 °C, then heated to 50 °C and 65 °C, finally to 79 °C and terminated after transformation of starch into sugars. At least 25 minute long break periods had been planned at each temperature step during the mashing process. Making the wort finally took 120 minutes; the temperature values measured in the brewing glasses are shown in Figure 3.

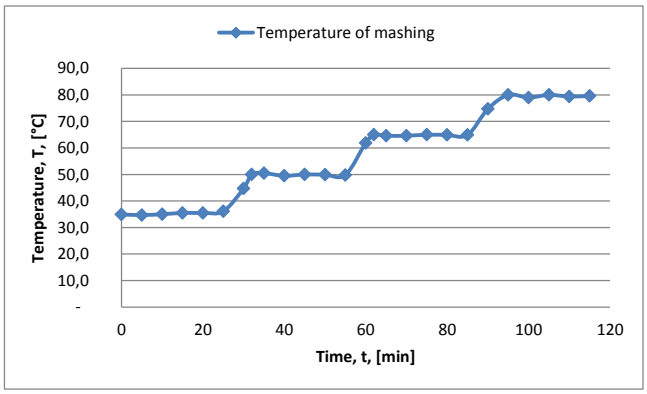

Figure 3 Temperature-rise of mashing as a function the time measured in water-bath thermostat

Separating the wort from the brewers' grains was carried out by sudden overturning of the brewing glass which was covered with filter canvas, gravity way. The quality of brewers' grains forming layers during filtering, the chronological build-up of flow rate presented the filterability. During the filtering experiment we measured filtered amount as the function of time and determined the volume of wort flowing through in 90 seconds. Determining the extract content of wort was performed by refractometer (OG-101/A, Hungary), which was also checked by analytical density measurement as well (SHIMADZU AY220M, Japan).

RESULTS AND DISCUSSION

The quantified results of making wort under laboratory circumstances are presented in Table 3. The utilised grinding (milling) methods were classified into an ascending order based on the average grain size. It shows that the sample containing the finest grains produces the highest extract content and it gradually decreases aligning with the increasing average grain size. The amount of filtrate is quite the opposite, it is less in case of fine grains, and it is more with large grains. The filtrate amount flowing through in 90 seconds is displayed in the filtrate column, but the gravity-driven flow ceased in all cases due to the brewers' grains forming layers. Further amount of filtrate could be gained by moving and stirring up.

Table 3 Extract content and the filterability of wort mashed in laboratory.

Samples (Based on milling)	$X_{average}$ [mm]	Extract content [°Bx]	Volume of filtrate** [ml]
A – Laboratory mill	0.46	16.9	35
B – Poppy seeds grinder	0.78	15.7	45
C - Mortar	1.39	14.5	85
D – Walnut grinder	1.94	12.7	130
E – Grape press	2.21	7.4	140
F – Without milling	>2.5	1.9	240

** Volume of filtrate under 90 second

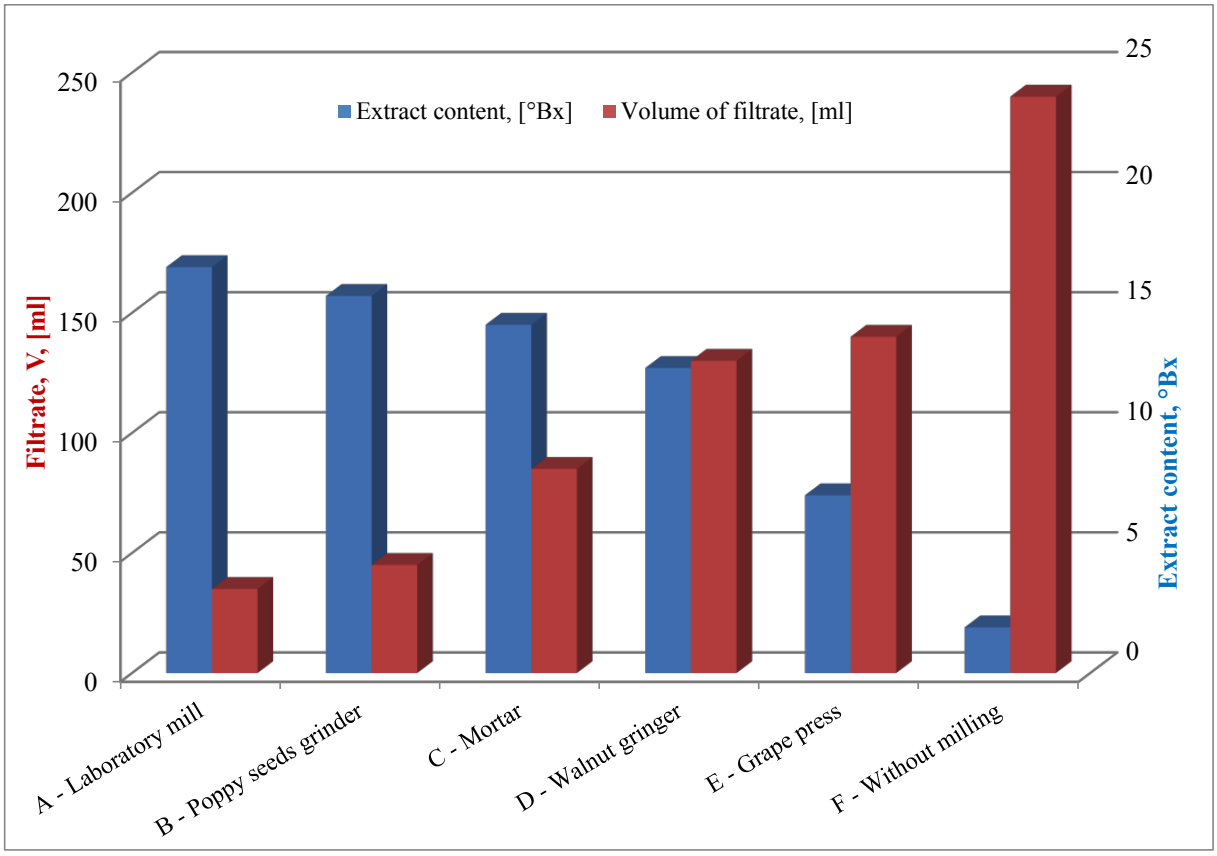

Figure 4 Extract content and filtrate volume of wort as a function of milling methods.

It would seem to be logical to establish an ideal grain-size distribution. Though we represented the results in a bar chart as well (Figure 4), however it would be an incautious statement to choose the grist fraction which is characterised by a "quite high" column from both filterability and extract content perspective. As the amount of filtrate and extract content are different parameters from units of measurement aspect as well, therefore comparing them with each other largely depends on the scale of diagram. Figure 4 nicely demonstrates the conclusions drawn from the table.

To repeat, malt grist of finer grain size can be characterised with larger extract and worse filterability. A better filterability can be achieved with larger grain fractions but from yield perspective it is not preferred. In the small-scale routine the favourable grain distribution can be realised by double triple milling of one portion of the malt, considering at the same time the experimental fact that milling of the malt husks must be avoided.

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EXAMINATION OF HEAT TREATMENTS AT PRESERVATION OF GRAPE MUST

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ABSTRACT

Heat treatment is a well-known process in food preservation. It is made to avoid and to slow down food deterioration. The process was developed by Louise Pasteur French scientist to avoid late among others wine further fermentation. The different heat treatments influence the shelf life in food production. In our article we present the process of grape must fermentation, as grape must is the base material of wine production. The treatment of harvested fresh grape juice has a big influence on end product quality. It is our experiments we examined the same grape must with four different methods in closed and in open spaces to determine CO_2 concentration change. There are four different methods for treatment of grape juice: boiling, microwave treatment, treatment by water bath thermostat and a control without treatment. As a result of the comparison it can be stated that the heat treatment delays the start of fermentation, thereby increasing shelf life of grape must. However, no significant differences were found between two fermentation of heat-treated grape must by the microwave and water-bath thermostat. The different heat treatment of grape must base materials was done at the laboratory in Faculty of Mechanical Engineering of Szent István University. The origin of the table grapes used for the examination was Gödöllő-hillside.

Keywords: grape must; preservation; CO_2 concentration; microwave; heat treatment

INTRODUCTION

Grape must is the juice squeezed from grape berries. This juice is the base material of good wine. Grape must is the favourite drink of harvest time, but the fermentation of its sugar content changes the flavour and it's other features. During fermentation the biggest part of sugar content (glucose) converted into ethyl alcohol and carbon dioxide.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 + \text{heat}$$

Figure 1 Fermentation of grape must
Source: verlieftwines.com by Dirk Roos **(URL 2)**

Fermentation is very important step in the process of wine production. The yeast in the must starts fermentation. But more and more producers try to preserve the grape must to sell it as a non-alcoholic drink **(Shea, 2008; Lafon-Lafourcade et al., 1984)**.

Heat treatment is a known process in food preservation. It is made to avoid and to slow down food deterioration. The process was worked out by Louise Pasteur French scientist to avoid late wine fermentation (among others). It is interesting, that at the same time as Pasteur, Preysz Móric Hungarian chemist worked out a similar process to avoid quality loss of Tokaj wines **(URL 1)**.

The mild heat treatment treatment (60 - 80 °C) delays the fermentation of grape must, so it is a frequent method at homemade preservation. Industrial heat treatments are made with tubular or plate heat exchangers by convective heat transfer. The heating of liquid food can be done by microwave energy transfer. At our previous researches we examined the effect of heat treatments on milk, egg fluid, beer and orange juice **(Géczi et. al. 2013, Korzenszky et al., 2013, Garnacho et al. 2012)**.

According to the previous mentioned, heat treatment for food preservation gives a good possibility also to examine microwave heat treatment **(Kapcsándi, 2012; Marsellés-Fontanet et al., 2009)**.

MATERIAL AND METHODOLOGY

The different heat-treating of grape must base materials was done at the laboratory in Faculty of Mechanical Engineering of Szent István University. The origin of table grape, which was used for the examination, was Gödöllő-hillside but the grape breed is unknown.

The comparisons were started with the preparation of the samples. The freshly squeezed table grape juice was

poured in different tanks according to the different heat treatments. For traceability the samples were marked with unique identifiers. The control sample (untreated) was marked with „U", the boiled with „B", the treated by microwave with „M", and treated by water bath thermostat with „T" according to the Figure 2.

The microwave food treatment researcher group made continuous operating experiment equipment, which is applicable for microwave heating, and heat-treating. In the last period with the development of this equipment we made a measurement circle which is applicable for making comparison experiments of fluid food heating with microwaves and with convective heat transfer (water bath thermostat) **(Géczi and Sembery, 2010)**.

The 'soul' of the comparison measurement circle is a glass spiral in which the liquid food (in our case grape must) is pumped with an adjustable volume flow STENNER 85M5 type (Stenner Pump Compony, Jacksonville, FL, U.S.A.) pump. The glass spiral is put in a Whirlpool AT 314WH (Whirlpool Corporation, U.S.A.) domestic microwave oven or a *T*-PHYWE (Lauda DR. R. Wobser GmbH, Lauda-Königshofen, Germany) water bath thermostat for heating.

We used 900 Watt output power of microwave oven and 70 - 95 °C temperature water bath in the thermostat. With this heat treatment (depending on the length of the glass spiral and the volume flow of the pump) liquid foods can be heated to the needed temperature in the glass spiral.

It is advantageous that with the utilization of glass spirals the heating is gradual and outcome temperature is constant. We managed to avoid temperature variation, which is common at microwave heating. The temperature - before and after the microwave equipment and water bath thermostat - can be easily controlled and regulated. We used an ALMEMO 2590-4S type (Ahlborn, Holzkirchen, Germany) thermometer and data logger system with NiCr-Ni heat elements.

We selected T_{target} = 70 °C target temperature for grape must heat treatment. We did not keep this temperature for any length of time, but we cooled it down immediately. The target temperature was achieved at Q =2.5cm^3.s^{-1} volume flow of peristaltic pump both of microwave and water bath thermostat heat treatment. In the latter case, we applied T_{bath} = 78 °C water temperature.

In case the making of boiled control sample the grape must was heated up gradual and after boiling was cooled.

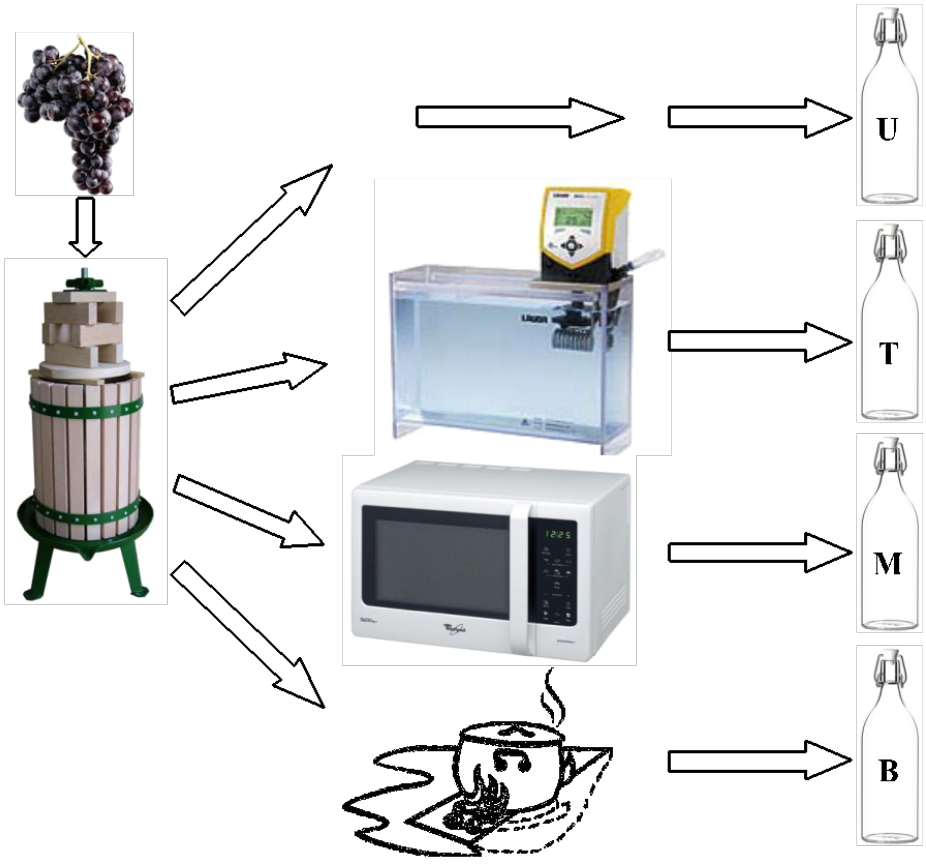

Figure 2 Forming of treated sample groups

„U" – untreated 'control'; „T" – heat treated with water bath;
„M" – microwave treated; „B" – heat treated with boiling 'control'

Figure 3 Examination methods for fermentation

We poured 1-5 dL of heat treated and untreated samples in bottles according to analysis. In all cases the samples were naturally cooled to 24 °C storage temperature.

The carefully prepared heat-treated samples were used for the definition of physical parameters in the laboratory with standardized methods. The analysis and control of grape must samples was done at the Department of Chemistry and Biochemistry, on Szent István University of Gödöllő. During the analysis of grape we must defined dry matter content, density, pH-level and conductivity. For the physical measures we used 0.5 - 0.5 L samples.

The fermentation process can be characterized to determine CO_2 concentration. It was done from closed bottle by sampling (Figure 3-I.) and by examination of airspace of opened barrels (Figure 3-II.). The analysis and control of samples were blind probes; the examiner person got coded and mixed samples. The person who heat-treated the samples did not take part in the measures.

For the examination I. we measured 170 - 170 mL of the samples with a measuring cylinder. These samples were put in gastight septum closed glass bottles. These were used for the CO_2 analysis.

During fermentation we took samples from the 1.2 L septum closed glass bottles, from the space above the fluid (with a 1ml Hamilton syringe). The CO_2 concentration of these samples was defined with a Hewlett Packard 5890 series II. gas chromatograph (Germany, SN.:3203616265), with an universal detector, TCD (used for measuring heat conduction) and with helium gas. For data visualization we used the HP GC Chem Station Rev. A. 08.03 software, which is able to present the values under the maximum of CO_2 concentration and retention time **(David Del Pozo-Insfran et al., 2006)**.

We analyzed the CO_2 concentration parallel at open tanks during fermentation (Figure 3-II.). CO_2 is heavier than air, so during the fermentation a constant increase of CO_2 could be measured inside the open barrels. For the measurement we used an ALMEMO 2590-4S type (Ahlborn, Holzkirchen, Germany) data logger with FYAD 00 CO2 B10 and FHA646E1C. We measured during 240 hours, and recorded the concentration. During the measures we examined storage temperature, humidity and air pressure (data not shown). The fermentation of different sample groups were examined parallel.

Table 1 Features of grape must treated with different heat treatments

Sample identifiers	**Untreated „U”**	**Mikrowave „M”**	**Thermostat „T”**	**Boiling „B”**
Dry matter content [%]	24.74	26.11	27.22	30.21
Density (20°C) [g/cm^3]	1.079	1.073	1.073	1.081
pH	2.76	2.81	2.98	3.05

*Average of three measurements

RESULTS AND CONCLUSION

We used standardized measurement methods for examining the grape must's physical features. The results of the experiments can be seen in Table 1. The physical parameters (density, dry matter content, pH) of grape must were changed slightly with heating and boiling. The change is explained with evaporation and CO_2 stripping. It can be seen that the dry matter content of grape must in case boiled sample "B" nearly 5% higher than in the untreated sample "U". The difference of samples density are minimum, the pH value in case the untreated sample "U" is 2.76, whereas for boiled sample "B" increased to 3.05. The values in the Table 1 are the average of three measurements.

The CO_2 concentrations of the samples were controlled during fermentation at laboratory conditions. (Fig. 3-I.) The CO_2 concentration change of the microcosmos closed samples can be seen on Figure.4. On the logarithmic scale it can be seen that CO_2-concentration of untreated grape must sample shows significantly bigger values as at the other samples.

In the untreated case CO_2 concentration starts from 1200 µg/L value and after 50 hours it reaches 550000 µg/L. The CO_2 concentration of boiled must does not change

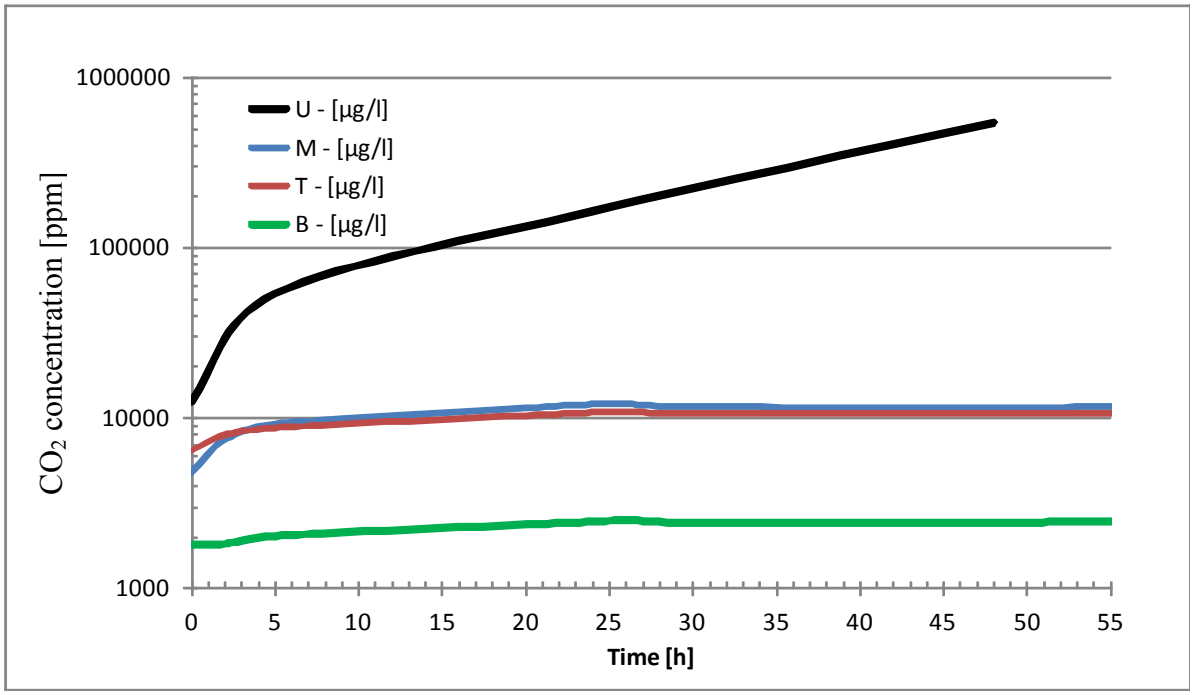

Figure 4 CO_2 concentration of grape must samples in sealed bottle as a function of the time

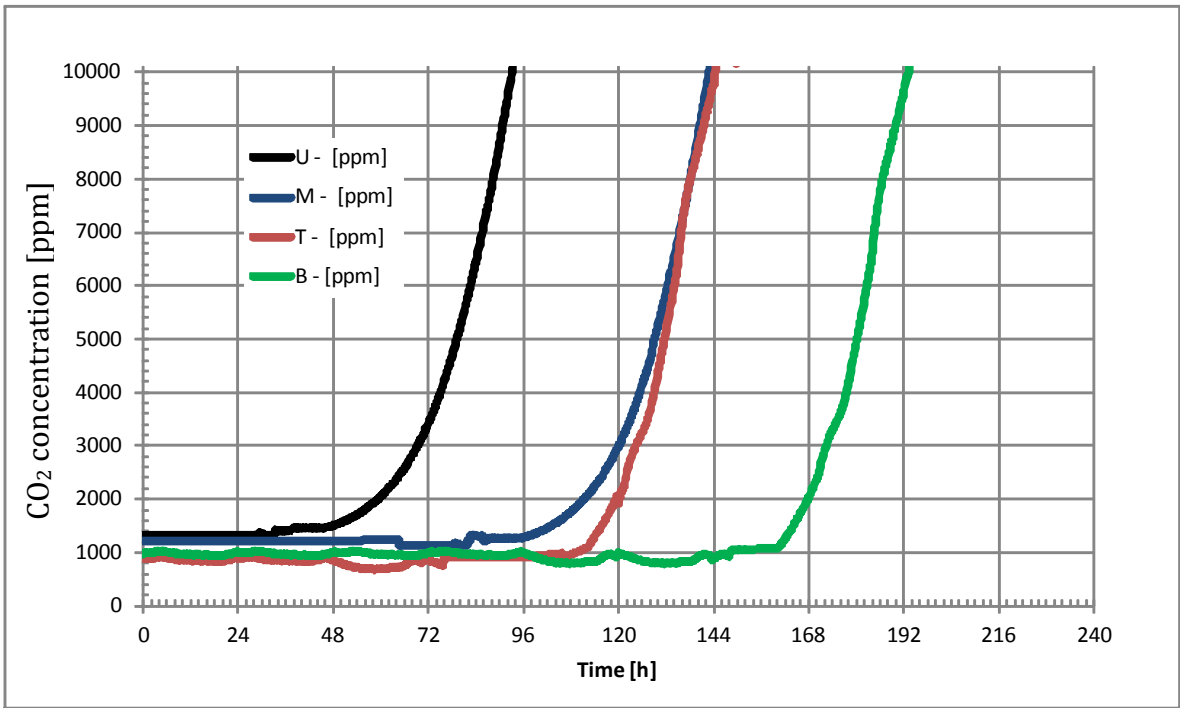

Figure 5 CO_2 concentration of must samples in airspace of opened barrels as a function of the time

significantly compared to the start value. The value changes from1750 μg/L to 2450 μg/L.

After microwave and water bath thermostat treatment the CO_2 concentration is similar. The starter value was 5000 μg/L and it changes to10000 μg/L.

In this treatment temperature (T_{target} = 70 °C) with two different processes no significant difference can be seen in CO_2 concentration growth of closed space.

The result of examination methods Figure 3-II for CO_2 concentration is shown on Figure 5. On the figure it can be seen that we measured different values at the four samples. The CO_2 concentration is showed from 0 to 10000 ppm - in accordance with the measurement range of the device - as a function of the time. The fermentation started earliest at the untreated sample "U", after 48 hours, CO_2 was observed intensive growth. The fermentation of more samples delayed by heat treatments, greatest extent was at boiled sample "B". The fermentation of 70 °C heat treated samples by microwave energy "M" and convective way in water bath thermostat "T" started on 5th day, while the fermentation of the boiled sample begin on 7th day only. The slopes which show the increase of CO_2 concentration are very similar all four cases, therefore we can conclude that the heat treatments do not influence for process of fermentation but delay the formation. Based on Figure 5 it can be stated that there is no difference between effect of the microwave and convective heat treatment methods for must preservation. We got similar results with the experiments of Nero and Bianca grape must (data not shown).

It can be stated that at similar treatment temperature the CO_2 concentration of "M" and "T" samples does not show significant difference during the fermentation process in open tank. We got similar results with the experiments of NERO must (data not shown).

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LEAD CONCENTRATION IN MEAT AND MEAT PRODUCTS OF DIFFERENT ORIGIN

Anetta Lukáčová, Jozef Golian, Peter Massányi, Grzegorz Formicki

ABSTRACT

Meat is very rich and convenient source of nutrients including also a large extent of microelements. Contamination with heavy metals is a serious threat because of their toxicity, bioaccumulation and biomagnifications in the food chain. The lead concentrations depend on the environmental conditions and the food production methods. Ingestion of contaminants, especially heavy metals, by animals causes deposition of residues in meat. The aim of this study was to determine the levels of lead in the Malokarpatska and Lovecka salami during the technological processing with comparison to the raw materials originating from domestic and foreign production. Lead content was determined by atomic absorption spectrometry. The starting materials in the Malokarpatska salami were found to contain the highest level of lead in the beef of foreign production (7.58 ppb), followed pork from foreign production (3.43 ppb), beef from domestic production (3.27 ppb), pork bacon from foreign production (2.41 ppb), pork from domestic production (1.63 ppb) and pork bacon from domestic production (1.57 ppb). The average concentration of lead was higher in homogenized samples with addition of additives and spices and ranged between 6.49 to 7.56 ppb. The lead concentration in final product Malokarpatska salami was in the range from 8.57 to 8.89 ppb. The highest mean Pb concentrations in the Lovecka salami in the starting materials were beef from foreign production, beef from domestic production, pork from foreign production, pork bacon from foreign production, pork from domestic production and pork bacon from domestic production (7.31, 3.77, 3.21, 2.40, 2.03, 1.97 ppb, respectively). Increasing concentration of lead was found after the addition of additives, spices and curing compounds causing a threefold increase in the concentration of lead in final products Lovecka and Malokarpatska salami. Technological process of meat processing can create a potential source of heavy metals in final products. Improvements in the food production and processing technology are increasing the chances of food contamination with various environmental pollutants, especially heavy metals.

Keywords: lead; meat; meat products; atomic absorption spectrophotometry

INTRODUCTION

Metals are found in all living organisms where they play a variety of roles **(Nuurtamo et al., 1980)**. Metal such as Fe, Cu, Mg, Co, Zn are essential for human body but chronic metabolic disturbances may occur due to the deficiency or excess of these metals. Non-essential elements such as Pb, Cd, Cr, Ni and As are considered to be toxic and their presence in the body can cause profound biochemical and neurological changes in the body **(Schroeder, 1991)**. Heavy metal is defined as a metal, which is neither essential nor has beneficial effect, on the contrary, it displays severe toxicological symptoms at low levels and is defined as a metal with a specific weigh more than 5 $g.cm^{-3}$ **(Gonzales-Waller et al., 2006)**. Environmental pollution associated with heavy metals has been of global concern over many decades. These metals are natural components of the environment but high rate of industrialization has been responsible for their wider diffusion and dispersal in the environment **(Rajaganapathy et al., 2011)**. The sources of toxic metals in the environment are the fossil fuels, mining industries, waste disposals and municipal sewage. Farming and forestry also contribute to the metal content in the environment due to the uses of fertilizers, pesticides **(Jayasekara et al., 1992)**. With increasing industrialization, more metals are entering into the environment. These metals stay permanently because they cannot be degraded in the environment **(Baykov et al., 1996)**.

The risk associated with the exposure to heavy metals present in food products had aroused widespread concern in human health **(Reilly, 1991)**. Improvements in the food production and processing technology had increased the chances of contamination of food with various environmental pollutants, especially heavy metals. Ingestion of these contaminants by animals causes deposition of residues in meat **(Sabir et al., 2003)**. Higher level of trace metals has been recorded in beef and mutton. Presence of substantiates levels of toxic metals lead and cadmium in meat products has been recorded **(González-Weller et al., 2006)**.

The main human exposure to heavy metals usually comes from food. After continuously evaluating studies on food additives and their toxicity, the WHO has came to the conclusion that even low levels of some metals, such as lead and cadmium, can give rise to diseases in humans **(WHO 2000, WHO 2001)**. This is produced by the capacity of these metals to accumulate in living organisms.

Lead is toxic heavy metal with widespread industrial use, but no known nutritional benefits. Chronic exposure at relatively low levels can result in damage to kidneys and liver and to immune, reproductive, cardiovascular, nervous and gastrointestinal systems **(Okoye et al., 2010)**. Lead, for example, bio-accumulates in plants and animals. Its concentration is generally magnified in the food chain **(Halliwell et al., 2000)**. Lead is metabolic poison and neurotoxin that binds to essential enzymes and several other cellular components and inactivates them **(Cunningham and Saigo, 1997)**. The main toxic effect of lead is nervous system dysfunction of the foetus and infants. In adults, it causes adverse blood effects, reproductive dysfunctions, damage to the gastrointestinal tract, nephropathies, damage to the central as well as the peripheral nervous system and interferences in the enzymatic systems **(Rubio et al., 2005)**. The health risks caused by lead and cadmium are well known and the levels in food as well as the migration of these metals from food containers are regulated **(Tahvonen and Kumpulainen, 1994)**. However, cases of accidental exposure to higher levels of lead and cadmium are often reported within the EU. For example, in Sweden in 2004, a man was poisoned by lead after using ceramics bought from another EU country, which had not been properly fabricated **(National Food Administraton in Sweden, 2004)**.

The risk of heavy metal contamination in meat is of great concern for both food safety and human health because of the toxic nature of these metals at relatively minute concentrations **(Santhi et al., 2008)**.

The aim of this study was to determine the level of lead in the traditional and popular meat products consumed in Slovak republic. This study is carried out to determine the levels of lead in Lovecka and Malokarpatska salami during the technological processing. The raw materials originating from domestic and foreign production were compared.

MATERIAL AND METHODOLOGY

Sample collection: To reach representative samples, average composition and characteristics of the goods were analysed. The concentration of lead was determined in 180 samples of raw materials and final products, respectively. The samples came from Slovakia Western Slovakia region and directly imported samples from European and American holdings. The collection of samples during the manufacturing process was carried out under the following scheme:

"*Malokarpatska salami*" - basic raw material (beef, pork and pork bacon) was collected; then samples of homogenized meat with additives (salt, spice extracts, sodium nitrite, highlighter flavour, *Lactobacillus*) and finally the actual sample of the final product after heat treatment was analysed;

"Lovecka salami" - basic raw material (beef, pork and pork bacon) was collected; than samples of homogenized meat with additives (salt, sodium ascorbate, erythorbic acid, ground black pepper, sugar, garlic, starter culture) and finally, the actual sample of the final product after heat treatment, cooling to 25 °C and drying in climates with aw = 0.95 was analyzed.

Sample preparation: Collected samples were packet to plastic bags, and frozen (-18 °C). Amount 30 - 50 mg of meat or homogenized meat samples and final products were used in the protocol.

The samples were dried at 105 °C in order to obtain dry mass of meat samples. All the samples were mineralized in the hot nitric acid (HNO_3 65% Ultranal®, POCH, Poland) at the temperature of 90 °C until complete dissolution of tissues using VELP Scientifica DK 20 (VELP Scientifica, Italy) mineralizator. Later the samples were thinned with spectrally pure water to cubic capacity of 10 mL. The mineralized samples were analysed by the AA spectrometer with the graphite furnace (PerkinElmer AAnalyst 800; MA, USA) to determine lead concentration. Final results were given in ppb ($\mu g.kg^{-1}$) for meat and other samples.

Statistical analyses: Data collected were presented as mean, standard deviation, coefficient of variation and standard error of mean. The results were subjected to statistical analysis using the Graph Pad Prism (ver. 6.0 for Windows; GraphPad Software, Inc.; USA) involving ANOVA tests and post hoc Tuckey analysis.

RESULTS AND DISCUSSION

The mean values, standard deviations, standard error of mean, coefficient of variation of lead concentration in Malokarpatska salami are listed in Table 1. The level of lead in beef from domestic production (3.27 ±0.827 ppb) was lower than in beef from foreign production (7.58 ±1.214). Pb content in the beef samples from foreign production was significantly higher ($p < 0.0001$) compared to those from domestic production. The lead concentrations in beef are similar to those reported by **Gonzales-Waller et al. (2006)** but lower than those described by **Akan et al. (2010)** in beef meat (25 ppb). **Oskarsson et al. (1992)** reported high concentrations of lead in the muscle of dairy cows raised on pasture than in the muscle of dairy cows kept indoors. Lead was present in pork from domestic and foreign production in the range from 1.63 to 3.43 ppb. There was a significant variation ($p < 0.05$) between Pb content in collected pork samples from domestic and foreign production. The most hazardous heavy metal monitored on the swine farms in the district of Hodonin, Czech republic in 1994-1999 was lead, the major source of which being paint coats (containing more than 0.6 g lead per kg), mineral components of commercial feeds, scrap lead batteries put away in barns and lead coated guide bars of electric lines **(Ulrich et al., 2001)**. Mean contents of lead in pork bacon from foreign production (3.43 ±1.147 ppb) was higher than in pork bacon from domestic production (1.63 ±0.276 ppb). Lead data showed noticeable insignificant difference between Pb content in pork bacon from domestic production and pork bacon from foreign production. The starting materials for the beef of foreign production had the highest mean lead level (7.58 ppb), followed by pork from foreign production (3.43 ppb), beef from domestic production (3.27 ppb), pork bacon from foreign production (2.41 ppb), pork from domestic production (1.63 ppb) and pork bacon from domestic production (1.57 ppb).

The average concentration of lead was higher in homogenized samples with addition of additives and

spices. The levels of lead in the homogenized samples from foreign starting materials ranged from 4.320 to 11.02 ppb. The average lead concentration 6.49 ppb was found in homogenized samples from domestic starting materials. **Larkin et al. (1954)** reported that pepper contains higher levels of lead (>2.5 ppm) as is added invariably to almost all types of meat products. **Nkansah and Amoako (2010)** reported that high value of Pb was registered for black and white pepper (0.965 and 0.978 $mg.kg^{-1}$, respectively).

Final product Malokarpatska salami was in the range from 8.57 to 8.89 ppb. The lead concentrations detected in Malokarpatska salami in this study are similar to those of **Gonzales-Waller et al. (2006)** who reported the mean concentration of lead in the pork meat products samples 6.72 ppb and in the beef products samples 9.12 ppb. **Santhi et al. (2008)** found lower concentration of lead in the salami (2.231 ±0.432 ppb). **Muller and Anke (1995)** reported that lead content in sausage was higher than that in the meat used for its production, presumably due to the spices used in sausage production. **Demirezen and Uruc (2006)** reported that the highest average lead concentrations were obtained from pastirma, meat and sausage (0.126, 0.115, 0.135 ppb, respectively). The values are lower in comparison to our outgoing products.

The concentrations of lead observed in the Lovecka salami are presented in Table 2. The mean Pb concentrations in beef ranged between 3.77 ppb from domestic production to 7.31 ppb from foreign production.

The mean level of Pb in the beef from foreign production was higher ($p < 0.0001$) than in beef from domestic production. **Oskarsson et al. (1992)** reported a high lead concentration (500 $\mu g.kg^{-1}$) in beef after accidental exposure to lead. **Humphreys (1991)** reviewed the effects of lead in animals and reported that due to its slow rate of elimination lead could accumulate in tissues after prolonged exposure to even low quantities of lead. Pork meat of the foreign production (3.21 ppb) appeared to accumulate more Pb than pork meat in the domestic production (2.03 ppb). Content of lead in the pork samples from foreign production was significantly ($p < 0.05$) higher than in pork samples from domestic production. Regarding lead concentrations in pork, our values are lower compared to data published by **Demirezen and Uruc (2006)**, but higher than reported by **Chowdhury et al. (2011).** Lead concentrations were significantly increased after the addition of additives to the homogenized samples. The concentration of lead detected in the homogenized samples

Table 1 Basic variation statistical characteristics of lead concentration in the raw materials and final product "Malokarpatska" salami

	Beef/Pb		Pork/Pb		Pork bacon/Pb		Homogenized samples/Pb		Final product/Pb	
	D	*F*	*D*	*F*	*D*	*F*	*D*	*F*	*D*	*F*
x	3.27	7.58	1.63	3.43	1.57	2.41	6.49	7.56	8.89	8.57
SD	0.827	1.214	0.276	1.147	0.368	1.096	1.715	1.696	2.243	1.739
Min.	1.980	5.330	1.110	1.980	1.150	0.420	4.320	5.210	4.210	6.120
Max.	4.420	9.330	1.920	4.990	2.320	4.120	9.920	11.02	11.65	12.89
Med.	3.260	8.110	1.600	3.120	1.570	2.420	6.480	6.970	8.700	8.560
SEM	0.249	0.366	0.083	0.346	0.111	0.331	0.517	0.511	0.676	0.524
CV	25.30	16.03	16.93	33.48	23.40	45.54	26.43	22.43	25.23	20.29
p	p <0.0001		0.014 (p <0.05)		0.622 (NS)		0.328 (NS)		0.994 (NS)	

Legend: $\bar{x}$ – mean, SD – standard deviation, CV(%) – coefficient of variation, SEM – standard error of mean, Min. – minimum, Max. – maximum, Med. – median, D – domestic and F – foreign production, NS - non significant, Pb – lead

Table 2 Basic variation statistical characteristics of lead concentration in the raw materials and final product "Lovecka" salami

	Beef/Pb		Pork/Pb		Pork bacon/Pb		homogenized samples/Pb		final product/Pb	
	D	*F*	*D*	*F*	*D*	*F*	*D*	*F*	*D*	*F*
x	3.77	7.31	2.03	3.21	1.97	2.40	6.41	6.61	7.62	7.88
SD	0.927	0.882	0.584	1.014	0.629	0.701	0.843	1.103	1.147	1.014
Min.	2.280	5.980	1.220	1.210	1.120	1.150	5.200	4.120	5.700	5.760
Max.	5.720	8.330	2.870	4.980	2.950	3.460	7.900	8.650	9.600	9.730
Med.	3.770	7.540	1.880	3.280	1.880	2.210	6.400	6.870	7.600	8.110
SEM	0.279	0.266	0.176	0.306	0.189	0.211	0.254	0.332	0.346	0.306
CV	24.62	12.07	28.79	31.55	31.95	29.26	13.15	16.68	15.06	12.86
p	p < 0.0001		0.028 (p <0.05)		0.883 (NS)		0.998 (NS)		0.988 (NS)	

Legend: $\bar{x}$ – mean, SD – standard deviation, CV (%) – coefficient of variation, SEM – standard error of mean, Min. – minimum, Max. – maximum, Med. – median D – domestic and F – foreign production, NS - non significant, Pb – lead

from foreign products (6.61 ±1.103 ppb) was higher than concentration of lead in homogenized samples from domestic products (6.41 ±0.843 ppb).

Al-Eed et al. (1997) pointed on the addition of spices that may be contaminated with trace and heavy metals to food as a habit may result in accumulation of these metals in human organs and can cause different health problems. **Nkansah and Amoako (2010)** warn that process of spices preparation and handling can make them a source of food poisoning. Lead may reach and contaminate plants, vegetables and fruits. Monitoring of the levels of heavy metals in spices would help ascertain the health impact of taking spices. **Ozkutlu et al. (2006)** reported highest concentration of lead in the samples of garlic (0.999 $mg.kg^{-1}$). In the case of our homogenized samples, they were mixed with additives (salt, sodium ascorbate, erythorbic acid, ground black pepper, sugar, garlic, starter culture).

In final product Lovecka salami from foreign production higher concentration of lead (7.88 ±1.014 ppb) than in final product Lovecka salami from domestic production (7.62 ±1.147 ppb) was found. The maximum lead, which is allowed in meat from different animals, is regulated by the **Commission Regulations (EC) No 466/2001**. The allowable level for lead is 0.1 $mg.kg^{-1}$. The concentrations of these metals measured in this work are all within the accepted limits. There is also an absolute need for good manufacturing practices - Hazard Analysis and Critical Control points to monitor and curtail the contaminants in meat and meat products.

CONCLUSION

The lead levels in meat and meat products analysed in this study were below the legal limits established by the current EU legislation. The obtained results suggested that the concentrations of lead are higher in meat product samples (final products) after homogenization than in raw materials. Technological process of meat processing can create a potential source of heavy metals in final products. Improvements in the food production and processing technology are increasing the chances of food contamination with various environmental pollutants, especially heavy metals. Steps have to be taken to control the environmental contaminants, as a primary and effective food safety control.

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MONITORING OF COLOR AND PH IN MUSCLES OF PORK LEG (*M. ADDUCTOR* AND *M. SEMIMEMBRANOSUS*)

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ABSTRACT

In order to identify PSE pork meat, pH and color testing was performed directly in a cutting plant (72 hours *post mortem*) in this research. Specifically pork leg muscles *musculi adductor* (AD) and *semimembranosus* (SM) from five selected suppliers (A, B, C, D, E) were examined. Twenty samples of meat for each muscle were examined from each supplier. The measured pH values ranged from 5.43 to 5.63, and the L* values from 46.13 to 57.18. No statistically significant differences in pH values and color were detected among the various suppliers with the exception of the a* and b* parameters for two suppliers, namely A and B ($p < 0.01$). On the contrary, a statistically significant difference ($p < 0.5$) was recorded between individual muscles (AD/SM) across all the suppliers (A, B, C, D, E) with the exception of a* parameter from suppliers B, C, D, E, and pH values for the E supplier. Our results revealed that individual muscles differ in values of pH and color. In comparison with literature, pH and lightness L* values in *musculus adductor* point to PSE (pale, soft and exudative) meat, while the values of *musculus semimebranosus* to RFN (red, firm and non-exudative). Use of PSE meat in production of meat products can cause several problems. In particular, it causes light color, low water-holding capacity, poor fat emulsifying ability, lower yield, granular or crumbly texture and poor consistency of the finished product. Therefore classification of the meat directly cutting plant may be possible solution for this problem. The finished product produces from muscles of *musculi semimembranosus* can obtain better quality than the finished product from *musculi adductor*.

Keywords: PSE; quality; pork meat; lightness

INTRODUCTION

In recent years, production of pork has been steadily decreasing in the Czech Republic. Meat processors import cheaper pork meat from abroad **(Línková, 2013)**. This fact entails certain disadvantages, including a high variability in the quality of pork meat. Variations in the quality of raw materials have a negative impact on meat processors and the quality of final products.

The quality of pork meat is defined as a combination of various characteristics of raw and cooked meat **(Joo et al., 2013)**. These characteristics relate to acceptability for consumers and technological aspects, such as color, water-holding capacity, and texture. Biochemical processes that take place in the muscle *post mortem* affect all of these characteristics. The consequence of these biochemical changes is influenced by pH value, which is considered one of the most important factors determining the quality of meat **(Van der Wal, Engel and Hulsegge, 1997)**. Based on the pH of meat and other characteristics, pork can be divided into different quality groups: RSE (red, soft, exudative), PSE (pale, soft, exudative), DFD (dark, firm, dry), PFN (pale, firm and non-exudative), whereas normal pork meat is considered to be RFN (red, firm and non-exudative), **(Kazemi et al., 2011; O'Neill et al., 2003; Van de Perre et al., 2010; Chmiel et al., 2011)**. For pork, the most commonly encountered defect is PSE **(Lesiów and Xiong, 2012)**. Pale, soft and exudative (PSE) pork is a defective product resulting from both preslaughter and postmortem factors, for example, animal genetics, nutrition, season of the year, stress during animal transportation, and carcass processing and storage conditions **(Barbut et al., 2008, Lesiów and Kijowski, 2003 and Scheffler and Gerrard, 2007)**. Genetic selection and pre-slaughter stress cause rapid postmortem glycolysis that results in increased lactic acid production and decreased pH. Decreased pH combined with high muscle temperature causes protein denaturation that exceeds that observed in normal muscle leading to the production of pale, soft, and exudative (PSE) pork. Because of this protein denaturation, there is an increase in water loss and paleness that is detrimental to product quality **(Schilling, et al., 2004)**. PSE meat has a huge economic impact on both, the supplier as well as meat industry. The paper by **Cannon et al., 1996** indicates that 10.2 per cent of carcasses in slaughterhouses are classified as PSE. A more recent study in slaughterhouses shows that the incidence of PSE ranges from 2 to 30% **(Owen, 2012).** In the research **Mlynek et al., (2013)** report and compare incidence of PSE in three countries – Slovakia, Netherlands and Hungary. The lowest incidence PSE meat was in the group of pigs imported from the Netherlands (13.8%). The highest frequency of PSE meat in the *musculus longissimus dorsi* (MLD) was in the group of pigs imported from Slovakia (24.13%). From these results can be concluded that the incidence of PSE meat in evaluated groups is relatively high **(Mlynek et al., 2013)**.

At present, there is a prevailing tendency to constantly reduce the incidence of PSE meat and to find reliable detection indicators (pH, color, texture, electrical conductivity, etc.), which might enable detection of this defect already before the processing itself. When PSE meat is separated from material exhibiting standard fresh meat characteristics, the final product reaches better characteristics that are acceptable for consumers **(Lesiów and Xiong, 2012)**.

In literature, the most commonly encountered classification of PSE meat is based on drip loss, lightness value L* and pH, e.g. drip loss >6 per cent and L* >50 for PSE **(Ryu et al., 2005)**. In accordance with **Šimek, et al. (2004)**, meat is considered PSE if characterized by drip loss >5 per cent, lightness L* >50 (or *L** >55), and pH_{1h} <5.6 **(Šimek et al., 2004).** Other authors identify PSE meat using pH_{45} (<5.7), whereas normal meat (RFN) reaches pH_{24} values within the range of 5.5 – 5.8 **(O'Neill et al., 2003)** and **Mota-Rojas, et al., (2006)** reports values 5.8 to 6.2.

Use of PSE meat in production of meat products results in several problems. In particular, it causes light color, low water-holding capacity, poor fat emulsifying ability, lower yield, granular or crumbly texture and poor consistency of the finished product **(Laville et al., 2005; O'Neill et al., 2003)**. These issues are described in a wide range of meat products including ham, bacon, dry fermented sausages, finely minced meat products, and smoked meat **(Severini et al., 1989; O'Neill et al., 2003)**. **Young, (1996)** stated that customers will not buy a gray, wet product, and that appearance of pork is the most important attribute to the consumer. The authors compared here the functional properties of finished products, using PSE and normal (RFN) meat and report that the PSE raw material produces final products of very low quality, compared with the normal raw material (RFN), **(Severini et al., 1989; O'Neill et al., 2003)**.

The biggest problem is caused by PSE meat in processing of cooked hams. A defect in hams due to the use of this raw material occurs in 5 – 20% of cooked hams **(Minvielle et al., 2001)**. The basic raw material for coked hams is meat of pork hind leg composed of several anatomically different muscles. Muscles that are most affected by variations in the quality of meat, include *musculi adductor* (AD), *semimembranosus* (SM) *and biceps femoris* (BF) **(Bucko et al., 2012; Hugenschmidt et al., 2010; Laville et al., 2005; Valous et al., 2010; O´Neill et al., 2003)**. *Musculus adductor* and *m. semimembranosus* are anatomically separated muscles of the topside of pork leg, and they may exhibit different characteristics in the production of cooked hams. **Laville, et al., (2005)** report that the incidence of PSE meat affects the integrity of white muscle and the so-called PSE zones are limited mainly to *AD* and the inner parts of *SM*. It is of prime importance to clearly distinguish the various kinds of PSE, because they differ in important traits such as tenderness or flavour beyond the most evident deficiencies common to all of them. Moreover, as they result from different mechanisms, they require different remedies. Visually, meat from PSE zones resembles serious cases of PSE induced by high rates of *post mortem* pH fall, as encountered in halothane-sensitive pigs for instance. Overall, meat from PSE-zones and fast pH fall-PSE meat show numerous histological and biochemical similarities, particularly in their protein characteristics **(Laville, aetal., 2005)**. PSE meat can be reliably detected at the slaughterhouse using pH_{45} or pH_1, but processors, who purchase the meat from slaughterhouses, do not have this opportunity because they get meat 48 hours or more after the slaughter. Thus, there must be other determination methods applied.

The aim of this study was to evaluate and compare the differences in meat quality from five foreign suppliers based on selected indicators (pH and color) in muscles of pork leg, namely *musculus adductor (AD), musculus semimembranosus (SM),* and to evaluate the differences between these two muscles.

MATERIAL AND METHODOLOGY

Samples of examined meat

Meat quality monitoring was performed directly in the cutting plant in pork legs (72 hours *post mortem*) from five different suppliers (A, B, C, D, E). Measurement of pH and color were performed in 20 samples of *m. adductor* muscles and in 20 samples of m. semimembranosus.

Measurement of pH and color of meat

Measurement of pH and color of meat was performed directly in the cutting plant. The pH values were measured using a pH-meter of WTW pH 340i (WTW GmBh, Germany) with a needle probe Double Pore (Hamilton Bonaduz AG, Switzerland). The instrument was calibrated to the pH values of 4 and 7 prior to the measurement itself. The pH was determined by inserting the probe into the sample to be analyzed for each of the muscles (AD, SM) at two different points. The color was measured in the CIEL*a*b* system using Minolta CM 2600d spectrophotometer (Konica Minolta, Japan). Instrument calibration was performed on black and white colors. The most commonly used value to measure the quality of color deviation of meat is L* – lightness or the values of a* – redness and b* – yellowness.

Results of the color (L*, a*, b*) and pH measurements were statistically analyzed using Statistica CZ 7 (Statsoft, Czech Republic).

RESULTS AND DISCUSSION

pH

Table 1, 2 and Figure 1 shows that the pH values for individual muscles differ. In AD, the measured pH for all suppliers ranged from 5.43 to 5.46 and, in SM, it ranged from 5.56 to 5.63. When comparing the pH of pork meat, pH values for individual muscles were significantly lower in AD ($p < 0.05$) than in SM for suppliers A, B, C, D, with the exception of the E supplier where there was no statistically significant difference between AD and SM. Different characteristics of AD and SM may affect the quality of final products and may cause some defects in them, as described e.g. by **Hugenschmidt, et al. (2010)**.

Table 1 Comparison of pH and color (L*, a*, b*) of the five selected suppliers in *m. semimembranosus*

Muscle	Supplier	n = 20	L* (means ±SD)	a* (means ±SD)	b* (means ±SD)	pH (means ±SD)
SM	A	20	47.38 ±2.57^{+}	6.17 ±1.36^{+}	9.53 ±0.99^{+}	5.63 ±0.22^{+}
SM	B	20	46.13 ±3.10^{+}	7.02 ±1.68	9.93 ±1.83^{+}	5.62 ±0.20^{+}
SM	C	20	46.99 ±3.43^{+}	6.53 ±1.99	9.83 ±1.10^{+}	5.59 ±0.17^{+}
SM	D	20	47.56 ±3.59^{+}	6.85 ±1.68	10.12 ±2.06^{+}	5.56 ±0.12^{+}
SM	E	20	48.53 ±3.02^{+}	5.72 ±2.51	10.14 ±2.33^{+}	5.57 ±0.19^{+}

SM – *m. semimembranosus*, L* – lightness, a* – redness, b* – yellowness, SD – standard deviation, $^{+}$ $p < 0.05$ significant between AD and SM

Table 2 Comparison of pH and color (L*, a*, b*) of the five selected suppliers in *m. adductor*

Muscle	Supplier	n = 20	L* (means ±SD)	a* (means ±SD)	b* (means ±SD)	pH (means ±SD)
AD	A	20	56.02 ±3.31^{+}	$^{+}$4.11 ±1.72^{++}	12.26 ±1.56^{++}	5.47 ±0.15^{+}
AD	B	20	56.81 ±3.92^{+}	7.49 ±2.49^{++}	15.26 ±2.43^{++}	5.47 ±0.12^{+}
AD	C	20	57.17 ±3.71^{+}	5.02 ±3.10	13.21 ±2.31^{+}	5.45 ±0.09^{+}
AD	D	20	55.81 ±2.56^{+}	6.85 ±1.68	10.12 ±2.06^{+}	5.43 ±0.06^{+}
AD	E	20	57.18 ±1.83^{+}	5.72 ±2.51	10.14 ±2.33^{+}	5.46 ±0.11^{+}

AD – *m. adductor*, L* – lightness, a* – redness, b* – yellowness, SD – standard deviation, $^{+}$ $p < 0.05$ significant between AD and SM ,$^{++}$ $p < 0.01$ significant between suppli*ers*

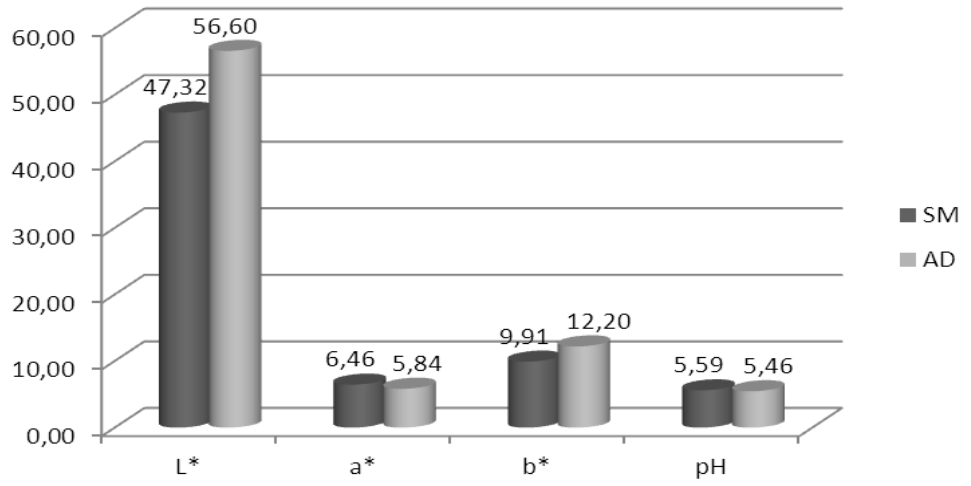

Figure 1 Average value of pH and color (L*, a*, b*) in *musculus semimembranosus* and *musculus adductor*

Comparison of pH values with other works in our case is limited because in focusing on our issue, monitoring of pH, e.g. pH_1, pH_{45} is impossible to be implemented. The meat is available for us 72 hours *post mortem*, so we have to work with this figure. Our results disagree with the work by **Hugenschmidt, et al. (2010)** who measured a higher pH value in AD (5.78) than in SM (5.61) after 72 hours. In the work by **Bucko et al., (2012)**, pH values of 5.72 in AD and 5.73 in SM are reached and there is no significant difference between the muscles as in our results. Values of pH as an indicator of pork meat quality differ in a number of studies and the boundaries between PSE and RFN meat is not uniform **(Chilling at al., 2004; Lesiów and Xiong 2013; O´Neill et al., 2003)**. For example, van **Laack and Kauffman, (1999) and Lien, et al., (2002)**, state that PSE meat has pH_{24} below 5.32. According to these authors, we identified the examined meat as normal – RFN from all the suppliers (A, B, C, D, E) with no differences in muscles. However, in some cases muscles exhibit characteristics of PSE even when the pH is relatively high – 5.48, as shown in **Ryu, et al., (2005)**. In a study by **Nam, et al., (2001)**, the PSE meat pH_{24} is considered below 5.47 which, compared with our results, corresponds to pH values measured for SM. **Kuo and Chu, (2003)**, on the other hand, report that the average value of pH_{24} in PSE meat reaches 5.6 and in RFN 5.96. In

this case, we would classify all the meat, without regard to the differences between muscles, as PSE. The measured pH values for AD and SM correspond to the work by **Chilling, et al., (2004)** wherein the pH ranges from 4.9 to 6.3. These authors also state that all samples with a pH below 5.5 are PSE while samples with a pH above 5.6 are RFN. **Chmiel, et al., (2011)** classifies PSE and RFN meat using a combination of pH_{24} and lightness L*. The average pH value of the meat is described as 5.49 for PSE meat and an average pH of 5.64 points to RFN meat. Furthermore, raw material having a low pH value is characterized by low moisture and high values of proteins as well **(Chmiel et al., 2011)**. The work by **Hugenschmidt, et al., (2010)** confirms that the lower the pH, the higher the incidence of defects in the final product. It is necessary to mention that in this work we compared the results of the pH_{ult} value and the pH measured 72 hours *post mortem*. An important role in the classification of deviations in the quality of meat is also played by pH monitoring during the entire process after the slaughter (pH_1, pH_{45} and pH after 2, 4, 8 hours). This fact is described by **Lesliów and Xiong (2013)**, where the meat was classified as PSE and RFN based on the color and pH, while the final pH_{ult} here was very similar – ranging between 5.35 and 5.38.

Color

Color is a significant indicator of the pork quality, because it is one of the most important features influencing evaluation of meat by the consumer **(Valous et al., 2009)**. Measurement and subsequent evaluation of color can be done with determining the *L**, *a**, *b**values in CIELAB color space and computer image analysis **(Du and Sun, 2004)**. The most frequently used methods of detection of PSE meat are instrumental methods, in particular pH measurement in combination with measurement of the color of meat in the CIEL*a*b* system **(van Laack and Kauffman, 1999; Lien et al., 2002; Nam et al., 2001; Kuo and Chu, 2003; Hugenschmidt et al., 2010; Lesiów and Xiong, 2013)**. **Scheier et al., (2013)** state that the color (L* - value) influences the consumerś purchasing decision more than any other quality factor. On the other hand, tenderness is deemed the most important quality parameter in determining consumer acceptance **(Damez and Clerjon, 2008)**. However, tenderness is an inherent property which cannot be estimated visually and which is often replaced by shear force measurements as a physical method **(Scheier et al., 2013)**. The muscles investigated in our research reached L* values on average from 46.13 to 57.18 (Table 1, 2). No statistically significant difference in the value of L* in both investigated muscles was determined among individual suppliers (A, B, C, D, E). A statistically significant difference ($p < 0.01$) was detected in the value of a* and b* between suppliers A and B. When comparing the values of L*, a*, b* between muscles, i.e. between AD and SM (Figure 1), a statistically significant difference ($p < 0.05$) was detected for all suppliers (A, B, C, D, E) with the exception of the a* parameter for suppliers A, B, C, D. The most commonly used parameter for the classification of pork meat quality groups (PSE, RFN) is L* **(van Laack and Kauffman, 1999; Nam et al., 2001; Lien et al., 2002; Kuo and Chu, 2003; Hugenschmidt et al., 2010; Lesiów and Xiong, 2013)**. For SM muscle, L* values were measured between the average values of 46.13 to 48.53 and, for AD muscle, these values were statistically significantly higher ($p < 0.05$) ranging between 55.81 – 57.18. When comparing the values of lightness L* in SM muscle with other studies, our results are similar. **Scheier, et al., (2013)** report that the average value of L* for SM reaches the values of 48.8, **Weschenfelder, et al., (2013)** reported 49.45 and the work by **Hugenschmidt, et al., (2010)** publishes the lightness values L* ranging from 47.1 to 48.9 depending on the pH. Our results, however, disagree with the work by **Bucko, et al., (2012)**. Here L* for SM reaches higher values of 61.43 than for SM of 40.87, i.e. in comparison with our work, these results are the opposite. In our case, we detected higher values for AD than for SM.

Identification of PSE and RFN meat using the L* value by a number of authors is inconsistent, as it is the case of pH values. For example, L* values for PSE/RFN (normal) published in literature reach the following values: 55.9/45.1 **(van Laack and Kauffman, 1999)**; 61.9/54.6 **(Lien et al., 2002);** 54.9/48.1 **(Nam et al., 2001)**; and 51.5/44.8 **(Kuo and Chu 2003)**. In accordance with the results of the work by **Chmiel, et al., (2011)**, based on the lightness L*, we would include all the investigated SM muscles, regardless of the supplier, among the normal (RFN) and all the AD muscles among the PSE. The author states that meat with PSE characteristics reaches average values of L* 56.01 and RFN 48.44 **(Chmiel et al., 2011).** This fact is confirmed by the work by **Scheier, et al., (2013)**, which indicates the boundary between the PSE and RFN L* 50 (<50 RFN, >50 PSE) or by the work by **Lesiów, et al., (2013)**, in which the L* value for PSE averaged at 56.5 and for RFN at 51.0. These differences demonstrated by experts in PSE meat are attacked by failing to define the PSE meat with similar qualitative characteristics, and to develop ingredients or technologies for utilization of PSE meat. Therefore, it is necessary to continue to focus on this research to generate control samples for fundamental studies **(Chilling et al., 2004).**

CONCLUSION

During the monitoring of the pork meat quality based on the examination of pH and color, no difference among the various suppliers of pork meat was detected. A statistically significant difference was observed between the individual muscles (*m. adductor* and *m. semimembranosus*) from all suppliers in the examination of pH and color. From the above results, it can be summarized that, in terms of pH and color (L* parameter), *musculi adductor* tend to be more PSE compared to *musculi semimembranosus*. The classification of meat based on pH and color directly in the cutting plant would help to separate the low-quality meat. The using of quality raw meat from *musculi semimembranosus* can to obtain finished products with the better properties. The results of this research show, that the quality of meat from suppliers of various Europe countries is on the low level and the detection of PSE meat after 72 hours *post mortem* is difficult. Detection of PSE meat according to pH value and color is possible, but it is

desirable and important rely on the experience of exeminer.

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GOOSE`S EGGSHELL STRENGTH AT COMPRESSIVE LOADING

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ABSTRACT

The paper deals with the study of the goose eggs behaviour under compressive loading between two plates using testing device TIRATEST. The influences of the loading orientation as well as the effect of compressive velocity are studied. 226 eggs from *Landes* geese were chosen for the experiment. Eggs have been loaded between their poles and in the equator plane. Five different compressive velocities (0.0167, 0.167, 0.334, 1.67 and 5 mm.s^{-1}) were used. The increase in rupture force with loading rate was observed for loading in all direction (along main axes). Dependence of the rupture force on loading rate was quantifies and described. The highest rupture force was obtained when the eggs were loaded along their axes of symmetry (X-axis). Compression in the equator plane (along the Z-axis) required the least compressive force to break the eggshells. The eggshell strength was described by the rupture force, specific rupture deformation and by the absorbed energy. The rupture force is highly dependent on compression speeds. The dependence of the rupture force on the compression velocity can be described by a power function. The same is valid for the rate dependence of the energy absorbed by the egg up to the fracture. The rate sensitivity of the Goose`s eggshells strength is significantly higher than that reported for the hen`s eggs.

Keywords: goose's egg; compressive velocity; ruptures force; deformation; absorbed energy

INTRODUCTION

Eggs can be regarded as naturally packaged food. When examining the quality of the packaging, one primarily considers the strength of the eggshell. For table eggs, shells must be strong enough to prevent failure during packing and/or transportation. For hatching eggs, shells have to be thick and strong for preservation of the embryo as well as thin for gas exchange and weak enough to allow the chick to crack the shell when hatching **(Narushin and Romanov, 2002)**. Except common eggs (hens, quails) are also consumed waterfowls eggs producing by small-scale farmers and in Asian countries.

Resulting eggshell strength is influenced by material and structural strength **(Bain, 1992)**. The material strength depends on the association of the mineral and the organic components of the shell. **Fraser et al. (1998)** mentioned that the organic matrix is considered to play a role in the regulation of various stages of crystal growth (i.e., the deposition of calcium carbonate on organic aggregates), to have the same function as steel in reinforced concrete, or both. Macroscopically is the material strength characterized by the Young`s modulus $E$, Poisson`s ratio ν and namely by the fracture stress. Structural strength, on the other hand, is related to the interaction among the building units and depends on several variables, namely egg dimensions, egg shape, eggshell thickness, and distribution of the shell components. Most techniques that aim at quantifying eggshell strength measure eggs as a whole and thereby make no distinction between these two properties.

The most common technique for the measurement of the shell strength consists in the compression of the egg between two plane plates. This technique has been mostly applied for the study of the mechanical properties of the hen`s eggs **(De Ketelaere et al., 2002; Lin et al., 2004; Narushin et al., 2004, Altuntas and Sekeroglu, 2008, Nedomová et al., 2009)**. There are also some papers on the mechanical behaviour of Japanese quail eggs **(Polat et al., 2007)**, on the strength of the Ostrich`s eggs **(Cooper et al., 2009)** and many others. The data on the mechanical behaviour of goose`s eggs are very scarce in the literature.

The aim of this study was to determine the mechanical behaviour of the goose`s eggs under their compression between two plane plate. The main emphasis has been given on the effect of the compression velocity on the parameters describing the eggshell strength, i.e. on the rupture force, eggshell deformation at the rupture, on the energy absorbed during the loading process etc. The study of this effect is limited only to the hen`s eggs. It has been found that the eggshell strength has been significantly depended on the compression rate **(Voisey and Hunt 1969; Carter, 1979, Altuntaş and Şekeroğlu, 2008)**. The investigation of the loading rate on the strength properties of the goose`s eggs should extent our knowledge on this phenomenon.

MATERIAL AND METHODOLOGY

226 eggs (3 days old) from *Landes* geese were chosen for the experiment. Geese were kept in free-range technology at a commercial breeding farm in the Czech Republic. Eggs were collected from 3 years old geese.

In this paper the analytical description of the eggshell contour curve was also obtained. This description enables to evaluate the radius of the curvature R, egg volume and egg surface. The radii were evaluated at the sharp end, blunt end, and at the maximum width of the egg (equator).

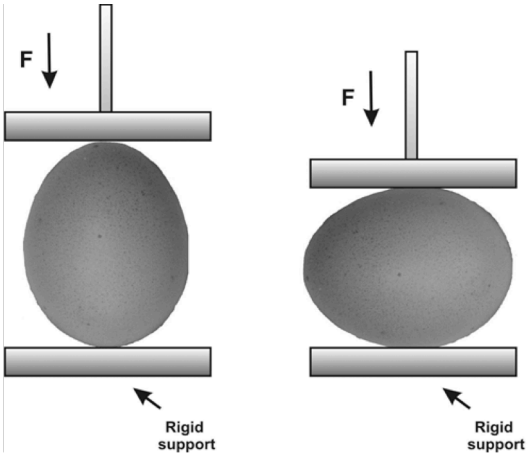

Figure 1 Schematic of the egg compression. On the left side the loading along the X-axis is shown. The loading along the Z-axis is displayed on the right part. This orientation is also termed as the loading in the equator plane

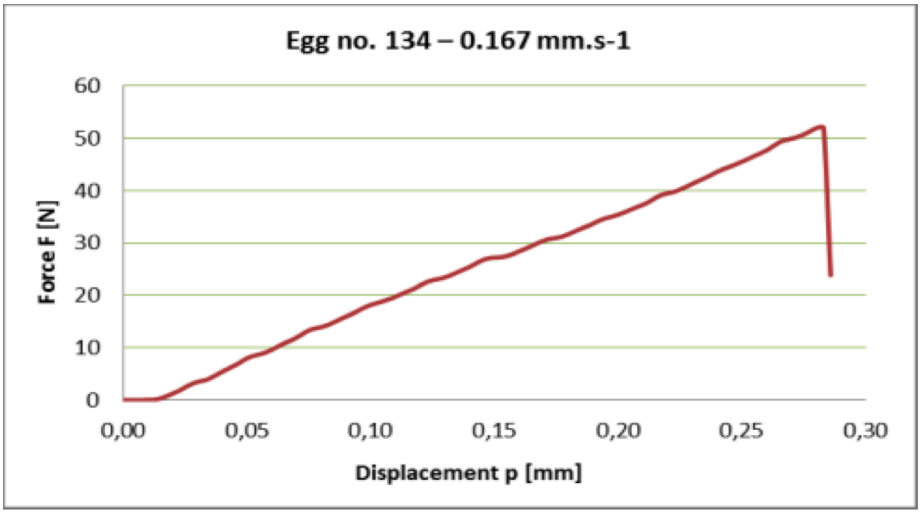

Figure 2 Example of the experimental record of the force during the compression of egg along the Z axis

Table 1 Thickness of the eggshells of the tested eggs

	Sharp End [mm]	Blunt End [mm]	Equator [mm]	Average [mm]
Minimum	0.248	0.267	0.321	0.313
Mean	0.505	0.460	0.511	0.492
Maximum	0.722	0.659	0.681	0.642
St. deviation	0.0838	0.0821	0.0681	0.0698

In order to complete the basic data the values of the eggshell thickness are given in the Table 1.
These quantities have been measured at the sharp end of the egg, at the blunt egg and at the maximum of the egg width (at the equator).

The eggs have been compressed between the two plates using testing device TIRATEST 27 025 (TIRA GmbH, DE). The egg sample was placed on the fixed plate and loaded at the compression velocities 0.0167, 0.167, 0.334, 1.67 and 5 $mm.s^{-1}$ and pressed with a moving plate connected to the load cell until the egg ruptured. Two

mutually perpendicular compression axes (*X, Z*) corresponding to main geometrical axes were used – see Figure 1.

The *X*-axis represented loading axis along the length dimension and the *Z*-axis represented the transverse axis covering the width dimension. Two more orientations were considered in case of *X*-axis. If the egg sharp end is in contact with the moving plate the symbol X_s is used. The symbol X_b corresponds to the orientation where egg blunt end is in contact with the moving plate.

Response of the egg to compression loading between two parallel plates is characterized by nearly linear increase in the loading force, *F*, with moving plate displacement *p*. At the moment of eggshell break the loading force rapidly decreases – see Figure 2.

This behaviour was observed in number of researches and described in many; see e.g. **De Ketealere et al. (2004)** and **Lin et al. (2004)**. Maximum of the loading force is than defined as the rupture force, F_m. Specific rupture deformation is defined by the following equation:

$$\varepsilon_f = \frac{p_m}{L}, \qquad (1)$$

Where *L* (mm) is the undeformed egg length measured in the direction of the compression axis and p_m (mm) is the displacement at the point of rupture of the eggshell **(Braga et al., 1999)**. Energy absorbed (E_a) by an egg at the moment of rupture is defined as:

$$E_a = \int_0^{D_r} F(x)dx. \qquad (2)$$

The series of 10 eggs was tested for each orientation.

RESULTS AND DISCUSSION

The experimental records force – displacement have been used to the evaluation of the quantities described in the previous charter. The results are summarized the Tables 2 – 4, where the basic statistics of the obtained data is presented.

The rupture force increases with the compression velocities as shown in the Figure 3.

For all used orientation of the egg compression the rupture force exhibits its maximum in such loading orientation, when the moving plate is in contact with the sharp end of the egg (X_s axis). The experimental data can be fitted by power function:

$$F_m = Ap^n. \qquad (3)$$

The parameters are given in the Table 5.

These results are generally similar to those obtained by **Altuntaş and Şekeroğlu (2008)** and **Trnka et al. (2012)**. Contrary to the results obtained for the hen`s eggs the goose`s eggs exhibit higher sensitivity of the rupture force to the loading rate. This rate sensitivity can be described by:

$$\frac{dF_m}{dv} = Anv^{n-1}. \qquad (4)$$

Owing to the values of n – see Table 4 – the increase in the loading rate v the rate sensitivity decreases. The same tendency as the rupture force exhibits also absorbed energy E_a – see Figure 4.

These data can be also fitted by the function (3). The parameters of this fitting are presented in the Table 6.

The displacement p_m at the egg rupture increases with the compression rate v. Its dependence on the orientation of the loading is not the same as for rupture parameters F_m and E_a – see Figure 5.

The egg shape can affect the obtained results. In **(ASAE, 2001)** method for compression tests of food materials of convex shape is described. According to this theory the loading force should be dependent on the main curvature of the eggshell. If we denote the radii of the curvature at the sharp end as R_1, at the blunt end as R_2 and at the equator as R_3 than for the loading in the X_s, X_b and Z-axes we obtain the curvatures k_1, k_2, k_3:

$$X_s \qquad k_1 = \frac{2}{R_1},$$

$$X_b \qquad k_2 = \frac{2}{R_2},$$

$$Z \qquad k_3 = \frac{1}{R_3} + \frac{1}{W},$$

Where W is the egg width.

In the Figure 6 the dependence of the rupture force on the eggshell curvature is displayed for the compression velocity 0.0167 mm.s^{-1}.

The rupture force F_m increases with the eggshell curvature *k*. Experimental data can be fitted by the power function:

$$F_m = ak^b + c. \qquad (5)$$

The same conclusions have been obtained for all remaining loading velocities. Parameters of the Eq.(5) are given in the Table 7.

The remaining eggshell strength characteristics also increase with the eggshell curvature. The obtained data can be fitted only by polynoms of order 6 and more.

The effects of the eggshell thickness on rupture parameters were not statistically significant. These characteristics are also independent on the egg shape index SI. This independence may be consequence of a relatively low scatter both of the thickness and SI.

As it has been mentioned in the introduction the rupture force obtained at the compression of the whole egg consists from the material and structural strength. In order to distinguish these two components some numerical simulation of the egg compression should be used. One of the possible approaches has been presented by **Trnka et al. (2012)**.

The explanation of the increase in the eggshell strength with the loading rate can be probably explained only in terms of the eggshell microstructure like in the case of many engineering materials (metals, ceramics, polymeric materials etc.).

Table 2 Compression in the X_s direction

	Compression velocity				
	0.0167 mm.s^{-1}	0.167 mm.s^{-1}	0.334 mm.s^{-1}	1.67 mm.s^{-1}	5 mm.s^{-1}
	F_m [N]				
Minimum	60.25	75.45	93.36	118.14	132.38
Mean	64.05	83.33	101.92	122.41	136.67
Maximum	67.36	88.21	106.48	127.75	150.55
Standard deviation	2.030	3.734	3.744	2.876	4.943
	p_m [mm]				
Minimum	0.23	0.24	0.40	0.40	0.51
Mean	0.25	0.30	0.43	0.48	0.55
Maximum	0.28	0.37	0.46	0.56	0.71
Standard deviation	0.014	0.046	0.020	0.057	0.056
	p_m/L [%]				
Minimum	0.260	0.271	0.453	0.426	0.539
Mean	0.286	0.338	0.488	0.530	0.610
Maximum	0.315	0.420	0.524	0.632	0.798
Standard deviation	0.018	0.050	0.022	0.064	0.079
	E_a [Nmm]				
Minimum	6.30	8.23	17.82	21.69	30.80
Mean	7.25	11.41	19.99	26.86	34.11
Maximum	8.05	14.44	22.26	32.52	48.59
Standard deviation	0.50	2.10	1.48	3.74	4.99

Table 3 Compression in the X_b direction

	Compression velocity				
	0.0167 mm.s^{-1}	0.167 mm.s^{-1}	0.334 mm.s^{-1}	1.67 mm.s^{-1}	5 mm.s^{-1}
	F_m [N]				
Minimum	39.40	51.32	70.56	81.65	107.25
Mean	43.21	53.70	72.82	84.81	111.43
Maximum	48.10	57.26	75.21	88.67	118.14
Standard deviation	2.97	.98	1.30	2.38	3.20
	p_m [mm]				
Minimum	0.22	0.20	0.28	0.29	0.30
Mean	0.28	0.30	0.32	0.32	0.33
Maximum	0.33	0.33	0.36	0.36	0.36
Standard deviation	0.03	0.03	0.02	0.02	0.02
	p_m/L [%]				
Minimum	0.238	0.210	0.308	0.330	0.191
Mean	0.309	0.331	0.358	0.356	0.361
Maximum	0.378	0.375	0.413	0.430	0.436
Standard deviation	0.041	0.043	0.029	0.029	0.076
	E_a [Nmm]				
Minimum	4.28	5.21	9.42	10.78	14.95
Mean	5.45	7.24	10.55	12.28	16.67
Maximum	6.43	8.19	11.55	14.51	19.33
Standard deviation	0.75	0.85	0.74	1.06	1.45

Table 4 Compression in the *Z* direction

	Compression velocity				
	0.0167 mm.s^{-1}	0.167 mm.s^{-1}	0.334 mm.s^{-1}	1.67 mm.s^{-1}	5 mm.s^{-1}
	F_m [N]				
Minimum	23.68	40.26	60.02	70.25	82.18
Mean	37.35	50.71	63.80	73.02	85.24
Maximum	45.71	59.55	67.03	77.11	88.32
Standard deviation	7.99	7.13	2.51	2.08	1.90
	p_m [mm]				
Minimum	0.16	0.20	0.20	0.44	0.51
Mean	0.22	0.36	0.30	0.49	0.54
Maximum	0.29	0.83	0.39	0.59	0.59
Standard deviation	0.05	0.19	0.06	0.05	0.03
	p_m/W [%]				
Minimum	0.284	0.335	0.355	0.546	0.829
Mean	0.375	0.604	0.520	0.817	0.919
Maximum	0.494	1.409	0.660	1.040	0.996
Standard deviation	0.075	0.324	0.093	0.137	0.055
	E_a [Nmm]				
Minimum	1.83	3.66	5.65	14.50	19.66
Mean	3.76	8.48	8.68	16.28	20.87
Maximum	5.49	21.03	10.86	19.13	22.61
Standard deviation	1.36	5.18	1.73	1.57	0.92

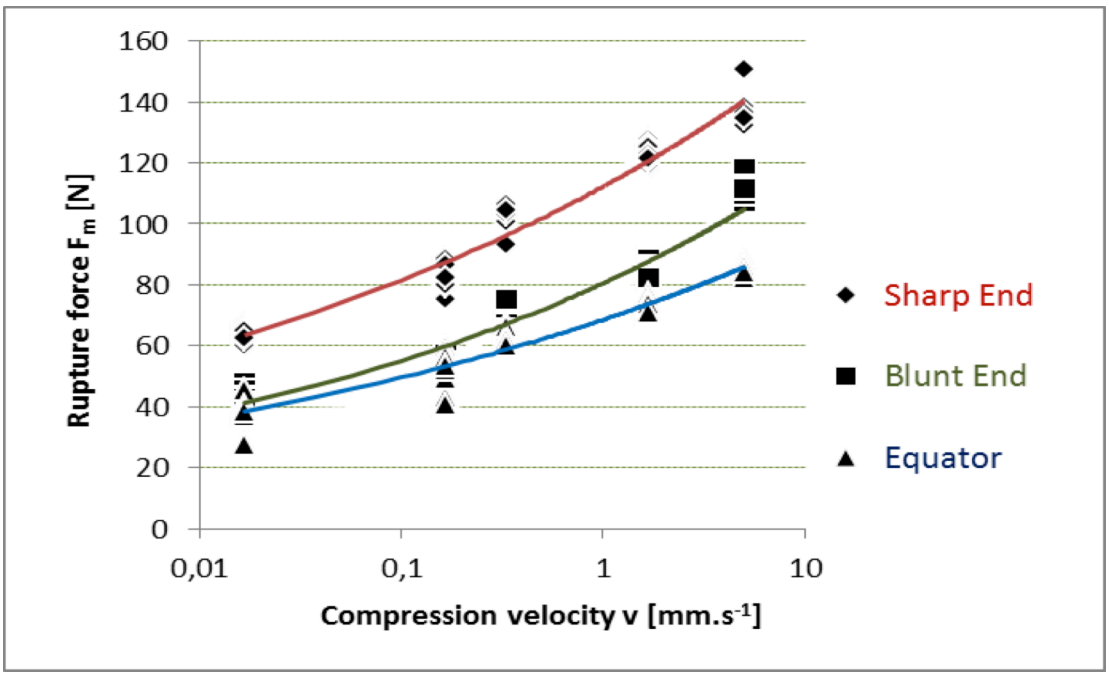

Figure 3 The influence of the compression velocity on the rupture force

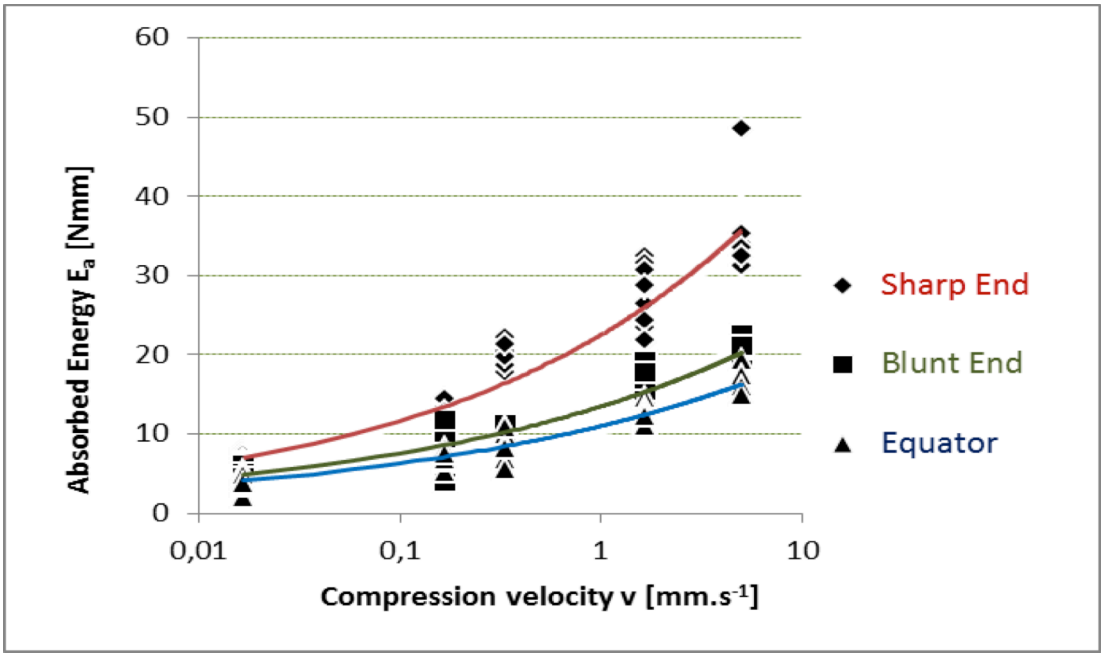

Figure 4 Energy absorbed up to the eggshell fracture

Table 5 Rupture force - parameters of Eq.(3). r^2 is the correlation coefficient

Compression orientation	A [n]	N [1]	R^2
	112.1	0.1341	0.9757
X_b axis	81.9	0.1754	0.9506
Z	68.6	0.1404	0.9408

Table 6 Absorbed energy - parameters of Eq.(3). r^2 is the correlation coefficient

Compression orientation	A [n]	N [1]	R^2
xis	22.80	0.2638	0.9631
X_b axis	11.78	0.2025	0.9611
Z	13.28	0.2919	0.9880

Table 7 Rupture force - parameters of Eq.(5). r^2 is the correlation coefficient

Compression velocity [mm.s^{-1}]	a [Nmm^{-1}]	b [1]	c [N]	r^2
0.0167	9726	2.899	35.94	0.6386
0.167	52210	3.695	49.42	0.6039
0.334	13010	2.778	70.87	0.8757
1.670	8881	2.638	61.99	0.8334
5.00	40030	3.33	80.59	0.7233

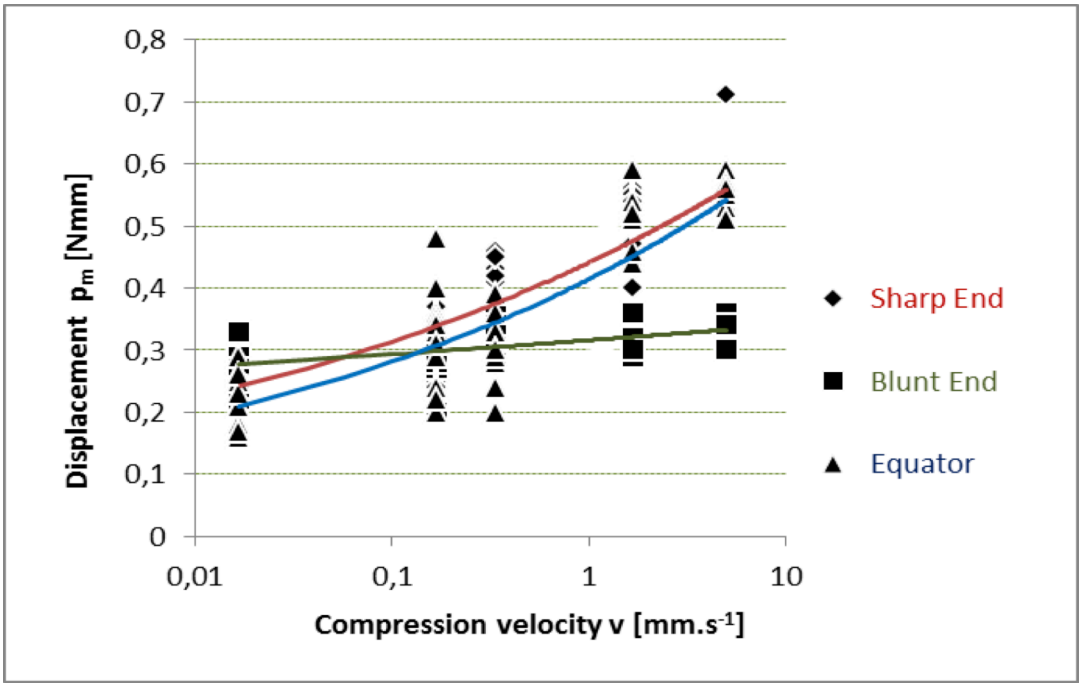

Figure 5 Eggshell displacements at the moment of the eggshell breakage

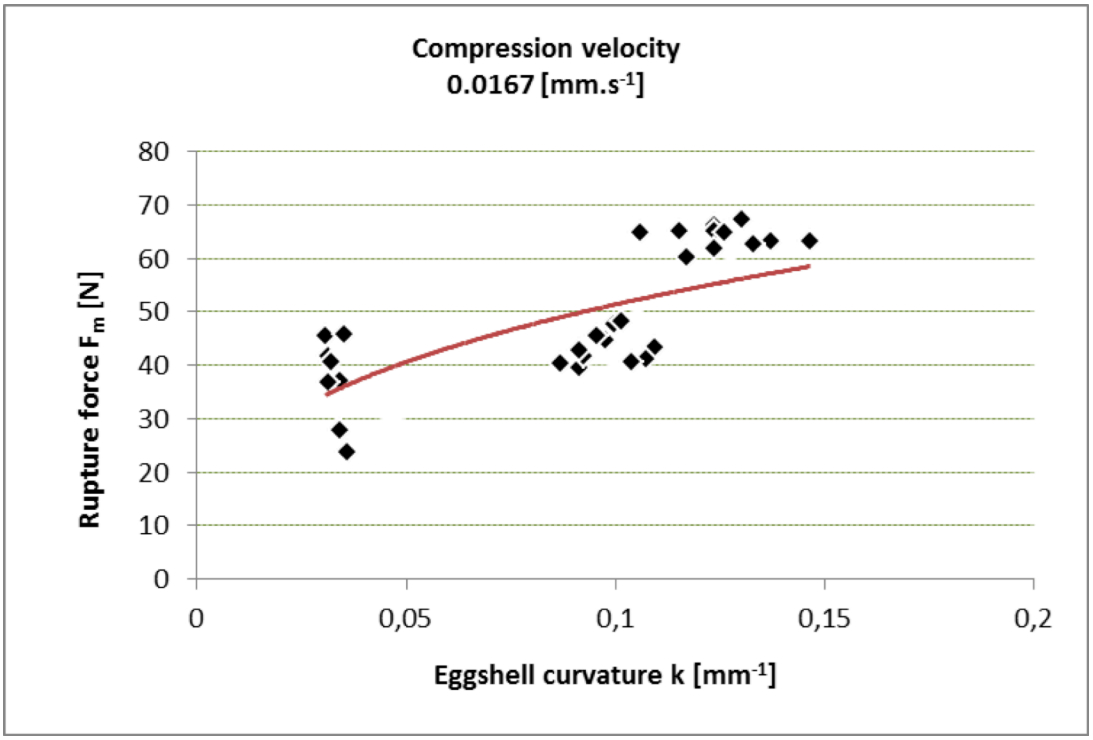

Figure 6 The influence of the eggshell curvature on the rupture force

CONCLUSION

The results obtained within presented research show on significant influence of the compression rate on the eggshell strength. The eggshell strength was described by the rupture force, specific rupture deformation and by the absorbed energy. The rupture force is highly dependent on compression speeds. The dependence of the rupture force on the compression velocity can be described by a power function. The same is valid for the rate dependence of the energy absorbed by the egg up to the fracture. Specific rupture deformation depends on the compression velocity by the same way as the rupture force. At the same time the dependence of this deformation on the loading orientation is different from that observed for the rupture force.

The highest rate dependence was observed for loading in the X_a direction, while the lowest in the Z direction.

The dependence of the rupture force on the orientation of compression axis can be quantitatively described as function of the main curvature of the eggshell surface, which is in the contact with the loading plate. This parameter is thus more important than the egg shape index SI which is widely used for the hen`s eggs description.

The increase of the rupture force with loading velocity is higher than that observed for the technical materials (metals, ceramics, polymeric materials etc.). The explanation of these phenomena represents a challenging problem for the next study.

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ANALYSIS OF CONSUMER BEHAVIOR AT CHOCOLATE PURCHASE

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ABSTRACT

At food purchase consumer is affected by several factors. In this work analysis of consumer behavior at chocolate purchase was performed involving 277 respondents. Statistical testing of results was performed by Chi - Square statistic, correlations have been tested with use of the Cramer's coefficient. It was found, that 86% of respondents consume chocolate. Factors affecting respondents at purchase were recommendations of friends, acquaintances (32%), brand of chocolate (24%), price (16%), personal experience (12%), health restrictions and allergies (11%). Less important factors when choosing chocolates are flavor (4%), nutritional quality (3%), country of origin (2%) and chocolate packaging (1%). In the consumption of chocolate moderate correlation among various categories of economic activity of respondents was confirmed. Chocolate was consumed mainly by respondents whose monthly income ranges from 801 to 1001 €. We found that consumers prefer milk chocolate followed by dark and white at the end. In terms of gender the most commonly was chocolate consumed by women, once to three times a week. The same frequency of chocolate consumption dominates at the categories of students and employee. Expenses frequently spent to buy chocolates were from 1-3 € per week by young people (18-23 years) and middle age generation of people (46-55 years).

Keywords: chocolate; purchase; preferences; consumption; obesity

INTRODUCTION

Chocolate is a very popular food due to its unique organoleptic properties such as sweet taste and pleasant characteristic aroma **(DeBrito et al., 2004)**. Chocolate consumption has a special relationship with stress: when surveyed about eating during stress, less than half admitted to eating more, and yet the vast majority would admit that they usually ate more chocolate when stressed **(Gibson, 2011)**. Individuals who experience guilt chocolate consumption reported more obsessive thoughts. Precisely why this occurred may depend upon the specific target of obsession, whether over personal failures, diet maintenance, a preoccupation with weight or appearance, etc. **(Tuomisto et al., 1997)**.

Protective effect of cocoa flavonoids on the heart and blood vessels is declared for longer time, and is associated with their ability to change the course of many pathological processes at the development of cardiovascular diseases **(Adriefdjohan et al., 2005; Ding et al., 2006)**.

There is strong evidence that high cocoa intake lowers blood pressure, improves vascular endothelial function, and potentially increases insulin sensitivity. With increased calories in chocolate consumption, further careful risk-benefit analysis is needed to assess whether consuming cocoa in the form of energy-dense chocolate products may yield a net benefit on cardiovascular risks **(Bauer et al., 2011)**.

Consumer behavior at food purchase was observed by several authors. **Zentková and Hošková (2009)** state that the expenditure of households' development in the Slovak Republic follows the development of their income and almost all of their incomes are consumed in a given year. Consumer demand is shifting towards higher prices for better food quality.

Expenses on food and soft drinks in the years 2004 till 2011 were analyzed by **Kubicová et al. (2013)**. They found that during the last 5 years expenses in all population groups decreased. The highest expenses on food in 2011 were spent by retirees (26.15%), followed by employees (20.45%) and the lowest proportion was recorded in the category of self-employed person (19.74%).

Consumers at food purchase in the supermarkets are influenced by several factors. The aim of this paper is an analysis of selected factors in purchasing behavior of consumers at chocolate purchase.

MATERIAL AND METHODOLOGY

The survey was realized from December 2011 till March, 2012. In the questionnaire respondents were asked for 4 questions relating to their opinions on chocolate. Sample of 277 respondents was interviewed. In the structure of respondents by gender, women represented 63% and men 37%. Respondents were divided into six age categories. The sample consisted of 22% of respondents aged 18-23 years, 18% of respondents aged 24-35 years, 12% of respondents aged 36-45 years, 23% of respondents aged 46-55 years, 17% of respondents aged 56-65 years and 8% of respondents aged over 65 years. By economic activity the studied group consisted of 35% of employees, 31% of students, 18% of entrepreneurs, retirees 9% and 7% unemployed.

Basic approaches of descriptive statistics had been used, as well as methods of association measurement. Statistical

significance had been tested by Chi - Square test based on the p-values. Correlations were proved by the Cramer 's V coefficient. Statistical analysis was performed with software package Statgraphics Centurion version XV.

RESULTS AND DISCUSSION

In the first question we were interested in whether respondents consume chocolate. In the questionnaire, 86% of respondents denoted that they consume chocolate and the remaining 14% said the opposite. When consuming chocolate in terms of gender it was confirmed correlation, there is a difference in chocolate consumption between the genders, more chocolate is consumed by women. Based on the Cramer's V coefficient we can state only slight dependency.

Similarly, among categories of economic activity, there were found significant differences. Cramer's V coefficient value is 0.28, which states moderate correlation. Cramer's V coefficient for age is 0.39, which indicates the existence of a higher correlation than at the economic activity of respondents (Table 1). Chocolate is consumed mainly by young people aged 18-23 years, respectively by middle-aged people from 46 to 55 years. Correlation was also confirmed in terms of their income, classification with moderate correlation can be done. Chocolate is consumed mainly by respondents whose monthly income ranges from 801 to 1001 €.

Respondents had the option to indicate the most important factors with the greatest influence at their chocolate purchase (Fig.1). Analysis of the first and the most important factor showed that respondents are mostly affected by the recommendations of friends, acquaintances (32%), followed by brand of chocolate (24%), price (16%). Other factors are personal experience (12%), health restrictions and allergies (11%). Less important factors when choosing chocolates are flavor (4%), nutritional quality (3%), country of origin (2%) and the appearance of chocolate packaging (1%).

The typical chocolate aroma is characteristic for dark, bitter-tasting chocolate and depends on its high proportion of cocoa butter. Dark chocolate and cocoa powder contain several hundred volatile constituents, including pyrazines, thiazoles, oxazoles, pyrrole derivatives, pyridines and furans **(Afoakwa et al., 2008; Bonvehí, 2005)**, and it is still difficult to assess which components really contribute to the specific chocolate aroma **(Voigt, 2013)**. Essential cocoa-specific aroma precursors are generated during fermentation of cocoa seeds by proteolysis of the vicilin-like globulin **(Kratzer et al., 2009)**.

At the evaluation of chocolate quality, crystal structure of cocoa butter causes the characteristic "crunchy" bursting. When held in the hand chocolate should melt away after a few seconds, otherwise it contains too many vegetable fats **(Coady, 1995)**. In our study we found that consumers prefer milk chocolate (Fig. 2).

Consumption of cocoa powder in Slovakia in 2011 was 0.5 kg / person / year and consumption of chocolate and chocolate confectionery 4.3 kg / person / year **(Meravá, 2013)**. In relation to consumption of chocolate, it is necessary to note that some consumers do not consume chocolate at all because of various health restrictions e.g. the incidence of lifestyle diseases such as diabetes, obesity and others. On the other side, in vitro and animal studies suggest that polyphenols in cocoa and chocolate show the potential to protect against diabetes and diabetes-related complications via a variety of molecular and physiological mechanisms. However, data from human and epidemiological studies remain limited **(Almoosawi and Al-Dujaili, 2013)**. Two recent meta-analyses of randomized controlled trials (RTCs) confirmed that flavanol-rich chocolate has a beneficial influence on endothelial function and reduces systolic and diastolic blood pressure (BP) **(Hooper et al., 2008; Hooper et al., 2012)**.

Wolz et al. (2009) performed a questionnaire survey of 274 patients with Parkinson's disease and 234 consumers, who formed the control group. They found that the consumption of chocolate by patients with this disease is higher than in the control group.

In terms of consumption frequency of chocolate it was confirmed statistically significant correlation on gender, economic activity and age of the respondents. By the Cramer's V coefficient we can state the moderate correlation, while the highest correlation (0.24) was determined at age, followed by gender (0.023) and economic activity (0.20). In terms of gender the most commonly is consumed chocolate by women once up to three times per week, respectively occasionally. The same frequency of chocolate consumption (1-3 per week, occasionally) dominates in the categories of student and employee.

In terms of age the highest share was found at young people aged 18-23. In terms of income it has not been confirmed any existence of a statistically significant correlation, e.g. income insignificantly influenced the respondents in terms of frequency of consumption of chocolate.

Childhood and adolescent obesity has been increasing in most middle- and high-income countries, and, as with adult obesity, this has been driven by increasingly obesogenic environments, especially the food environment. This constitutes a “market failure,” signaling the need for government interventions with policies, programs, and social marketing. Population prevention strategies are critical, and children and adolescents should be the priority populations. Food marketing to children is a central policy issue for governments to address, and comprehensive regulations are needed to provide substantive protection for children. The social and cultural shifts that support healthy eating and physical activity occur differentially, and special efforts are needed to reduce the socioeconomic gradients associated with childhood obesity. A positive public health approach encompassing environmental, regulatory, sociocultural, and educational strategies offer the best chance of reducing obesity without increasing disordered eating patterns **(Swinburn, 2009)**. Chocolate generally marketed to children contains approximately 30% fat and up to 45% sugar, giving it a very high energy density (2200 Kj/100 g) likely to engender satiety, making it likely those children will acquire a taste for it without difficulty.

Table 1 Results of statistical analysis at question "Do you eat chocolate?"

	p-value	correlation	Cramer´s V coefficient
gender	0.0017	yes	0.1885
economic activity	0.0005	yes	0.2819
age	0.0000	yes	0.3926
income	0.0241	yes	0.2156

Table 2 Results of statistical analysis at question „How often do you eat chocolate?"

	p-value	correlation	Cramer´s V coefficient
gender	0.0064	yes	0.2268
economic activity	0.0025	yes	0.1952
age	0.0000	yes	0.2365
income	0.2306	no	0.1477

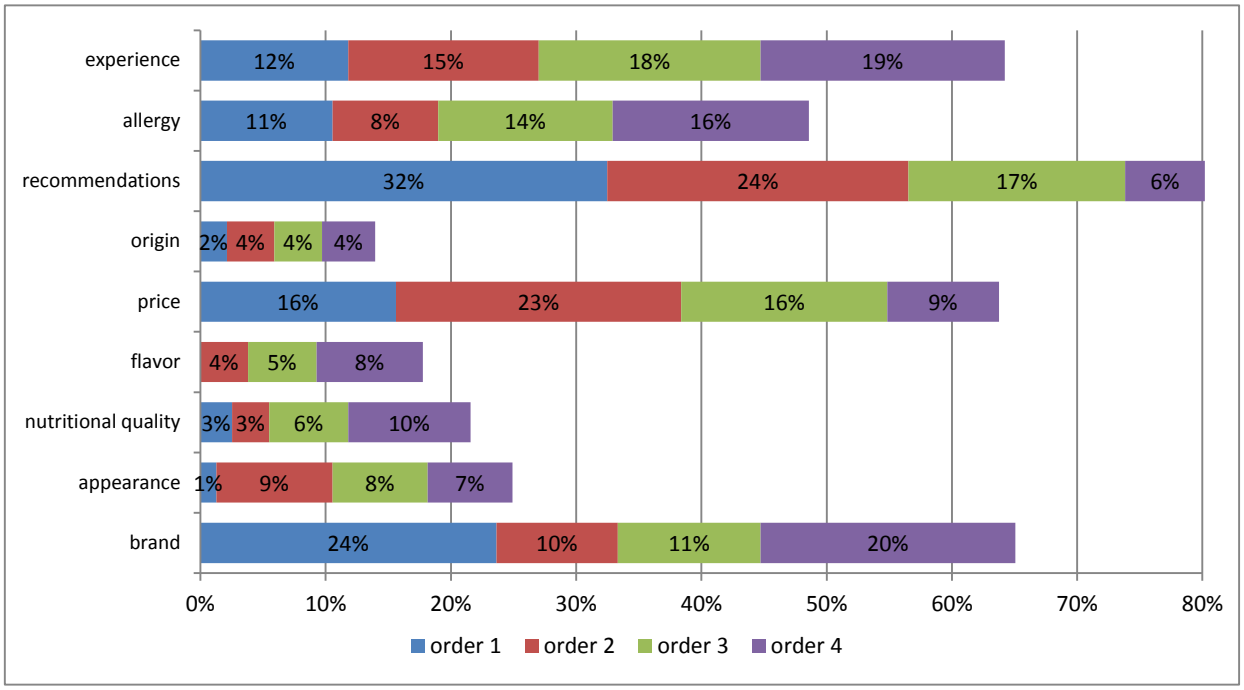

Figure 1 Factors affecting respondents at chocolate purchase
Notice: Respondents had the possibility to select four the most important factors and arrange them in order. Order 1 means that for the respondent is the most important factor.

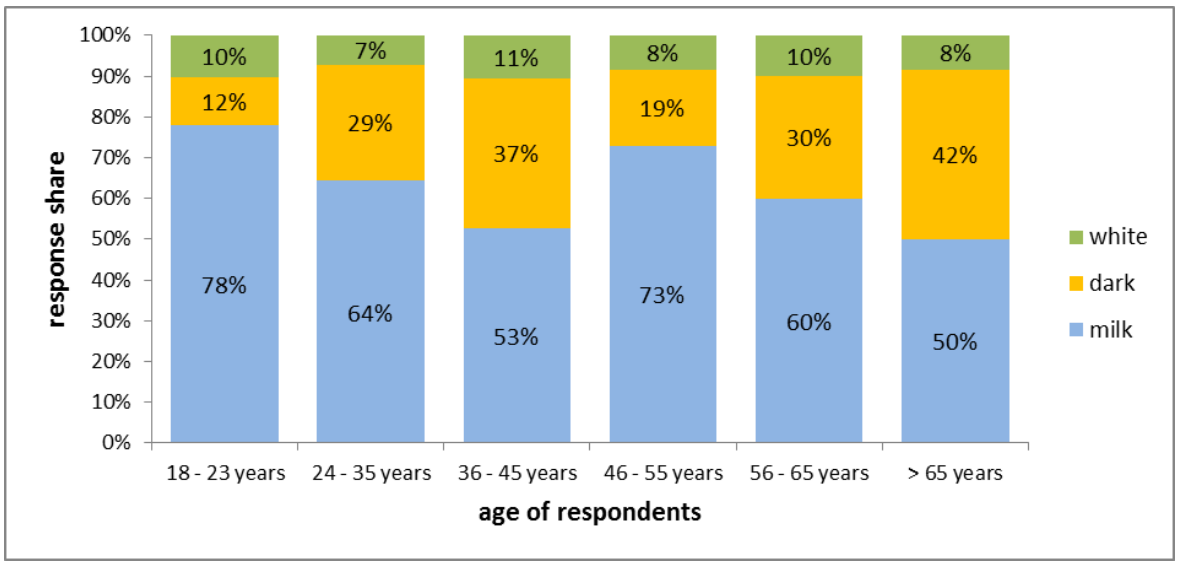

Figure 2 Preferences by type of chocolate and age

Table 3 Results of statistical analysis at question „How much money do you spend per week to buy chocolate?"

	p-value	correlation	Cramer´s V coefficient
gender	0.3036	no	0.1430
economic activity	0.3583	no	0.1512
age	0.0116	yes	0.1976
income	0.0467	yes	0.1828

Severe parental restriction of sweet foods may increase children's liking and desire for them, which can lead to increased consumption when parents are absent **(Standen-Holmes and Liem, 2013)**.

At evaluation of chocolate effect on respondents statistically significant relationship in terms of gender, age and economic activity was demonstrated. Moderate correlation has been demonstrated at age (0.33), followed by economic activity (0.26) and less moderate correlation was detected at gender (0.17). We can therefore conclude that women state the positive action of chocolate. Similarly, positive effect of chocolate declared students and employees. In terms of age positive effect of chocolate is stated by young people aged 18-35 years and people aged 45-65 years. In terms of income it has not been confirmed any statistically significant correlation, income did not significantly affect the respondents in their perception of chocolate.

Results of previous study by **Cramer and Hartleib (2001)** present finding that males felt less guilty after chocolate consumption than females; but unlike past investigations, new study found no evidence for craving differences between male and female respondents.

Assessing the amount of expenses that respondents are willing to spend on chocolate purchase per week, it was not confirmed any statistically significant correlation of the gender and economic activity. Men and women spend almost the same amount of money. Among different categories of economic activity of respondents there are no important differences. Statistically significant difference was determined at age and monthly income. In terms of correlation intensity taking into account both cases, we can state only mild correlation (Cramer's V coefficient = 0.20 for age and 0.18 for income). By age, to buy chocolates € 1-3 is frequently spent by young people (18-23 years) and middle age generation (46-55 years). In terms of income, the same amount of money (€ 1-3 per week) was spent by respondents with monthly income from 801 to 1000 €.

CONCLUSION

Factors affecting respondents at chocolate purchase are the recommendations of friends, brand of chocolate and its price. The others are personal experience, health restrictions and allergies. Less important when choosing chocolates are flavor, quality, and country of origin and the packaging of chocolate. 86% of surveyed respondents consume chocolate. In the consumption of chocolate it was confirmed moderate correlation among various categories

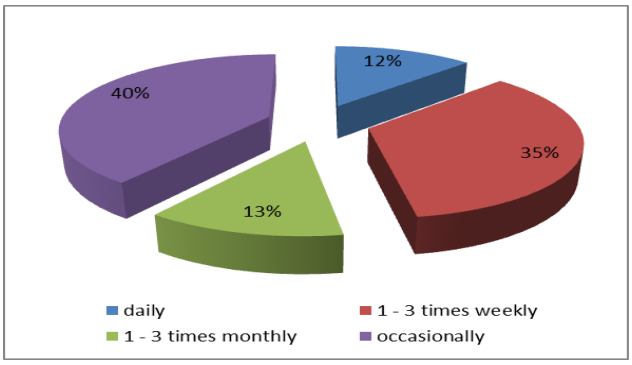

Figure 3 Frequency of chocolate consumption

of economic activity of respondents. In terms of gender the most commonly is chocolate consumed by women and the same frequency of chocolate consumption prevails in categories student, employee. To buy chocolates, expenses spent are € 1-3, once up to three times per week at young people and at middle age generation of people (46-55 years). In terms of income the same amount of money (€ 1-3 per week) is spent by respondents with monthly income from € 801 to € 1000.

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THE IMPACT OF CHILLING METHODS ON MICROBIOLOGICAL QUALITY OF BROILER CARCASSES

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ABSTRACT

The aim of this work was to compare two chilling methods, combined (aerosol) and water chilling, in terms of their effectiveness in chilling of different weight categories of broiler chickens. At the same time microbial associations of different weight categories of broiler chickens were evaluated. Samples were collected in an approved establishment and poultry carcasses were divided according to weight and chilling methods into five categories. The first four categories were chilled using combined chilling method and fifth category was chilled with water. The temperature of the breast muscle before and after chilling and microbiological parameters (total viable count, *Enterobacteriaceae*, *Salmonella*) was measured. By comparing the temperature of the breast muscle after combined chilling method was not achieved in the breast muscles temperature below 4 °C in all weight categories. In any case, the lowest average temperature has been reached in the weight category <1.2 kg (4.9 °C) and with increasing weight, the average temperature was rising, and the highest was in weight category 1.8 to 2.5 kg (10.8 °C). Poultry carcasses were subsequently divided into portions and after cutting were chilled up to a temperature below 4 °C. In poultry carcasses chilled by water, the average temperature of the breast muscle after 20 minutes in the water bath was even higher (19.6 °C) compared to combine chilling. Thus chilled poultry carcasses were frozen up to -18 °C in a core of muscles. Comparing the microbiological contamination in different weight categories and chilling techniques, we found that the lowest total viable count (TVC) before and after chilling was in the lowest category and the difference before chilling was significantly lower comparing with all other categories. Conversely TVC after chilling by water was decreased. In comparing the number of *Enterobacteriaceae* before and after chilling, a similar pattern of contamination as above was found. Microbiological examination of samples of poultry carcasses did not detect the presence of *Salmonella*.

Keywords: poultry carcasses, microbiological parameters, cooling

INTRODUCTION

An important advantage of the production of poultry meat and eggs is relatively short reproductive cycle, which can be purposefully focused on seasonal market and thereby contribute to regulation of market supply and demand. Poultry consumption has increasing worldwide trend and poultry meat and products are also affordable for consumers **(Nagy, 2007)**.

Poultry farming has an irreplaceable role in ensuring rational nutrition of the population. In terms of nutritional value, poultry meat, but especially meat of gallinaceous poultry, is very important because of the high content of protein, essential amino acids, low proportion of fat, and a high proportion of essential unsaturated fatty acids, minerals, and vitamins.

Chilling of meat along with freezing are included among the priority methods used for extension of shelf life of foods. Chilling carried out immediately after slaughter and freezing minimises the growth of bacteria and therefore the microbiological contamination. As soon as the meat is cut and, where appropriate, packaged, it must be chilled to a temperature of not more than 4 °C. Meat must attain a temperature of not more than 4 °C before transport, and be maintained at that temperature during transport. Meat derived from poultry intended for freezing must be frozen without undue delay **(Commission Regulation 558/2010)**.

When carcases are subjected to an immersion chilling process, account must be taken of every precaution to avoid contamination of carcases, and taking into account parameters such as carcase weight, water temperature, volume and direction of water flow and chilling time **(Regulation 853/2004)**.

Among the indications which may optionally be used on the labelling are those concerning the method of chilling and particular types of farming **(Commission Regulation 543/2008)**.

For chilling of poultry are currently used water chilling, air chilling and combined (aerosol) chilling.

During immersion in cold water the chilling baths reduces temperature of eviscerated poultry to the desired maximum temperature of 4 °C. In case of severe microbial contamination of water may cause cross contamination of large amounts of poultry. The most common cause of contamination is poultry, in which during evisceration the digestive tract has been disrupted. In addition, cross-contaminated water (about 4-5%) is absorbed during chilling of poultry; thereby the level of microbial contamination in poultry carcasses is increased. In this way, not only increasing the number of contaminating

bacteria, but also the risk of pathogenic bacteria is occurred **(Pipová, 2011)**.

Weight loss during air chilling achieves 0.5 to 1.0%, but the hygiene benefit in comparison with chilling in water is significant. Combined chilling takes into account advantages and disadvantages of above mentioned methods of chilling. Aerosol chilling is combined with spraying of water and it is technologically identical with air chilling. Preferably, the water evaporation does not lose weight, but in the case of incomplete set of chilling parameters may take place water absorption **(Nagy et al., 2011)**.

The aim of this work was to compare two methods of chilling, combined and water chilling, in terms of their effectiveness in chilling of different weight categories of broiler chickens. At the same time microbial associations of different weight categories of broiler chickens were evaluated.

MATERIAL AND METHODOLOGY

The experiment was conducted in an approved establishment, which is subjected to the slaughtering of poultry, poultry meat cutting, production of poultry meat products, mechanically separated poultry meat and poultry freezing, chilling, subsequent storage and marketing in Slovakia and the EU. Technological line for poultry slaughtering has maximum output 6000 pcs/hour. An experiment was conducted at standard line speed 4000 pcs/hour. Poultry used in the experiment was intended for cutting after combined chilling, respectively, whole carcasses were frozen after chilling in water bath.

Combined (aerosol) chilling was carried out at a temperature of -1 to +3 °C at a pressure in the inlet jet 0.2-0.4 MPa and 1 MPa at the outlet. The capacity of the cold tunnel is 6200 pieces, which at the speed of the production line 4000 pcs/hour representing chilling time 1.5 hours, regardless of carcase weight.

Water chilling was carried out in cold water (0-4 °C) for 20 minutes; the water consumption was 1.5 litres/pc/day.

Sampling

Sampling was conducted in the summer (May-September), when the ambient temperature in operation room reached 17 to 18.5 °C. Samples were collected from broiler carcasses divided, based on weight and method of chilling, into five categories, and in each category were tested six broilers. The first four categories were chilled with combined (aerosol) method and the fifth category was chilled with water:

1. Carcasses weight <1.2 kg chilled with combined (aerosol) method.
2. Carcasses weight 1.2 - 1.5 kg chilled with combined (aerosol) method.
3. Carcasses weight 1.5 - 1.8 kg chilled with combined (aerosol) method.
4. Carcasses weight 1.8 - 2.5 kg chilled with combined (aerosol) method.
5. Carcasses weight 1.5 - 1.8 kg chilled with water.

Temperature of carcasses was measured inside the breast muscle before and after chilling respecting the line speed and the mass of broiler carcasses using Testo 105 Hand-held T-Bar thermometer (United Kingdom). Samples from carcasses were taken also for microbiological examination to assess microbial associations before and after chilling. Methods and sampling points of the carcasses for microbiological testing, as well as rules for the storage and transport are provided in **ISO 17604 (2003)**. Broiler carcasses for microbiological examinations were chosen at random, according to the selected weight categories and methods of chilling. At each sampling session samples were taken from six carcasses in each weight category and method of chilling. Samples were taken before and immediately after chilling.

Microbiological examination of samples

Samples from broiler chickens were taken aseptically. Total viable count (TVC) was determined using the pour plate method according to **ISO 4833 (2003)** and plates were incubated at 30 °C for 24-48 hours. Plate count method was used also for *Enterobacteriaceae* **(ISO 21 528-2 2004)**, and colonies were counted in a solid medium after incubation at 37 °C. Analyses were performed in two parallels. The results of all the counts are expressed as the mean values of replicates. *Salmonella* was determined in accordance with the standard procedures **(ISO 6579 2002)**.

Statistical analysis

The mean values and standard deviations were calculated by using column statistics with processing of six values for each analyzed group. Statistically significant differences between groups were calculated using t-test and one-way ANOVA analysis by Tukey comparative test in the program GraphPad Prism 5 (2007). Differences were evaluated as statistically significant when P value was <0.05.

RESULTS

Comparing breast muscle temperature before chilling, the lowest temperature was measured at the lowest weight category (<1.2 kg) and with increasing weight raised also the initial temperature of broiler carcasses. After 1.5 hours of combined chilling in a cold tunnel has not been reached in the breast muscle temperature below 4 °C in any weight category. The lowest average temperature has been reached in the weight category <1.2 kg (4.9 °C) and with increasing weight, the average temperature was rising, and in the heaviest category (1.8 to 2.5 kg) was 10.8 °C. Poultry carcasses were subsequently cut into parts, and after cutting and packing were chilled at a temperature below 4 °C. Broiler carcasses, chilled 20 minutes in water bath, reached the average temperature of breast muscles 19.6 °C. Thus chilled poultry was packed and then frozen until the meat cores had reached -18 °C.

Sampling for microbiological tests was conducted from May to September at ambient temperature in operation room from 17 to 18.5 °C and total viable count (TVC), the count of *Enterobacteriaceae* (ENT) and the presence of *Salmonella* spp. were tested.

Comparing the average values of total viable count (TVC) before and after combined chilling, except the lowest weight category (<1.2 kg), an increase in TVC was recorded ($P < 0.05$). Statistically significant increase ($P < 0.05$) in TVC and ENT microbiological parameters was found only in the heaviest weight category. Microbiological examination of the surface of poultry

carcasses chilled in water revealed decrease in TVC, while the count of ENT was similar to the combined (aerosol) chilling, and increase of microbiological contamination

Table 1 Comparison of breast muscle temperature in different weight categories of poultry before and after different methods of chilling.

Temperature °C	Combined (aerosol) chilling				Water chilling
	<1.2 kg	1.2 - 1.5 kg	1.5 - 1.8 kg	1.8 - 2.5 kg	1.5 - 1.8 kg
Before chilling	30.68	34.37	36.47	40.20	37.85
After chilling	4.97	6.73	7.85	10.88	19.67

Table 2 Comparison of microbiological parameters (log) in different weight categories of poultry before and after different methods of chilling.

Microbiological parameters log CFU.g^{-1}	Combined (aerosol) chilling				Water chilling
	< 1.2 kg	1.2 - 1.5 kg	1.5 - 1.8 kg	1.8 - 2.5 kg	1.5 - 1.8 kg
TVC before chilling	3.52^{1a}	3.99^{1b}	3.93^{1b}	3.98^{1b}	4.15^{b}
TVC after chilling	3.92^{1a}	4.28^{2ab}	4.33^{2ab}	4.42^{2b}	4.09^{ab}
ENT before chilling	2.64^{1a}	3.11^{1ab}	3.07^{1ab}	3.65^{1b}	2.79^{1a}
ENT after chilling	3.04^{1a}	2.97^{1a}	3.13^{1a}	4.01^{2b}	3.19^{2a}

[1,2] within TVC and ENT rows, different superscript numbers indicate significant differences ($P < 0.05$)

[a,b] within columns, different superscript letters indicate significant differences ($P < 0.05$)

TVC - total viable count

ENT - *Enterobacteriaceae*

CFU - colony forming units

($P > 0.05$) was found.

Comparing the average values of microbiological contamination between different weight categories and chilling methods, the lowest TVC before and after chilling was in the lowest weight category (<1.2 kg) and prior to chilling the difference was significantly lower when compared to all other categories ($P < 0.05$), while after chilling was significant difference ($P < 0.05$) only in comparison with the heaviest weight categories, in which was the largest increase of bacterial contamination. When comparing the count of ENT before chilling, statistically significant difference between the lowest and heaviest weight categories ($P < 0.05$) was found, while after chilling the difference was between the heaviest weight categories and all other categories.

The presence of *Salmonella* was not detected in either of the test samples.

DISCUSSION

The primary objective of chilling poultry is to reduce microbial growth to a level that will maximize both food safety and shelf life. Chilling, required for poultry, has been an accepted processing step in the preservation of many food commodities for numerous years **(Caroll and Alvarado, 2008)**. The two most common methods of chilling broilers are immersion chilling, in which the product is immersed in chilled (0 to 4 °C) water, and air chilling, in which carcasses are misted with water in a room with circulating chilled air **(Crews, 2006)**. Air-chilling methods use forced cold air circulation (usually 0 to 1.7 °C) to chill chicken carcasses in a tunnel-room for 90 to 150 minutes to an end carcass temperature of less than 4.4 °C. Air-chilling methods can be classified into dry and wet air-chilling (complemented with chilled water spray). Air chilling consists of two phases: during the first phase of approximately 30 minutes, very cold air is blown onto the carcasses at high velocities and during the second phase, which lasts approximately two hours, carcasses are chilled further by relatively low-velocity air at 0 °C **(Barker et al., 2004)**. Air chilling has been claimed to be the safest chilling technology and delivers a much higher-quality, better tasting, and more tender chicken, however sensory flavour and texture profiles of air-chilled broiler breast meat do not differ from those of immersion-chilled samples when the muscles are deboned at the same time after the initiation of chilling **(Zhuang et al., 2009)**.

Previous research has shown that each of the chilling methods results in a different quality of finished products, such as microbial contamination, moisture content, flavour, appearance, and meat texture **(James et al., 2006)**. Chilling of poultry carcasses is necessary to prevent microbial growth, and the United States federal regulations require that the carcass temperature must reach 4.4 °C or less within four to eight hours, depending on the post slaughter carcass weights **(USDA, 2009)**. Air chilling, although inferior to water chiling in chilling efficiency, offers great potential for quality improvement (less cross contamination and a better taste), minimizes water consumption, reduces waste water management, and is labour saving during or after chilling **(McKee, 2001)**.

The combined method of chilling by air and aerosol spraying has been developed to combine the advantages of water and air chilling methods. Cold water is sprayed onto the surface of the bodies at regular intervals, resulting in improving of heat conduction, minimizing losses and reducing the weight and the range of colour changes in the skin **(Barbut, 2002)**. **Mielnik, et al., (1999)** found that chicken chilled by aerosol chilling had a lighter and less intense yellow colour than those chilled by air chilling because the sprayed water prevented the surface from dehydrating and maintained a lighter skin colour. The internal carcass temperature was 39.9 °C at the beginning and decreased to 4 °C, with average chilling times of 55, 155, and 120 min for water chilling, air chilling, and aerosol chilling, respectively. It is commonly known that immersion carcass chilling in water (45 to 50 minutes) is more efficient and faster than chilling in air (130 to 150 minutes) **(Huezo et al., 2007)**. **Zhuang, et al., (2009)** reported that the average of initial carcass temperature, when carcasses were commercially obtained and transported to their laboratory, was 32.1 °C and reached 4 °C in 45 minutes for water chilling and in 130 minutes

for air chilling. In the current study, we noticed slightly longer times, probably because of the high initial carcass temperature (39.9 °C) upon processing on-site, different processing factors (e.g. water-to-ice ratio, velocity, or air temperature), and different carcass weights. **James, et al., (2006)** showed that the chilling time of poultry carcasses was affected by various factors, such as the carcass weight, water-ice mixture, starting temperature, air velocities, hanging conditions, temperature and humidity of the chilling room, and chilling method.

Poultry meat has a high risk of contamination during its processing. Storage temperature, type of packaging, and types and numbers of psychrotrophic bacteria are the major factors determining the spoilage of poultry meat **(Tuncer and Sireli, 2008)**. In general, the microbiological quality of air chilling poultry is better than poultry chilled water **(Barbut, 2002)**. On the contrary, in our work, we found that the water chilled poultry carcasses had a lower total viable count after chilling. Our results are supported by **Caroll and Alvarado (2008)**, when during immersion chilling, cold water flows in a counter-current direction, creating a continuous clean water system for the birds during chilling. This process provides a greater reduction in total bacterial load and results from the washing action achieved with immersion chilling. Total aerobic bacteria, coliforms, *Escherichia coli*, and *Campylobacter* were also enumerated by **Berrang, et al., (2008)** and data showed that immersion-chilled carcasses had lower numbers of bacteria; however, the difference was not large.

Allen, et al., (2000) evaluated six commercial poultry chilling systems in relation to factors affecting microbial contamination of carcasses. These systems included water immersion chilling, air chilling and air chilling with evaporative cooling using water sprays. Samples of neck skin and body cavity were taken from carcasses, together with samples from the chilling environment. These were examined for total aerobic mesophilic microbes and counts of presumptive coliform bacteria and *Pseudomonas* spp. at specific points in the chilling process. Physical measurements included surface and deep-muscle temperatures of carcasses, water temperatures and chlorine concentrations in the immersion system and air speed and temperature during air chilling. The results obtained for water immersion chilling confirmed previous experience that the washing effect reduces microbial contamination of carcasses, although initially the numbers of pseudomonads tended to increase. However, the use of water sprays tended to increase contamination of the cavity, while relatively heavy spraying using non-chlorinated water, resulted in a substantial increase in the numbers of pseudomonads. Microbial levels in the air were extremely low during all the chilling process.

CONCLUSION

Comparing temperature of breast muscles after chilling, temperature below 4 °C has not been reached in any category. In practice this means that to achieve the temperature below 4 °C, in accordance with **Regulation 853/2004**, it is necessary to adjust the speed of technological line for each weight category, and the initial classification of carcasses according to weight is required. Practically, the speed should be adjusted to the heaviest weight category (about 2.5 kg). In terms of practical applicability, it seems to be more appropriate method, when the parts of slaughtered poultry are chilled after cutting to a temperature below 4 °C according to **Commission Regulation 558/2010**, and in compliance with the principle that the process must be completed as fast as possible.

To prevent contamination of poultry carcasses after slaughtering, respectively, during chilling is necessary to comply with the principles of Good Manufacturing Practice. If chilling in water is used, cold water and possibly ice addition must be applied, and the water flow must be directed in the opposite direction to the movement of carcasses in a chilling tank.

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AMINO ACIDS AND FATTY ACIDS PROFILE OF CHIA (*SALVIA HISPANICA* L.) AND FLAX (*LINUM USITATISSIMUM* L.) SEED

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ABSTRACT

The seeds of most plants are rich in various nutrients and can provide a lot of useful health benefits. The objective of this study was to determine and compare differences in fat, fatty acids, crude protein and amino acids concentrations for chia and flax seeds. Study was carried out using brown and gold seeds of Flax (*Linum usitatissimum* L.) and Chia (*Salvia hispanica* L.). The mean protein content in tested seeds ranged from 211.8 to 252.5 g/kg dry matter and in chia seed was about 13.10% higher than the average value of crude protein content in brown and gold flax seed (223.25 g/kg dry matter). Differences in the content of individual amino acids among the seeds were not statistically significant ($P < 0.05$), except that for glutamic acid. Percentage of the essential to the total amino acids, which is considered as indicator of protein quality, was 37.87%, 33.76% and 35.18%, for chia, brown and gold flax seed respectively, which demonstrates the high quality of these proteins. The average fat content of flax seeds was about 71.42 g/kg higher than that in chia seed (321.37 g/kg dry matter). The fatty acids composition showed the presence of palmitic, stearic, oleic, linoleic, α- linolenic and arachidic fatty acids in all tested samples. The α-linolenic acid constitutes on average 54.38% of the total fatty acids of flax seeds and 63.79% of chia seed, and for linoleic acid it was 15.30% and 18.89%. All seeds had low n-6 PUFA / n-3 PUFA ratio. Results of our study confirmed the excellent quality of protein and fat in chia seed, brown and gold flax seed samples. There was no significant effect of the flax seed coat colour for all measured values. Chia seed is the richest of n-3 PUFA α-linolenic fatty acid in the vegetable world. Both, flax seed and chia seed are the good choice of healthy food to maintain a balanced serum lipid profile. It must be pointed that flax seeds must be ground to release their nutrients, but chia seeds do not.

Keywords: amino acid; α-linolenic fatty acid; chia seed; fatty acid; flax seed

INTRODUCTION

Plant products are currently popular to combat various physiological threats. Scientific evidence has been provided that dietary phytochemicals can play important roles in the treatment and prevention of many diseases. With increasing public health awareness, demand for functional foods with multiple health benefits has also increased. It is possible to say that all foods are functional because they provide varying amounts of nutrients for growth or support of vital processes. Functional foods are generally considered as the foods which offer various benefits that may promote optimal health or reduce the risk of disease **(Hasler et al., 2000)**.

The seeds of most plants are rich in various nutrients and can provide a lot of health benefits. Flax seed and chia seed are renowned as good nutritional sources. These seeds originate from agricultural crops: Flax (*Linum usitatissimum* L.) and Chia (*Salvia hispanica* L.).

Flax is a member of the family *Linaceae*. It is a food and fibre crop (Flax varieties grown for human consumption are different from flax varieties grown to produce fibre) that is grown in cooler regions of the world. Flax is an annual plant growing to about 1.2 m tall, with slender stems. The flowers are usually blue, with five petals; they can also be bright red. The fruit is a round and dry capsule (5-9 mm), contains several glossy, flat, oval seeds with a pointed tip, 4 - 6 mm in length **(Daun, et al., 2003)**. The seeds have a chewy texture and a pleasant nutty taste **(Carter, 1996)**. There are two basic varieties, brown and yellow or golden. Seed colour is determined by the amount of pigment in the outer seed coat.

Salvia hispanica, or more commonly known as chia, has a long history of use as a food in South and Central America, not only for humans but for animals as well. It was one of the main foods of the old Aztecs and Mayas. Chia is a biannually cultivated plant which is a member of the family Labiatae. It is a low water user plant which is well adapted to arid and semiarid climates **(Ayerza, 1995)**. Chia can grow up to 1 m tall, has opposite arranged leaves and chia flowers are small and usually purple (3-4 mm) with small corollas. The seed coat colour ranges from black, grey, and black spotted to white and the shape is oval with size varying from 1 to 2 mm **(Ixtaina et al., 2008)**.

Polyunsaturated fatty acids (PUFA): linoleic acid (C18:2 n-6) and α-linolenic (C18:3 n-3) are essential nutrients, i.e. humans and animals must obtain them by food because the body requires them for many metabolic processes, but

cannot synthesize them **(Gorjao et al., 2009)**. Then highly unsaturated metabolites can be created from these fatty acids; arachidonic acid and γ-linolenic acid (n-6 PUFA) from linoleic acid (LA) and the most important metabolites: eicosapentaenoic acid and docosahexaenoic acid (n-3 PUFA) from α-linolenic acid (ALA).

The well-known source of n-3 PUFAs are marine fish **(Gorjao et al., 2009)**, but flax seeds and chia seeds are important plant sources as well. These are the two vegetal species having the highest concentration of ALA **(Ayerza, 1995; Coates and Ayerza, 1998; Oomah et al., 1995)**. Most of studies have been carried out with fish and fish oils, which are rich in eicosapentaenoic acid and docosahexaenoic acid, but also with various plant seeds and their oils as a source of ALA.

A low ratio of n-6 PUFA / n-3 PUFAs in daily food is the best way how to help many metabolic processes in the body. The problem is that for today's diets the high content of saturated fatty acids and n-6 PUFA and low content of n-3 PUFA is typical **(Simopoulos, 2004)**. Typically modern diets have greater ratio n-6 PUFA / n-3 PUFA than 15:1. This imbalance increases the risk of heart disease and support body's inflammatory processes. The ideal ratio is from 1:1 to 3:1.

The aim of this study was to determine and compare differences in nutrient content (fat, fatty acids, crude protein and amino acids concentrations) for chia and flax seed.

MATERIAL AND METHODOLOGY

The object of our analyses were six samples of brown and gold flax seed (Figure 1) and three samples of chia seed (Figure 2) which were obtained from health food stores.

Tested seeds were milled and analyzed for content of dry matter, crude protein and ether extract in accordance with **AOAC (1990)** standard procedures.

The amino acid composition of tested samples was analyzed by ion-exchange chromatography **(Llames and Fontaine, 1994)**. The content of amino acids after hydrolysis with 6 M HCl and Met with Cys after oxidative hydrolysis were determined using an automatic AA analyzer (AAA 400; Ingos, Prague, Czech Republic).

The content of long chain fatty acids we analysed after extraction of samples with petroleum ether and subsequent esterification with esterifying agent such as methyl esters of fatty acids by gas chromatography using gas chromatograph GC 6890N (Agilent Technologies).

Experimental data were analysed by ANOVA using Statgraphic Plus package (version 3.1; Statistical Graphics Corp.,Rockville, MD). Differences were considered statistically significant if $P < 0.05$. When a significant value for treatment means was observed, differences between means were assessed using Fisher's LSD procedure.

RESULTS AND DISCUSSION

The main values for studied nutrients: dry matter, fat, crude protein and amino acids are summarized in Table 1. The values of dry matter show a close similarity between the chia seed, brown and gold flax seed.

The concentration of crude protein in all samples ranged from 211.8 to 252.5 g/kg dry matter. Numerically highest content of crude protein was determined in chia seed (252.5g/kg dry matter) and this value was about 13.10% higher than the average value of crude protein content in brown and gold flax seed (223.25 g/kg dry matter). Difference in the content of crude protein between brown and gold flax seed was also close to ten percent (9.78%). Our values of crude protein content correspond to those in the literature. **Sammour (1999)** reported that the total proteins in flax seed represent about 20-30% of the seed meal, which makes it a good source of proteins.

The highest amount of total amino acids was in brown flax seed (202.0 g/kg dry matter). Crude protein content was higher in chia seed but total amino acid content was higher in brown flax seed, due to higher content of nonessential amino acids especially glutamic acid, glycine and aspartic acid (Table 1). There was only one statistically significant difference - for glutamic acid in chia and brown flax seed. The amount of total essential amino acids was the lowest in the gold flax seed (64.0 g/kg dry matter) and almost practically the same in chia and gold flax seed (68.6 and 68.2 g/kg dry mater). All the above mentioned differences were not statistically significant ($P < 0.05$).

Figure 1 Flax (*Linum usitatissimum*) and flax seed

Figure 2: Chia (**Salvia** *hispanica*) and chia seed

Table 1 Content of studied nutrients in analysed seeds, g/kg DM

	Chia seed	Brown Flax seed	Gold Flax seed	SEM
Dry matter	930.3	935.0	925.1	0.1
Fat	321.37	383.44	402.13	0.1
CP*	252.5	234.7	211.8	0.1
Arginine	20.0	24.0	20.7	0.1
Phenylalanine	11.6	10.2	9.2	0.1
Histidine	6.1	5.1	4.8	0.0
Isoleucine	7.4	8.6	7.7	0.1
Leucine	14.2	12.9	11.7	0.2
Lysine	9.3	9.1	8.8	0.3
Methionine	6.7	4.9	5.1	0.3
Threonine	5.4	7.1	7.5	0.5
Valine	7.9	10.3	9.2	0.1
Alanine	9.4	9.9	9.1	0.1
Aspartic acid	12.8	14.1	11.3	1.1
Cystine	4.2	3.2	2.8	0.0
Glutamic acid	28.7[a]	45.1[b]	39.6[b]	0.8
Glycine	9.1	13.3	12.0	0.1
Proline	12.8	9.1	8.3	0.2
Serine	9.4	10.2	9.4	0.2
Tyrosine	6.1	4.9	4.7	0.0
Total AA*	181.1	202.0	181.9	1.4
Total EAA*	68.6	68.2	64.0	1.1
Total NEAA*	112.5	133.8	117.9	2.7

[abc] means in row are significantly different ($P < 0.05$)

*CP - crude protein, AA - amino acids, EAA - essential amino acids, NEAA - non-essential amino acids

When comparing the proportions of amino acids in brown and gold flax seed, except methionine and threonine, all other amino acids in gold flax seed were lower compared with brown flax seed, but differences were not statistically significant ($P < 0.05$). Proteins of flax seeds are limited by lysine, threonine and tyrosine **(Thompson and Cunnane, 2003)**. Our values of lysine content in both colour varieties of flax seeds were lower than the content of lysine in chia seed (Table 1). Proteins of flax seeds are characterized by a high coefficient of digestibility (89.6%) and biological value (77.4%) **(Martinchik, 2012)**. Brown flax seed proteins contain relatively higher levels of aspartic acid, glutamic acid and arginine (Table 1). These values indicate the high content of amides **(Ayad, 2010)**. The amino acid pattern of flax protein is similar to that of soybean protein, which is viewed as one of the most nutritious of the plant proteins **(Oomah and Mazza, 1993)**. Flax seed proteins, brown and gold variety, contain 33.76% and 35.18% as percentage of the essential to the total amino acids. The value of this indicator for chia seed was 37.87%. Proteins with such high values are considered as a high quality protein. **Ayad (2010)** reported 36% for flax seed protein in his study.

The protein quality of chia has been demonstrated to be higher than that of common cereals and oil seeds **(Weber et al., 1991; Reyes-Caudillo et al., 2008)**, which is in accordance with our results. All tested seeds were rich in fat (Table 1). The average fat content of both varieties of flax seed was about 71.42 g/kg higher than that in chia seed, but there were no significant differences in total fat content. There was close similarity to the results of **Capitany et al. (2013)**, which present 327 ±8.0 g/kg for chia seed in his study.

Gas chromatography analysis of the fatty acids composition showed the presence of palmitic, stearic, oleic, linoleic, α-linolenic and arachidic fatty acids in all tested samples. In addition, three more fatty acids were identified in all analyses: lauric, myristic and palmitooleic. However, all of them were present just in traces. Only one significant difference among fatty acids was detected, it was for oleic acid (Table 2). The fatty acid profile for

Table 2 Content of fatty acids, %

		Chia seed	Brown Flax seed	Gold Flax seed	SEM
12:0	Lauric acid	0.03	0.03	0.03	0.00
14:0	Myristic acid	0.06	0.06	0.04	0.01
16:0	Palmitic acid	7.04	6.14	5.39	0.04
16:1 n-7	Palmitoleic acid	0.03	0.05	0.02	0.01
18:0	Stearic acid	2.84	4.23	3.17	0.00
18:1 n-9	Oleic acid	7.30[a]	22.43[b]	18.70[b]	0.22
18:2 n-6	Linoleic acid	18.89	14.47	16.13	0.02
18:3 n-3	α- linolenic acid	63.79	52.38	56.37	0.16
20:0	Arachidic acid	0.02	0.21	0.15	0.00
	Σ n-6	18.89	14.47	16.30	0.02
	Σ n-3	63.79	52.38	56.37	0.16
n-6 PUFA / n-3 PUFA		0.30	0.28	0.29	0.04

[abc] means in row are significantly different (P <0.05)

tested seeds was similar to that reported by another authors **(Ayerza, 1995, 2009, 2010; Coates and Ayerza, 2009; Martinchik, 2012)**. ALA constitutes on average 54.38% of the total fatty acids of flax seeds and 63.79% of chia seed and for LA it was 15.30% and 18.89%. Our results are in accordance with **Bhatty (1993)** who reported the ratio of LA in chia seed with about 18% and ALA with about 64% as unique. There was 53.3% of ALA for flax seed in his study. All these dates are close to ours. Both chia and flax seeds are rich in ALA, but chia seed is the highest plant-based source of ALA **(Ayerza and Coates, 2011)**.

All seeds had low n-6 PUFA / n-3 PUFA ratio (Table 2). This observation has important health implications. The best way to lower the risk of coronary heart disease is to keep dietary n-6 PUFA / n-3 PUFA ratios as low as possible **(Jones et al., 2006)**.

The 2010 Dietary Guidelines for Americans states reported that an adequate intake of ALA ranges between 1.1 and 1.6 grams/day for adults. Since 12 to 18 grams (2 to 3 teaspoons) of chia contain between 2.5 and 3.6 grams of ALA, this is more than a sufficient amount to meet this recommendation.

The EFSA Journal (2009) published labelling reference value for the n-3 PUFA ALA which is 2 g per day. This amount is consistent with recommended intakes for individuals in the general population in European countries based on considerations of cardiovascular health.

Flax seeds and chia seeds can be also used for feeding to animals to enrich their eggs and meat with omega 3 fats. Eggs from hens fed with chia had higher ALA content as compared to hens fed with flax seed **(EFSA Journal, 2009; Coates and Ayerza, 2009)**.

It is necessary to know that chia seeds can be consumed directly and do not need to be ground unlike flax, which must be ground or milled prior to consumption. Since flax seed content is protected by a thick shell and to obtain benefits from flax seeds it is necessary to use not whole seeds. Whole seeds passing through the digestive system undigested.

CONCLUSION

The quality of protein and fat in chia seed, brown and gold flax seed samples is excellent. Chia seed is the best known plant source with the highest content of n-3 PUFA α-linolenic fatty acid. Both flax seed and chia seed are the good choice of healthy food to maintain a balanced serum lipid profile. These seeds can be an appropriate alternative to n-3 PUFA sources for vegetarians and people allergic to fish. Flax seeds must be ground to release their nutrients, but chia seeds do not.

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DETECTION OF NATIVE STARCHES IN MEAT PRODUCTS USING HISTOCHEMICAL LUGOL CALLEJA METHOD

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ABSTRACT

Starch has been still used in food industry today as one of the main additives in foodstuffs. The reason for the use of starches in foodstuffs is their ability to bind water and to contribute to the coherent structure of the final product. However, the presence of starch in some foodstuffs is limited by legislation. These are especially meat products where legislation prohibits using starches. This study deals with determination of native starches using histochemical Lugol Calleja staining in meat products. The targeted structures of this successive staining are starches and collagen ligaments. Other structures can also be detected, based on the knowledge of their morphology. Within the scope of this study, the possibility of histochemical proof on the basis of reaction between Lugol's iodine solution and starch amylose was demonstrated. From the samples analyzed, the following criteria for the method were determined: Repeatability and repeatability of intralaboratory results was 100%, selectivity was determined to be 1.03, specificity of the method was determined to be 0.9, limit of detection was established to be 100% for 0.001 $g.kg^{-1}$ of the addition, and 87.7% for the concentration of 0.0001 $g.kg^{-1}$ of the starch addition. Based on the results it is obvious that the method is suitable for determination of native starches in meat products, and, in combination with staining of other foodstuffs ingredients, it gives a complex view of the composition and structure of the meat product.

Keywords: histochemistry; food; addulteration; collagen; verification; microscopy

INTRODUCTION

Starches in food industry are widely used with both plant-origin and animal-origin foodstuffs. In meat industry, starches are ranked among effective additives which increase the binding qualities of the meat and meat product, improve the binding capacity of fat in the product and in this way they influence the texture, consistency and stability of the final product. Starches are used in meat products as connective additions with the aim of increasing the yield and reducing the losses during the cooking, for improving the structure and cutting qualities, enhancing the succulence and prolonging the shelf life of the product **(Eliášová et al., 2012)**. Starches from different plant species are used in meat products. Most often we can see potato starch, corn starch, wheat starch and tapioca starch. And in the frame of one plant species, there can be differences between starches as far as their chemical composition is concerned. They differ mainly in the proportion of amylose and amylopectin. In case of high content of amylopectin, they are labeled as waxy starches. Especially waxy corn starch and waxy potato starch are being used.

For demonstration of starches, we can use a number of methods. The enzymatic method is officially recognized for determination of content of starch in meat products, in accordance with **ISO 13965 (1998 (E))**. It is also possible to use polarimetric, titration **ISO 5554 (1978)** or microscopic methods. Microscopic methods are not very fast due to time-demanding processing of samples. On the other hand, with the use of successive staining (more stains in one protocol) it is possible to detect more structures in the product. From this point of view, microscopic methods can be ranked among screening methods. The purpose in using the screening methods could also be to distinguish samples with the presence or absence of the analyte. Only on the basis of this decision, qualitative analysis is then carried out for samples with the confirmed presence of the analyte **(Trullols et al., 2004)**. Here is the advantage of this solution, especially with expensive methods.

Histochemical Lugol Calleja method was selected for the screening analysis of starches in meat products. This method was selected because of the bond of iodine (KI/I2 solution) to glucan polymer's helices **(Saibene and Seetharaman, 2006)**. The suitability of the use of Lugol's iodine solution as one constituent in the staining was verified in a number of studies **(Ernst and Bufle, 1994; Kutík and Beneš, 1977)**. Staining according to Calleja was selected due to its suitability for meat products **(Sifre et al., 2013; Sifre et al., 2009)**. The staining also enables a histochemical proof of collagen ligaments and demonstration of other plant-origin or animal-origin ingredients on the basis of morphological knowledge. The obvious condition is that the bond to the analyte examined (starch) is specific and does not interfere with other ingredients in the meat product. The choice of staining methods of the foodstuffs is relatively difficult, due to the diversity of matrices and raw materials used.

The use of new methods in the analysis of the ingredients examined is possible only on the basis of verification of the method. Among microscopic methods, these are

mainly qualitative methods, i.e. screening methods. For these methods, different validation parameters are established than for quantitative methods **(Eurachem, 1998; European Commission, 2002)**.

The aim of this study was the validation of histochemical Lugol Calleja staining method for detection of native starches in meat products and the description of basic principles of validation of qualitative methods, among which this method ranks.

MATERIAL AND METHODOLOGY

To analyze the limit of detection, model samples were examined. They were ordered with respect to increasing concentration of the addition of potato starch (0.0001, 0.001, 0.01, 0.1, 1, 3 $g.kg^{-1}$) into ground pork and beef muscle (1:1 in proportion). A sample without any starch addition was used as a control sample. The samples were processed in Thermomix TM 31 mill (Germany, Vorwerk & Co. KG) with the addition of 1.5% of salt and 200 ml of water.

To analyze repeatability and reproducibility (repeatability of intralaboratory results), we examined two samples from the retail network with content of starch declared by the producer, and starch was also confirmed by microscopic examination. To verify repeatability, both analyzed samples (samples A and B) were repeatedly examined ten times. To verify reproducibility, samples A and B were examined by another trained evaluator.

To analyze specificity, we evaluated 20 products from the retail network with the content of starch declared by the producer twice with two trained evaluators.

Microscopic examination was carried out using the method of paraffin blocks (**Pospiech and Petrášová, 2013**). The thickness of each section was 4 μm and analysed area was 1 cm^2. Four blocks were taken from each sample analyzed (edge, inside, inside, edge) so as to cover all the sample. 8 sections with the trimming of 100 μm were taken from the paraffin blocks. After the deparaffinization, the samples were stained with Lugol Calleja staining following this procedure: (1) bath in nuclear red for 15 min; (2) wash in deionized water; (3) bath in Lugol's iodine solution for 5 min. (KI/I2 solution, 2:1 (w/w)); (4) wash in deionized water; (5) bath in the Bauer Calleja solution for 5 min.; (6) wash in deionized water; (7) wash in 96% aqueous solution (v/v) and eventually in absolute ethanol; (8) bath in xylene p.a. twice for 7 min. each. The chemicals used were of p.a. quality from a verified distributor (Fisher Scientific, Czech Republic).

All samples were during examination anonymized.

RESULTS AND DISCUSSION

Determination of starches using histochemical staining is possible with the help of Lugol's iodine solution, or eventually with other stainings such as PAS (periodic acid Schifs's) staining. Staining in Lugol's iodine solution is specific for starches. The ability to stain with Lugol's iodine solution is indirectly proportional to the degree of branching of starch **(Krisman, 1962)**. The lateral bonds in the starch macromolecule are caused by amylopectin and are thus also connected with its content. Another factor enabling the binding of iodine to glucan polymer's helices is water activity. Higher water activity enables hydration of starch and easier penetration of iodine into starch **(Saibene and Seetharaman, 2006)**. Also, temperature has a positive influence on the accessibility of iodine in glucan polymer's helices. It is due to the creation of surface pores and a fistula getting into starch particles, which results in enlarging the surface area and improving the transfer of iodine **(Fannon et al., 1992)**. High water activity and heat treatement of starch in the production process of most meat products enable the iodine in Lugol's iodine solution to bind to starch molecules contained therein and the creation of polymer-iodine complex. This assertion was also verified - see Table 2 where one examiner detected starch in all the samples with starch additions. Another examiner gave a positive evaluation to one sample, which was in contradiction with the declaration by the producer. However, in the sample given, just one starch particle was detected in one section. So the content of starch was low and on the edge of the limit of detection. Another cause of a contradictory result can be cross-contamination in the processing plant or laboratory. From the view of evaluating the presence or absence of starch, these findings can be avoided by establishing a minimum number of sections with the content of starch particles which will be taken as positive.

Starches in food industry are used mainly on the basis of suitability of their qualities. Food industry can use starches with high content of amylose but also starches with high content of amylopectin. Their mutual proportion also impacts the binding capacity of iodine in starch molecules, which also becomes evident through Lugol Calleja staining. The changes in stainability of starch depending on the proportion of amylose and amylopectin are commonly used for determination of this proportion. To calculate this, blue value is used **(Delrue et al., 1992)** or λ max is used together with Lugol's iodine solution. Low blue values and λ max, and thus low stainability of amylose for pure starches were confirmed in a study **(Kortstee et al., 1998)**. Another parameter expressing the proportion of amylose and amylopectin is the content of iodine. Differences in content of iodine (lower values for low content of amylose) were confirmed by **Manion, et al., (2011)**. Even with these starches, we can use the staining but the color of starch grains change from blue to reddish-brown with a blue hilum core **(Karlsson et al., 2007)**. The reason of this reduction of iodine ratio and blue value is the change in absorbance of starch which is measured for specific wavelengths (400-700 nm) **(Manion et al., 2011)**. In case of microscopic examination, the evaluation is carried out for the whole spectrum of visible light and what occurs is the change in stainability of the starch particle. Stainability depends also on the plant species from which the starch comes. Corn, high amylose corn, potato, rice, chick pea starch, all of them show a similar intense dark blue, almost black, color that is indicative of the color formed from the amylose-iodine complex (Fig. 1). With waxy potato, tapioca, waxy rice, chick pea, and mung bean starch - all within a narrower range of 0.30-0.43 % of iodine - the colors vary from dark blue, to dark brown, to light blue, to faint pink, to dark blue and to dark blue, respectively **(Manion et al., 2011)**.

Among other histochemical stainings, also PAS staining can be used for detection of starches. This staining can be used to determine the total amounts of amylose and amylopectin **(Atkin et al., 1998)**. However, in case of starch detection in foodstuffs and thus also in meat products, the PAS staining reacts with other polysaccharides also, and therefore this cannot be considered as a conclusive method for starches exclusively.

Another parameter of the analysis of starches is their morphology. The shape of a starch grain is typical for each plant and is dependent on its metabolism. The descriptive characteristics for distinguishing between starches are: shape, size, position of hilum and stratification **(Eliášová et al., 2012)**. Identification of starches according to their shapes is possible only in native starches or starches in the initial phase of gelatinization. In modified or fully gelatinized starches, their structure was changed into a fibrous one and species identification is not possible (Figure 1, Figure 2). For histochemical analysis we can also use the combination of individual staining methods. Based on laboratory experience, we chose the method of successive Lugol Calleja staining. With this staining, Lugol's iodine solution is targeted to demonstrate the presence of starches and the staining according to Calleja is aimed to prove collagen ligaments (Figure 2). The combination of these stains results in the black or faint pink color of starches, as described above. The collagen ligaments are stained in blue, the muscle tissue in green, elastic ligaments in yellow, and nuclei in red.

The utilization of qualitative methods, i.e. methods determining the presence/absence, is common for laboratory examination, especially as the first step with subsequent determination of the concentration for the substance examined. However, qualitative methods can also be applied as screening methods before a more expensive examination by a qualitative method. The advantage of their use is in the reduction of costs and production time **(Trullols et al., 2003)**. However, with qualitative as well as quantitative methods, their users must make sure that the results are suitable for their purpose, i.e. all methods must be validated **(ISO, 1994; European Commission, 2002)**. Procedures based on quantitative methods are commonly used for these validation processes. There are many validation procedures approved by regulatory bodies and professional public working in the field **(Trullols et al., 2003)**. In accordance with **Eurachem (1998)**, the following parameters should be evaluated: confirmation of identity, sensitivity, selectivity/specificity and precision. Similarly, in an official document of **European Commission (2002)**, the following parameters are established for the evaluation of quantitative methods: limit of detection (LoD), selectivity/specificity, stability, applicability and robustness. In accordance with the recommendation given, the following parameters were determined for the validation of the method: LoD, sensitivity, specificity. For reasons of verification of differences between evaluators of the samples analyzed, the repeatability test and the repeatability of intralaboratory results were performed.

As can be easily inferred, presence/absence is not considered to be an absolute measure related to a concentration level of zero but to a specific concentration level. Below this limit, the concentration of the analyte is considered insignificant.

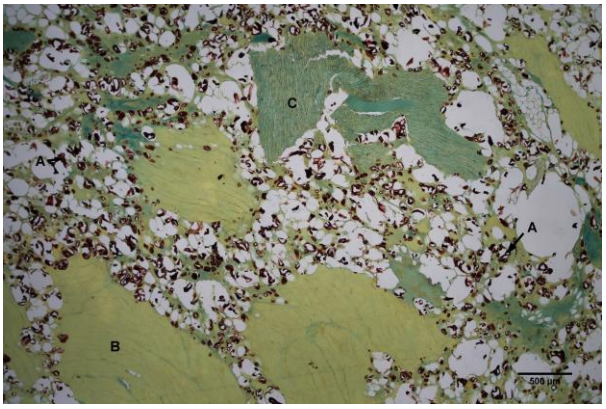

Figure 1 Frankfurters (Debrecínské párky), Lugol Calleja, A – potato starch, B - muscule, C - collagen ligaments

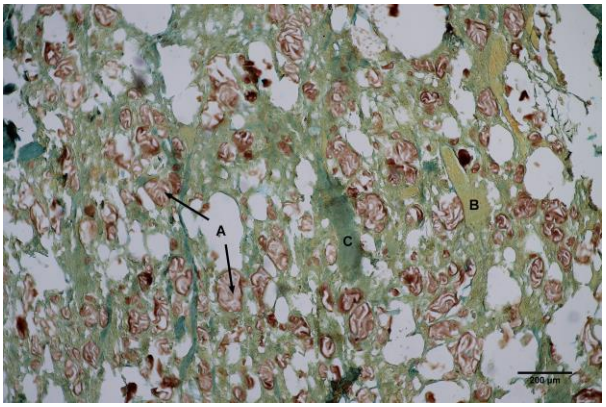

Figure 2 ESO Frankfurters, Lugol Calleja, A – not specified starch, B - muscule, C - collagen ligaments

LoD is most often used to determine the concentration limit for the analyte. LoD is defined as the lowest concentration of the analyte which can be reliably detected by the method as positive for a given matrix **(O' Rangers and Condon, 2000)**. For the Lugol Caleja method, the LoD was established on model samples with growing concentration of the starch addition. As stated in Table 1, going up from the concentration of 0.001 $g.kg^{-1}$, starch was reliably detected in all repetitions. For the concentration of 0.0001 $g.kg^{-1}$, starch was not detected in one case. According to European Commission (2002), the percentage of recommended false results for qualitative methods is less than 5%. For the method examined, starch concentration can be reliably detected for the addition amount of 0.001 $g.kg^{-1}$ and higher.

Table 1 Limit of detection for the Lugol Calleja method

Concentration [$g*kg^{-1}$]	No. of Repetitions	Positive /negative
0	7	0/7
0.0001	7	6/1
0.001	**7**	**7/0**
0.01	7	7/0
0.1	7	7/0
1	7	7/0
3	7	7/0

Table 2 Sensitivity/specificity for the Lugol Calleja method

Samples	*S1*	*S2*	*S3*	*S4*	*S5*	*S6*	*S7*	*S8*	*S9*	*S10*	*S11*	*S12*	*S13*	*S14*	*S15*	*S16*	*S17*	*S18*	*S19*	*S20*
Declaration	*N*	*N*	*N*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*N*	*N*
Examiner A	*N*	*N*	*N*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*N*	*N*
Examiner B	*N*	*N*	*P**	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*N*	*N*

N – negative (grey), P – positive content of starch
* different results

Table 3 Repeatability for the Lugol Calleja method

Measurement No.	***Repeatability***	
	Sample A	***Sample B***
1	*Yes*	*No*
2	*Yes*	*No*
3	*Yes*	*No*
4	*Yes*	*No*
5	*Yes*	*No*
6	*Yes*	*No*
7	*Yes*	*No*
8	*Yes*	*No*
9	*Yes*	*No*
10	*Yes*	*No*
Correspondence	***100%***	***100%***

Table 4 Reproducibility for the Lugol Calleja method

Measurement No.	***Examiner***	***Sample A***	***Sample B***
1	***A***	*Yes*	*No*
2	***A***	*Yes*	*No*
3	***A***	*Yes*	*No*
4	***A***	*Yes*	*No*
5	***A***	*Yes*	*No*
6	***B***	*Yes*	*No*
7	***B***	*Yes*	*No*
8	***B***	*Yes*	*No*
9	***B***	*Yes*	*No*
10	***B***	*Yes*	*No*
Correspondence		***100%***	***100%***

Other evaluation criteria for qualitative methods are sensitivity and specificity. By sensitivity for qualitative methods we understand the ability of a method to detect truly positive samples as positive **(O' Rangers and Condon, 2000)**. Therefore, sensitivity rate is the probability for the concentration given that the method will classify the examined sample as 'known' positive **(Massart et al., 1997)**. On the other hand, specificity is defined as the ability of a method to detect truly negative samples as negative **(O' Rangers and Condon, 2000)**.

Therefore, specificity rate is the probability for the concentration given that the method will classify the examined sample as 'known' negative **(Massart et al., 1997)**. For the Lugol Calleja method, sensitivity was established to be 1.03. Specificity was established to be 0.9 in accordance with the methodology **(Trullols et al., 2004)** (Table 2). The results thus show that the method is highly sensitive and specific for verification of starch in meat products.

Repeatability of the method is an optional parameter for qualitative methods. This parameter is suitable for histochemical methods because sample fixing, sample processing and histochemical staining are performed on different days. So the parameter is recommended to eliminate the influence of the environment. The repeatability was established to be 100% for positive as well as negative samples, following the methodology **(Suchánek, 1999)**.

Based on the finding that different results can be caused by different examiners (Table 2), the method was also evaluated for the repeatability of intralaboratory results (Table 3 and 4).

Two examiners assessed ten positive and ten negative samples with the content of starch higher than the established LoD. The agreement of the examiners was 100%. This finding confirms the hypothesis that for S3 sample, which gave different results due to sensitivity, the difference may have been caused by low content of starch in the product.

CONCLUSION

The microscopic method Lugol Calleja for determination of native starches in meat products was validated. The parameters of LoD, sensitivity/specificity, repeatability, and repeatability of intralaboratory results were selected for the validation. The results determined for the individual parameters were: 0.001 $g.kg^{-1}$ LoD, 1.03 sensitivity, 0.9 specificity, 100% of repeatability and 100% of repeatability of intralaboratory results. The percentage of false results was less than 5%, as recommended by current methodology. For full validation of this method should be results compared with other validated method. On the basis of these results, the method can be recommended as a screening method which can be in case of positive findings further supplemented by another result of qualitative methods.

The Lugol Calleja method is suitable also due to the successive staining with two stains and, besides starch, the samples can be examined for the presence/absence of other constituents such as collagen ligaments, muscle tissue, skin, others organs, spices or other raw materials for which the evaluator knows their morphological structure.

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IMMUNOFLUORESCENT DETERMINATION OF WHEAT PROTEIN IN MEAT PRODUCTS

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ABSTRACT

In food industry nowadays, there are various plant-origin protein additives which are meant for production of meat products. Among the most frequent additives of this type there are different kinds of flour, starch, fiber, and plant-origin proteins. Their usage at present is limited by the existing legislation not to prevent consumer deception but also for reasons of possible influence on consumer health. Therefore, this problem is paid a lot of attention not only in the Czech Republic but also all over the world. The main risk is seen in the impossibility to choose a suitable foodstuff for an individual prone to allergic reactions. Potential allergens are also often plant-origin raw materials which are added into foodstuffs for their technological qualities and low price. Wheat is widely cultivated cereal as well as an important source of proteins. After ingestion or inhalation, wheat proteins may cause adverse reactions. These adverse effects include a wide range of disorders which are dependent on the method of contact with wheat protein. These adverse effects can then take the form of various clinical manifestations, such as celiac disease, T-cell mediated inflammatory bowel disease, dermatitis, skin rash, breathing difficulties, allergy to pollen or to wheat flour or food allergy to foodstuffs containing gluten. The only possible protection against adverse immune reactions for those with food allergies is strictly excluding the allergen from their diet. Although the number of studies dealing with the reduction or loss of allergenicity is increasing, yet these practices are not common. Most of the population suffering from food allergies is thus still dependent on strict exclusion of foodstuffs causing adverse allergic reactions from their diet. In order to avoid misleading consumers and also to protect allergic consumers, analytical methods applicable to all types of foodstuffs have been developed. Unfortunately, detection of allergens in foodstuffs is relatively difficult because of the fact that they occur in trace amounts and are often masked by various parts of the product. This paper deals with detection of wheat protein in meat products bought in the retail network of the Czech Republic. Ten cooked meat products, especially types of sausages and soft salami which stated wheat protein in their composition, were examined. The samples were processed using the method of immunofluorescence and stained with Texas Red fluorochrome. The presence of wheat protein was demonstrated in all the examined meat products. From the results it follows that the method of immunofluorescence is suitable for detection of wheat protein in meat products.

Keywords: fluorescence microscopy; plant allergens; meat; adulteration; celiac disease

INTRODUCTION

Wheat protein is, inter alia, used in meat production where it contributes to the reduction of total production costs of meat products. On the other hand, wheat ranks among foodstuff ingredients given in **Directive 2003/89/EC** amending **Directive 2000/13/EC** as regards indication of the ingredients present in foodstuffs. Annex IIIa there to contain a list of ingredients of foodstuffs and products of them classified as possible allergens or substances with potential risk of intolerance. Food allergy is an abnormal immune response to foodstuffs **(Bruijnzeel-Koomen, et al., 1995)**. This type is characterized by an inappropriate reaction of one's immune system to the stimulus of the allergen which can be a protein or carbohydrate, for example **(Ferguson, 1992)**. In addition, food allergens contained in foodstuffs naturally are resistant to high temperatures, low pH in the stomach, and enzymatic digestion in the digestive tract **(Hefle, et al., 1996)**. However, it has been reported that there is no correlation between in vitro digestibility and protein allergy **(Fu, et al., 2002)**. Allergies to specific foodstuffs may in some cases exhibit also after ingestion of foodstuff of similar origin, which is known as a cross-reaction. This occurs when IgE antibodies originally produced against one allergen are produced also upon contact with a similar protein from another source **(Aalberse, et al., 2001)**. Food allergies have become a major health problem worldwide. Adverse health effects due to allergic reactions to food products or food ingredients occur in about 1-3% of population and in about 4-6% of children (including food intolerance). Food allergy is therefore more common in children than in adults. In recent years, wheat protein has also been more and more recognized as a cause of anaphylaxis due to foodstuffs **(Hischenhuber, et al., 2006)**. Sensitization to proteins of wheat grain may be caused in three different ways: inhalation, direct contact, and ingestion **(Battais, et all., 2006)**. Depending on the method of allergen exposure and based on immunological mechanisms, an allergy to wheat protein may exhibit as asthma and rhinitis, as well as contact urticaria or it may

occur as a classic food allergy affecting the skin, intestine and airways and is also known as anaphylaxis **(Sicherer, et al., 2000)**. Baker's asthma is one of the most common forms of occupational asthma. Baker's asthma is an occupational disease affecting 4-10% of bakery workers in European countries **(Baur, et al., 1999)**. Food allergy which is defined as an adverse immune response to food proteins, affects 6% of children and 3 to 4% of adults **(Sicherer and Sampson, 2000)**. Any protein from foodstuffs can cause an allergic reaction. In France between 2002 and 2004, wheat protein was responsible for 6% of anaphylaxis occurrences caused by foodstuffs **(Moneret-Vautrin, et al., 2005)**. In Iran, wheat protein is considered to be the most frequent trigger of anaphylaxis in children **(Pourpak, et al., 2007)**. In France, wheat ranks the eighth out of all allergies to foodstuffs in children and it ranks the twelfth in adults **(Rance et al., 1998)**. Allergy to wheat protein more often occurs in northern than southern Europe **(Rasanen et al., 1994)**. Data on the percentage of allergies to wheat protein vary. In his study, **Sicherer, et al., (2000)** states that 20% of children population suffers from wheat allergy. In **Niggemann's study of 2001**, the number of 14% is reported. **Moneret-Vautrin, et al., (2003)** states that allergy to wheat protein affects 10.9% of children and 25% of adults. On the other hand, in an American study focused on allergies in children, only 2.5% of children suffered from allergies to wheat protein **(Rance, et al., 2005)**. These numbers could have been underestimated because they represent only the most serious cases when hospital treatment was necessary. Clinical symptoms of wheat allergies are similar to those of other allergies to foodstuffs, with signs occurring in the skin, digestive system, and respiratory system **(Sicherer, 2000)**. The main symptom in children is atopic dermatitis, occurring either independently or in connection with respiration symptoms and digestion problems **(Moneret-Vautrin, et al., 2000)**. Various clinical symptoms were identified in adults, such as angioedema or eosinophilic esophagitis **(Scibilia, et al., 2006)**. Anaphylactic reaction caused by the contact with wheat protein was demonstrated in adults as well as in children **(Lehto, et al., 2003)**. For an allergic consumer it is especially important to know the exact composition of a foodstuff, especially as far as the presence of wheat protein is concerned. Analytical methods for the detection of allergens require high sensitivity, specificity, and sufficient robustness **(Battais, et al., 2006)**. As it follows from the paper of **Talandová, et al., (2013)** where the method of immunofluorescence was successfully applied to the products containing wheat protein, this method seems to be sufficiently sensitive and specific. Article focused an identity wheat protein in meat products.

MATERIAL AND METHODOLOGY

10 cooked meat products were examined, mainly types of sausages and soft salami bought in the retail network of the Czech Republic which, with respect to their composition, were supposed to contain wheat in various forms, such as wheat starch or protein, or they were labeled with a note saying "This product can contain traces of wheat". Four samples sized 1 cm^3 were taken from each meat product and then frozen. The samples were subsequently processed at the Department of Vegetable Foodstuffs Hygiene and Technology at FVHE (Faculty of Veterinary Hygiene and Ecology), UVPS Brno. The samples were cut into 4-μm-thick sections using HM 550 cryostat (Germany, Microm). These sections were then transferred to Thermo Superfrost slides (Germany, Thermo Scientific). Nine sections were cut for each meat product. Each sample was represented by four frozen blocks. From these blocks, microscopic sections were taken with trimming after 50 μm each. Immunofluorescent microscopy was selected as the method of detection as it is more sensitive and more selective than histochemical methods. The immunofluorescent procedure itself was started by inserting the sections into acetone. After the preparations were rinsed in PBS (phosphate buffer saline) for the period of 5 minutes, the blocking of endogenic peroxidase was carried out using a 3% solution of hydrogen peroxide. After repeated rinsing in PBS (2 x 5 min), the samples were placed into a wet chamber where the blocking of non-specific binding ran for 30 minutes using Goat Diluent Normal serum (GB, Vector Laboratories). Then the biotinylated primary antibody Anti-wheat (GB, Sigma-Aldrich A1052) was applied to the sections and the wet chamber was left in the fridge overnight. On the following day, the samples were rinsed in PBS (2 x 5 min). Then they were placed again into a wet chamber and secondary antibody (GB, Vector Laboratories BA-1000) was applied to the sections for the period of 30 min at room temperature. Rinsing in PBS (2 x 5 min) and application of fluorochrome followed. The Texas Red (GB, Vector Laboratories) was used as fluorochrome. Then the sections were mounted and examined using Leica DM3000 fluorescence microscope (Germany, Leica), and they were further processed with the help of Leica IM50 program (Germany, Leica). Nine sections were examined in this way for each meat product with the magnification of 40x and 100x.

RESULTS AND DISCUSSION

Meat products, on the cover of which their producers declared the use of wheat protein, were bought in the retail network and examined. Wheat, together with cow milk, eggs, soybean, nuts and peanuts, fish, shellfish, crustaceans and molluscs, is the cause of approximately 90% of allergies to foodstuffs and it is also the primary foodstuff causing anaphylaxis **(Sicherer and Sampson, 2000)**. In the interest of consumer protection, the European Commission issued **Directive 2003/89/EC amending Directive 2000/13/EC** as regards indication of the ingredients present in foodstuffs. Annex IIIa thereto contains a list of ingidients in foodstuffs and products of them classified as possible allergens or substances with potential risk of intolerance, such as gluten-containing cereals, crustaceans, eggs, fish, peanuts, soybean, milk (including lactose), nuts, celery, mustard, sesame seeds, sulphur dioxide and sulphites. **Directive 2003/89/EC** demands that each of the twelve above stated potentially allergenic constituents is labeled even if it forms less than 25% of the foodstuff. Therefore, the objective of this study was to verify a method suitable for demonstration of gluten in meat products.

Table 1 Wheat protein detected for individual measurements

Meat Product	Producer's Declaration	Block			
		A	B	C	D
1	**content of wheat protein**	+++	++	++	++
2	**content of wheat protein**	++	++	++	+++
3	**traces of wheat**	+	+	++	++
4	**content of wheat**	++	++	+++	+
5	**traces of wheat**	++	++	++	++
6	**content of wheat starch**	+	+/-	+	++
7	**content of wheat**	+++	+	++	++
8	**content of wheat protein**	++	+++	++	++
9	**content of wheat**	+	+	++	+++
10	**traces of wheat**	++	+	++	+

Explanatory notes: + to +++ shows the power of fluorescence intensity of wheat protein, +/- shows a dubious result.

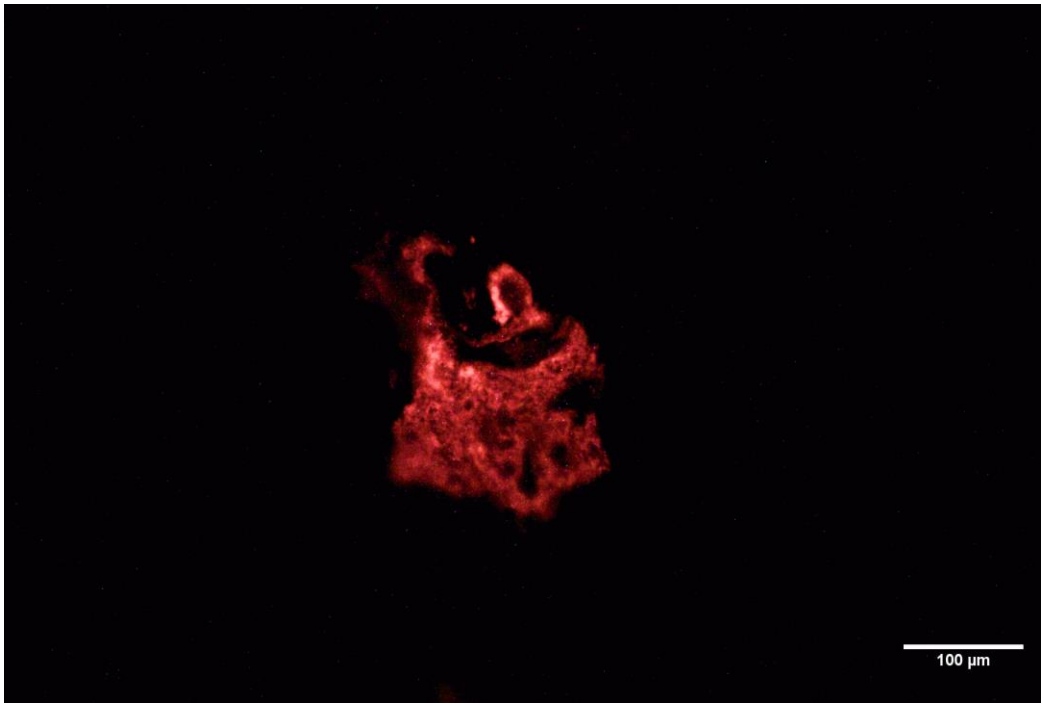

Figure 1 Sample no. 4: Wheat protein (red) in a meat product (black), fluorescence intensity +++, magnification 200x

Fluorescent immunohistochemistry was selected as the examination method. Wheat protein was detected on the basis of its specific structure and with the help of fluorescence which was created using the binding of Texas Red fluorochrome and excitation of light at the wavelength of 596 nm (Fig. 1). The results are given in Table 1. The number of crosses represents the power of fluorescence intensity of wheat protein. From the table it follows that wheat protein was detected in all the blocks for each meat product analyzed. From the results it follows that it is possible to confirm the presence of wheat protein using the method of immunofluorescence in products with targeted addition of wheat protein for improving texture and binding qualities which were labeled with "content of wheat protein" by the producer. The method of immunofluorescence was also sensitive for meat products labeled "traces of wheat" and the use of wheat was confirmed in all the products labeled in this way. For the meat product number 6, a dubious result was found in block B, which might have been caused by the low amount of gliadin due to the use of wheat starch.

CONCLUSION

Wheat protein ranks among allergens in foodstuffs which represent a serious health problem. The correct detection of the presence of these allergens in foodstuffs is absolutely necessary from the allergic consumer's point of view. Fluorescent immunohistochemistry could be used as one of these methods. With the help of this method, 10 meat products, for which the producers declared the presence or traces of wheat, from the retail network were examined. The presence of wheat protein was confirmed in all of these products. The method of immunofluorescence is therefore a sensitive method suitable also for the analysis of meat products for the presence of wheat protein. The results also show that producers are responsible in declaring the presence of the allergen in their products.

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THE EFFECT OF YUCCA ON PROLIFERATION, APOPTOSIS AND STEROIDOGENESIS OF PORCINE OVARIAN GRANULOSA CELLS

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ABSTRACT

Yucca shidigera is a medicinal plant native to Mexico. Is a plant widely used in folk medicine to treat a variety of ailmentary disorders, but its action on reproductive processes and possible mechanisms of such action remains unknown. *Yucca schidigera* extract contains a number of steroidal saponins that, because of their biological activity, have attracted attention from the food industry for many years. Yucca extract is used as a natural feed additive with positive effect to microflora, digestion, metabolism and to improve animal muscle growth. Its extract has been used as a foodstuff and folk medicine to treat a wide variety of diseases for many years. Nevertheless, it remaines unknown, whether consumption of yucca can affect reproductive system. The aim of this study was to examine the effects of yucca on basic ovarian cell functions – proliferation, apoptosis and steroidogenesis. Porcine ovarian granulosa cells were cultured with and without yucca extract (added at doses 0; 1; 10 and 100 $\mu g.mL^{-1}$ of medium). Markers of proliferation (% of PCNA-positive cells) and apoptosis (% cells containing bax) were analysed by immunocytochemistry. Release of steroid hormones (progesterone and testosterone) was measured by EIA. It was observed, that addition of yucca inhibited proliferation (expression of PCNA), increased apoptosis (expression of bax), stimulated progesterone and inhibited testosterone release. The ability of yucca to reduce ovarian cell proliferation, to promote ovarian cell apoptosis and affect steroidogenesis demonstrates the direct influence of yucca on female gonads. Furthermore, our observations suggest the multiple sites of action (proliferation, apoptosis, steroidogenesis) of yucca on porcine ovarian cell functions. It is not to be excluded, that consumption of yucca can suppress female reproductive functions.

Keywords: yucca; proliferation; apoptosis; steroidogenesis; porcine granulosa cell

INTRODUCTION

Medicinal plants contain a variety of molecules with potent biological activities **(Coran et al., 2001)**. Changes in nutrition can affect human and animal reproductive processes **(Sirotkin, 2010)**. Herbal medicine and functional food provides plentiful alternatives to synthetic compounds in the treatment of almost any condition, as it is believed to be less toxic. *Yucca schidigera*, know as yucca, is a plant belonging to the *Agavaceae* family, native to the South-Western United State and Mexico. Indians recognized yucca as one of the nicest desert plants, "a tree of life" with health promoting activity. Its extracts have been used for centuries in folk medicine to treat a wide variety of inflammatory disorders, especially headaches, gonorhea, arthritis, and rheumatism **(Cheeke, 1998)**. Yucca powder has powerful anti-inflammatory activity, mediated via inhibition of NFkB activation. Yucca polyphenolics inhibit NFkB, a transcription factor which stimulate iNOS, an inducible enzyme which produced the inflammatory agent nitric oxide **(Cheeke et al., 2006)**. In recent time, this plant and its extract have been used to produce drugs that are applied to treat various human diseases. Furthermore, yucca is used as a food additive increasing the performance in sportsmen. It also improves performance and health of the livestock in addition to feed in various concentrations **(Duffy et al., 2007)**. The ability of yucca to affect food digestion and metabolism are well known. It has been showed that *Yucca schidigera* and yucca-supplemented diets had lower rumen ammonia N concentrations compared to the control diet. *Yucca schidigera* can be used to modify rumen fermentation in order to decrease ruminal ammonia concentrations and reduce urinary N excretion thereby reducing the environmental impact of ruminant production systems **(Santoso et al., 2006)**. The mode of action of *Yucca schidigera* is believed to be related to their steroidal saponins (sarsapogenin, smilagenin, markogenin, samogenin, gitogenin and neogitogenin) and phenolic compounds (resveratrol and yuccaols) were found in the yucca plant bark **(Abaza and Said, 2005)**. Yucca polyphenols are potent antioxidants **(Oleszek et al., 2001)**. Yucca phenolics also are antioxidants and free-radical scavengers, which may aid in suppressing reactive oxygen species (ROS) that stimulate inflammatory responses **(Cheeke et al., 2006)**. Yuccaols inhibit the generation of free radicals in blood platelets may be beneficial in protecting against cardiovascular diseases **(Olas et al., 2003)**. According to **Jaques (1989)** saponins contained in the yucca plant it just one of the many natural biosecurity substances which increase the efficiency of farm animals. Steroidal saponins in yucca were reported to exhibit antiyeast or antifungal activities **(Tanaka et al., 1996)**. Saponin-containing yucca extracts are currently used in the feed industry for control of ammonia and odour **(Cheek,**

1999). Yucca saponins are known to reduce iron absorption **(Southon et al., 1988)** and may reduce fatty acid absorption by sequestering bile acids necessary for micelle formation and fat absorption **(Oakenfull and Sidhu, 1989)**. The evidence demonstrates that saponins enhance feed efficiency and weight gain in pigs, as well as feed efficiency, weight gain, and increased egg production in chickens, increase production of milk in dairy cows. Livestock clearly benefits when the extract from this plant is included in the feed. Because it is 100% natural, the *Yucca schidigera* dry extract is environmentally safe and is the best answer to naturally lowering toxic ammonia levels in housing and improving the quality and potential output of animals **(Cheeke et al., 2006)**. **Balazi, et al., (2013)** suggests that the addition of Yucca shidigera plant into the normal feed had positive effects on male's spermatozoa parameters. Effect of yucca on ovarian functions and steroidogenesis has been unknown. Progesterone (P) is the ovarian steroid hormone that is needed for ovarian functions, embryonic development and in mammary gland development **(Hagan et al., 2009)**. It is produced by porcine **(Sirotkin et al., 2008; Kolesarova et al., 2010 a, b)**, rabbit **(Sirotkin et al., 2009)**, sheep **(Al-Dabbas et al., 2008)** and goat **(Blaszczyk et al., 2009)** and other animal ovarian cells. Progesterone governs ovarian functions of pigs **(Sirotkin et al., 2008, Kolesarova et al., 2010a,b)** and rabbits **(Sirotkin et al., 2009)**. Testosterone (T) is a steroid hormone that is produced in the testes, ovaries and a small amount in adrenal gland **(Cox et al., 2005; Reed et al., 2006)**. It is important for healthy development of the individual and the establishment of secondary sexual characteristics **(Swaab et al., 2009)**. Progesterone and testosterone can affect ovarian functions via influence on ovarian cell proliferation and apoptosis, whose in turn define ovarian follicullogenesis, ovulation and fecundity **(Sirotkin, 2014)**. The influence of yucca on ovarian cell proliferation, apoptosis, steroidogenesis, the interrelationships between these processes and their mechanisms remain unknown yet.

MATERIAL AND METHODOLOGY

Isolation and culture of granulosa cells

Ovaries of non-cycling pubertal gilts, about 180 days of age, were obtained after slaughter at a local abattoir. They were washed several times in sterile 0.9% NaCl and 95% alcohol. Granulosa cells were aspirated by syringe and sterile needle from follicles 3-5 mm in diameter and granulosa cells isolated by centrifugation for 10 min at 200 g. Cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittakerTM, Verviers, Belgium), resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittakerTM) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 106 cells/ml medium. Portions of the cell suspension were dispensed to 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 μL/well) and incubated at 37.5 °C in 5% CO_2 in humidified air until 60-75% confluent monolayer was formed (3-5 days), at which point the medium was renewed. Further culture was performed in 200 μL/medium in the same chamber slides, as described previously.

After medium replacement experimental cells were cultured in the presence of yucca (KONFIRM, Brno, Czech Republic) alone at concentrations of 0; 1; 10 and 100 $\mu g/mL^{-1}$. Yucca was dissolved in culture medium immediately before its addition to the cells. Control cells were cultured without yucca. After two days in culture, the medium from the 24-well plates was gently aspirated and frozen at -24 °C to await EIA. After removing the medium from chamber slides, cell were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2-7.4; 60 min) and held at 4 °C to await immunocytochemi. cal analysis.

Immunocytochemical analysis

Following washing and fixation, the cells were incubated in the blocking solution (1% of goat serum in phosphate-buffered saline - PBS) at room temperature for 1 h to block nonspecific binding of antiserum. Afterwards, the cells were incubated in the presence of monoclonal antibodies against either PCNA (marker of proliferation) and bax (marker of apoptosis) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:500 in PBS) for 2 h at room temperature at overnight at 4 °C. For the detection of binding sites of primary antibody, the cells were incubated in secondary swine antibody against mouse IgG labeled with horse-radish peroxidase (Servac, Prague, Czech Republic, dilution 1:1000) for 1 h. Positive signals were visualized by staining with DAB-substrate (Roche Diagnostics GmbH, Manheim, Germany).

Following DAB-staining, the cells on chamber-slides were washed in PBS, covered with a drop of Glycergel mounting medium (DAKO, Glostrup, Denmark); then coverslip was attached to a microslide. Cellular presence and localization of PCNA and bax positivity in cells was proved on the basis of DAB-peroxidase brown staining. A ratio of DAB-HRP-stained cells to the total cell number was calculated.

Immunoassay

Concentrations of P4 and T were determined in 25-100 μL samples of incubation medium by EIA. Previously validated for use in culture medium by using antisera against steroids produced in the Institute of Animal Science, Neustadt, Germany. P4 concentrations were measured by using EIA as described previously **(Prakash et al., 1987)**. Rabbit antiserum against P was obtained from Research Institute for Animal Production, Schoonoord, Netherlands. It cross-reacted <0.1% with 17 β-estradiol, dihydrotestosterone, testosterone and 17 β-hydroxyprogesterone. Sensitivity was 12.5 pg/mL. Inter and intra-assay coefficients of variation did not exceed 3.3% and 3.0% respectively. T was assayed by using EIA according to **Münster (1989)**. Sensitivity was 10 pg/mL. The antiserum cross-reacted <96% with dihydrotestosterone, <3% with androstenedione, <0.01% with progesterone and estradiol, <0.02% with cortisol and <0.001% with corticosterone. Inter and intra-assay coefficients of variation were 12.3% and 6.8% respectively.

Statistical analysis

Each experimental group was represented by three Chamber-slide wells. The proportions of cells containing specific immunoactivity were calculated from at least 1000 cells per chamber. The percentage of cells containing antigen in different groups of cells was calculated. Each series of experiments was performed twice. The data shown are the means of values obtained in these two separate experiments performed on separate days with separate groups of granulosa cells, each obtained from 9 animals.

Significant differences between the experiments were evaluated using Student´s T-test and one/two-way ANOVA folowed by paired Wilcoxon-Mann Whitney test, by using Sigma Plot 11.0 software (Systat Software, GmbH, Erkhart, Germany). Differences from control at $P < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

These data constitute first reports concerning the influence of yucca on reproduction. Moreover, this is the demnstration of direct influence of this medical plant on ovarian cells. We have shown, that yucca significantly increased the percentage of cells containing apoptosis marker (bax) at doses 10 and 100 $\mu g.mL^{-1}$. Dose 1 $\mu g.mL^{-1}$ did not affect the apoptosis. The percentage of cells containing proliferation marker (PCNA) was significantly increased at all doses added. The effect of yucca on both proliferation and apoptosis has a dose-dependent manner (Table 1).

We have been observed yucca has effect on steroidogenesis. In our experiment yucca stimulated release of P and inhibited T release by a dose-dependent manner (Table 2).

This study demonstrated effect of yucca addition on porcine granulosa cells. It was find dose-dependent effect on hormone release of yucca. Yucca at the highest dose (100 $\mu g.mL^{-1}$) significantly stimulated the release of P4, while doses 1 and 10 $\mu g.mL^{-1}$ did not effect on secretion. In our experiment, T release was inhibited by yucca at doses 1 and 10 $\mu g.mL^{-1}$ and the highest dose (100 $\mu g.mL^{-1}$) did not affect secretion. This is the first finding, that yucca can influence not only P4, but also androgen output. P4 and T have antiproliferative and proapoptotic properties. They can suppress growth of ovarian follicles. Therefore, it might be hypothesized, that yucca through promotion of P4 and T can inhibit porcine ovarian development. This hypothesis was supported by the ability of yucca to affect markers of ovarian cell proliferation and apoptosis. We have been observed that yucca can directly suppress the accumulation of proliferative peptide PCNA and promote the expression of apoptotic peptide bax. Physiological influence of yucca on ovarian granulosa cells could be important practical viewpoint. It is not to be excluded the yucca may used in the regulation of functions including fertility in pigs, other animals and humans, although this hypothesis should be verified by further, *in vivo* experiments. An inhibitory effect of yucca on porcine ovarian functions observed in our experiments could indicate that this plant substance could be potentially useful for synchronisation of porcine ovarian cycles. If the negative effect of yucca on ovarian functions occurs not only in pigs, but also in humans, and not only in-vitro, but *in vivo* too, it could be hypothesised, that yucca could jeopardize human reproduction and fertility.

Table 1 The percentage of granulosa cells containing markers of proliferation (PCNA) and apoptosis (Bax) in the porcine granulosa cells exposed by yucca

Supplement	PCNA	bax
Control (no adition)	49.94 ±1.92 (1700)	35.06 ±1.48 (1600)
Yucca 1 $\mu g.mL^{-1}$	33.29 ±2.17* (850)	40.47 ±3.15 (850)
Yucca 10 $\mu g.mL^{-1}$	26.00 ±1.92* (800)	45.00 ±2.84* (800)
Yucca 100 $\mu g.mL^{-1}$	22.71 ±1.60* (900)	54.38 ±2.53* (800)

All the values represent P or T release, means ± SEM, * - significant ($P < 0.05$) differences with control (cells not treated with yucca).

Table 2 The secretion of steroid hormones in the porcine granulosa cells treated and not treated with yucca (EIA)

Supplement	P4 secretion ng/106 cells/day	T secretion pg/106 cells/day
Control (no adition)	19.98 ±1.22	392.80 ±33.23
Yucca 1 $\mu g.mL^{-1}$	17.28 ±2.31	108.18 ±48.81*
Yucca 10 $\mu g.mL^{-1}$	20.42 ±3.22	171.71 ±11.47*
Yucca 100 $\mu g.mL^{-1}$	31.53 ±1.71*	375.58 ±31.23

All the values represent P or T release, means ± SEM, * - significant ($P < 0.05$) differences with control (cells not treated with yucca).

CONCLUSION

This study is the first evidence possible stimulatory effect of yucca on the release of progesterone, inhibitory effect on the release of testosterone, inhibitory impact on proliferation (accumulation of PCNA) and stimulatory influence on apoptosis (accumulation of bax) on granulosa cells of porcine ovary. Our results suggest a direct effect of yucca on steroidogenesis, proliferation and apoptosis in porcine ovaries. It is possible, that consumption of yucca by animal and human females may suppress their reproductive functions.

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PRODUCTION OF ENTEROTOXINS OF *STAPHYLOCOCCUS* SPP. ISOLATED FROM SAMPLES OF SHEEP MILK

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ABSTRACT

In our study was followed occurrence of mastitis in herd of 430 sheep of breed zoslachtena valaska with hand milking technology examined two times during one lactation season. Individual examination consisted from clinical examination of udder and microbiological examination of milk samples. By PCR was determined presence of genes coding production of enterotoxins, and by ELISA methods production individual types of enterotoxins. From individual forms of mastitis were frequently detected subacute (6.7%), subclinical (5.7%) and acute (2.9%). The coagulase-negative staphylococci (CNS) were identified in 102 (65.4%) from all 156 positive isolates. The CNS and *S. aureus* caused subacute (5.1%), subclinical (3.9%) and acute (2.4%) forms of mastitis. The most frequently isolated were *S. epidermidis*, followed by *S. chromogenes* and *S. xylosus* from ewes with subacute and subclinical mastitis. From acute and chronical forms of mastitis were predominantly isolated *S. aureus*, *S. uberi*s and *S. epidermidis*. The production of staphylococcal enterotoxins (SE) - SEA, SEB, SEC, SED and the presence of genes *sec* (3), *sea* (2), s*eb* (2) and *sed* (2) were determined in *S. aureus*, *S. epidermidis*, *S. schleiferi* and *S. chromogenes*, respectively. The results suggested on the high occurrence (12.4%) of subacute and subclinical forms. Confirmed production of enterotoxins and presence of genes coding their production present a risk for human health and decreased a quality of milk and products from sheep´s milk.

Keywords: sheep; hand milking; mastitis; pathogens, staphylococcal enterotoxins

INTRODUCTION

Mastitis has been considered an economically important disease in the production of sheep. The occurrence of mastitis in sheep is in interval from 4.0 to 50.0%. Staphylococci are the main aetiological agents of small ruminant intramammary infections (IMI), and *Staphylococcus aureus* is the most frequent isolate from clinical mastitis cases and coagulase-negative species are the most frequent in subclinical IMI. The annual incidence of clinical IMI in dairy sheep is generally lower than 5%, but in a small percentage of herds the incidence may exceed 30-50% of the animals, causing mortality (gangrenous mastitis) or culling of up to 70% of the herd **(Fthenakis, 1994, 1995; Vautor et al., 2009)**.

Antibiotic treatment of mastitis leads to significant increase in milk quantity and quality, lower somatic cell count and is likely associated with reduction in prevalence of clinical mastitis among herds, which is economically beneficial **(Contreras et al., 2007)**.

The important factor of virulence of *Staphylococcus* spp. besides to antibiotic resistance is production of enterotoxins, which showed high health risk for human. Milk and other dairy products are reported to be frequently associated with SE food poisoning. It was supposed that milk of infected animals constitute the main source of staphylococcal enterotoxigenity of animal origin **(Omoe et al., 2002; Scherrer et al., 2004)**.

During the many years the production of enterotoxins was connected only with *S. aureus*. Many authors report, that other species of CNS (*S. intermedius*, *S. hyicus*) may producing of enterotoxins **(Becker et al., 2001, Beatriz et al., 2006)**.

The aim of our study was the observed the occurrence of mastitis in herd of sheep with hand milking and determined of enterotoxigenic bacteria of *Staphylococcus* spp. isolated from milk samples during one milking season.

MATERIAL AND METHODOLOGY

Animals and milking

The experiment was carried out in herd of 430 sheep of breed zoslachtena valaska, which during winter season were stabled in bricked shed with deep bedding. During milking season (from April to September) was hand milking carrying out in cover strung cage with three fixation places two times per day.

Examination of health status of sheep and milk samples

Complex examination of health status of udder in sheep was carried out at the start (April) and at the end of milking (September). The clinical examination was carried out according to **Hariharan et al. (2004)** and milk from individual halves was evaluated by NK-test (Bioveta a.s., Inovice na Hané, Czech Republic) according to **Fthenakis (1994).**

Laboratory analyses

From the every individual milk sample were inoculated 0.05 ml, onto blood agar (Oxoid LTD, Hampshire, UK) and cultivated at 37°C for 24h. Based on the colony morphology, bacteria *Staphylococcus* spp. was selected for

the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspect colonies *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriacae* spp. were isolated on blood agar, cultivated at 37°C for 24h and identified biochemical using the STAPHYtest, STREPTOtest, ENTEROtest (Erba-Lachema, Brno, Czech Republic) and identification by software TNW Pro 7.0 (Erba-Lachema, Brno, Czech Republic).

Identification of genes coding staphylococcal enterotoxins *(sea, seb, sec, sed, see)* was carried out by PCR method according to **Becker et al. (1998)**. DNA was isolated by QiAMP tissue kit (Oiagem, Hilden, Germany). For control were used reference strains for types of SE: SEA, SEB SEC, SED, SEE (Bergdoll; CNCTC, Brno, Czech Republic). For the detection of genes was used oligonucleotid primers *sea - see* **(Becker et al., 1998)**. Validation of production of SE *in vitro* in every strain was confirmed by ELISA method, by set Ridascreen ® Set A, B, C, D, E (R-Biopharm AG, Darmstad, Germany).

RESULTS

From total number of sheep (n = 820) in Figure 1, on the base of complete examination were determined 19.5% occurrence of mastitis (n = 160) during one milking season. The most frequently were found subacute (6.7%), subclinical (5.7%) and acute (2.9%) forms of mastitis in 126 sheep (15.4%).

The highest ratio had CNS, which occurrence was determined in 102 cases (12.4%), at which caused subacute (4.8%) and subclinical (3.8%) mastitis. From CNS, *S. epidermidis* (16.0%), *S. chromogenes* (11.9%), *S. simulans* (7.0%) and *S. schleiferi* (6.4%) predominantly caused subacute and subclinical forms of mastitis. Bacteria *S. aureus* (6.4%) and *S. uberis* (4.5%) were isolated from acute and subacute forms of mastitis (Table 1).

The presence of genes coding production of enterotoxins in bacteria *Staphylococcus* spp. (n = 115) described Table 2. Four strains of *S. epidermidis* showed presence of gene *sea* (1), *seb* (2) and *sed* (1), but by ELISA method was confirmed only production SE types A (1) and B (1) in two strains of staphylococci. In *S. chromogenes* and *S. schleferi* was determined presence of one gene *sec*, and gene *sed* in *S. schleferi* was conjugated with production correspondent type of SE. Three strains of *S. aureus* was characterized by presence of genes *sea* (1) and *sec* (2), but the production of SEC was determined only in one strain.

DISCUSSION

How common mastitis is in sheep is extremely variable. Studies of cull ewes at slaughter in Britain show a very high prevalence ranging from 13 to 50%, indicating that clinical mastitis is likely an important cause of culling of ewes in the UK **(Conington et al., 2008)**.

In our study we confirmed 19.5% occurrence of mastitis, predominantly subacute (6.7%), subclinical (5.7%) and acute (2.9%) forms caused by CNS, *S. aureus* and *S. uberis*. According to **Ozenc et al. (2011)**, the general pathogens, which caused clinical mastitis in dairy sheep herds, are *Staphylococcus aureus* and *Streptococcus agalactiae*. Subclinical forms are refers to CNS, which often grows to subacute and acute forms. In compare with *S. aureus* have CNS lower frequency of virulence factors, however their representation in clinical forms of mastitis in sheep is becoming increasingly a problem in the holdings as confirmed in our study. From 156 isolated bacterial pathogens, 102 (12.4%) were represented by CNS, which caused subacute (4.8%) and subclinacal (3.8%) forms of mastitis. Bacteria *S. aureus* (1.6%) and *S. uberis* (1.5%) were isolated predominantly from acute and subacute mastitis.

Several authors in their studies from France and Spain recorded, that the species of *Staphylococcus* spp. belongs to general aetiological agents of intramammary infections in small ruminants (*S. aureus* in clinical cases and CNS in subclinical). From the CNS is more frequently *S. epidermidis* what is also determined in our study. No less important bacterial pathogens are *Corynebacterium*

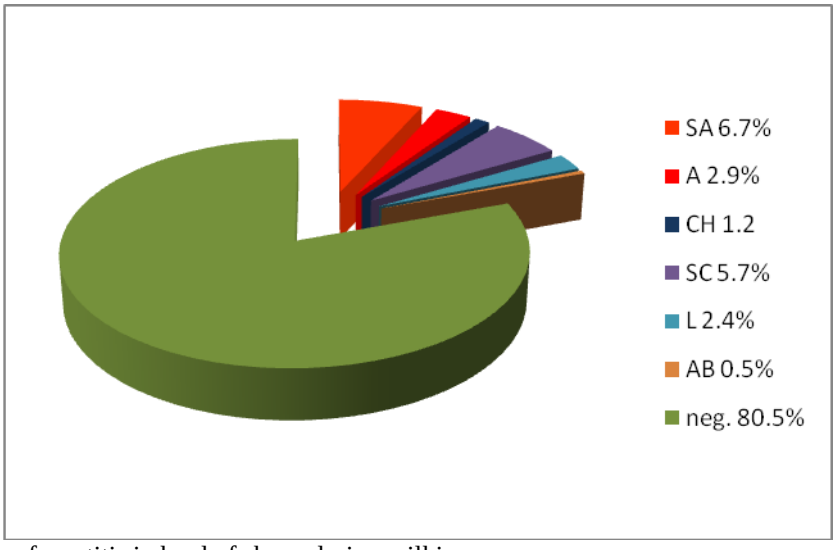

Figure 1 Occurrence of mastitis in herd of sheep during milking season
SA - subacute mastitis, A - acute mastitis, CH - chronic mastitis, SC - subclinical mastitis, L - latent mastitis, AB - abacterial mastitis, neg. - negative

spp., *Enterococcus* spp. and *Micrococcus* spp. **(Bergonier et al., 2003; Berthelot et al., 2006; Ozenc et al., 2011)**.

Subclinical and subacute mastitis are thought to have a prevalence of between 10% and 30% in Lowland flocks in Southern England **(Conington et al., 2008)**. From 430 sheep was determined 5.7% incidence of subclinical mastitis. Most frequently isolated were CNS, predominantly *S. epidermidis*, *S. chromogenes*, *S. simulans* and *S. schleiferi.*

Hariharan et al. (2004) showed 6.7% positive samples of milk from 492 examinated sheep in which were isolated CNS. The most frequently isolated were *S. equorum*, *S. xylosus* and *S. simulans* from ewes with subclinical mastitis during lactation.

Burriel (1997) reported that *S. simulans*, *S. xylosus*, *S. hyicus* were the predominant species of CNS in the milk from meat ewes, *S. epidermidis* and *S. schleiferi* was the predominant species in the milk of dairy ewes, what was also confirmed in our study. In addition to the high incidence of subclinical and subacute mastitis, which were caused by CNS and *S. aureus*, in some strains were determined production of SE.

The staphylococcal enterotoxins are recognised as being agents of intoxication such as staphylococcal food poisoning syndrome in man and they may be involved in other types of infections **(Zschöck et al., 2005)**.

Table 1 Bacterial agents of ovine mastitis

Bacterial pathogens	∑	**%**	**Mastitis form**									
			SA		**A**		**CH**		**SC**		**L**	
			n	%	n	%	n	%	n	%	n	%
S. aureus	13	*1.6*	3	1.9	7	4.5	2	1.3	1	0.6	-	-
S. epidermidis	37	*4.5*	14	9.0	7	4.5	2	1.3	11	7.1	3	1.9
S. chromogenes	22	*2.7*	8	5.1	3	1.9	2	1.3	7	4.5	2	1.3
S. schleiferi	17	*2.1*	6	3.8	2	1.3	1	0.6	4	2.6	4	2.6
S. simulans	14	*1.7*	5	3.2	1	0.6	-	-	6	3.8	2	1.3
S. caprae	7	*0.9*	4	2.6	-	-	-	-	1	0.6	2	1.3
S. xylosus	5	*0.6*	2	1.3	-	-	1	0.6	2	1.3	-	-
Bacilus spp.	9	*1.1*	3	1.9	1	0.6	-	-	4	2.6	1	0.6
S. sanguinis	3	*0.4*	2	1.3	-	-	-	-	1	0.6	-	-
S. uberis	12	*1.5*	4	2.6	3	1.9	-	-	4	2.6	1	0.6
E. coli	6	*0.7*	1	0.6	-	-	-	-	2	1.3	3	1.9
*Others**	11	*1.3*	3	1.9	-	-	2	1.3	4	2.6	2	1.3
positive	156	**19.0**	55	***6.7***	24	***2.9***	10	***1.3***	47	***5.7***	20	***2.4***
negative	664	81.0										
Total	820	100.0										

*Others** - *Arcanobacterium* spp., *Proteus* spp., *Corynebacterium* spp., *Enterococcus* spp., ∑ - number of sheep, SA - subacute mastitis, A - acute mastitis, CH - chronic mastitis, SC - subclinical mastitis, L - latent mastitis

Table 2 Staphylococcal enterotoxins and genes coding production of SE in bacteria *Staphylococcus* spp. (n=15) in herd of sheep

***Staphylococcus* spp.**	**production of SE**				**presence of genes**			
	SEA	SEB	*SEC*	*SED*	*sea*	*seb*	*sec*	*sed*
S. aureus	1		1		1		2	
S. epidermidis	1	1			1	2		1
S. chromogenes			1				1	
S. schleiferi				1				1
Total	**2**	**1**	**2**	**1**	**2**	**2**	**3**	**2**

Valle et al. (1990) tested 342 *Staphylococcus* spp. bacteria for their ability to produce enterotoxins, which were isolated from various parts of the body of small ruminants. Staphylococcal enterotoxins were produced by 74.3% of 70 coagulase-positive bacteria and 22% of coagulase-negative bacteria. Most enterotoxigenic bacteria were isolated from the teat skin and milk. These bacteria most frequently produced staphylococcal enterotoxin of type C, namely either alone (67.9%) or in combination with other type of SE. From our results it follows that within 115 *Staphylococcus* spp. bacteria the production of SEC and SEA was recorded frequently, than SEB and SED, all the same as a frequently presence of *sec* gene.

The production of enterotoxins SEA, SEC and SED by *S. aureus* field strains isolated from mastitis animals has been investigated in several studies **(Matsunaga et al., 1993; Zschöck et al., 2000).** In our study presence of genes coding SE in species of CNS (*S. epidermidis, S. chromogenes, S. schleiferi*) was also certified.

CONCLUSION

By complex examination in herd of sheep we determined 12.4% occurrence of subacute and acute forms of mastitis caused predominantly by CNS, *S. aureus* and *S. uberis.* Very important is the early diagnosis of mastitis in sheep during the milking season. At the start of the treatment of subclinical mastitis showing the subclinical forms of mastitis can significantly eliminate clinical stage of subacute and acute forms of mastitis. In bacteria *S. aureus, S. epidermidis, S. schleiferi* and *S. chromogenes* were by PCR detected the presence of enterotoxins genes *sec* (3), *sea* (2), s*eb* (2) and *sed* (2), as well as production of SE - SEA (2), SEC (2), SEB (1) and SED (1) by ELISA method. Because of the importance of these toxins for public health and food safety, an efficient screening for the prevalence of enterotoxic strains in mastitis is required.

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OCCURRENCE AND ANTIMICROBIAL RESISTANCE OF *STAPHYLOCOCCUS AUREUS* IN BULK TANK MILK AND MILK FILTERS

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ABSTRACT

This work is focused on the monitoring of *Staphylococcus aureus* prevalence in raw milk and milk filters, its antibiotic resistance and detection of methicillin resistant *Staphylococcus aureus* (MRSA). Samples of raw cow´s milk and milk filters were collected in the period from 2012 till 2014, from 50 dairy farms in the Czech Republic. The total of 261 samples (164 samples of raw milk and 97 milk filters) were cultivated on Baird-Parker agar. Both the typical and atypical colonies were examined by plasmacoagulase test and PCR method was used for detection of species specific fragment SA442 and *mec*A gene. Standard disk diffusion method was used to determinate resistance to antimicrobial agents. The bacterium *Staphylococcus aureus* was detected on 25 farms (50%). The antimicrobial resistance showed differences between the farms. Total of 58 samples were positive for *Staphylococcus aureus*, of which were 37 (14.2%) isolated from raw milk samples and 21 (8.1%) from milk filters. From these samples we isolated 62 *Staphylococcus aureus* strains, 41 isolates bacteria *S. aureus* from raw milk (66.1%) and 21 isolates *S. aureus* from milk filters (33.9%). The presence of antibiotic resistance in *Staphylococcus aureus* isolates was low, most of them were resistant to amoxicilin. According to the results obtained by the PCR method for the methicillin - resistant *S. aureus* (MRSA), the *mec*A gene was present in 6 strains (9.7%), 4 isolates obtained from milk samples (6.5%) and 2 isolates from milk filters (3.2%). These isolates can be considered as a possible source of resistance genes, which can be spread through the food chain. Nowadays, a globally unfavourable increasing trend of prevalence of methicillin resistant staphylococci strains especially *Staphylococcus aureus* is being observed worldwide. The improper hygiene and poor farm management practices contributed to the presence of *S. aureus* in the milk. This may have contributed to the high level of *S. aureus* isolated. Improving the hygienic conditions of the milking environment and utensils may reduce the prevalence of *S. aureus* in milk. Objective of this study was monitoring of *Staphylococcus aureus* prevalence and determine the prevalence rate of antimicrobial resistance of *S. aureus* isolated from raw milk and milk filters in the Czech Republic.

Keywords: *mec*A gene; MRSA; PCR; antibiotic resistance; disk diffusion method

INTRODUCTION

Milk and dairy products are basic components of human diet. However, raw milk consumption is accompanied by the risk of ingesting pathogenic bacteria that can pose an elevated health hazard **(Latorre et al., 2009)**. One of the pathogen is *Staphylococcus aureus*, which recognized worldwide as one of the most important foodborne pathogens associated with food poisoning **(Gundogan et al., 2005)**. The literature demonstrates the fact that it is considered the most common cause of illness caused due to consumption of raw milk **(Lee Loir et al., 2003)**. *S. aureus* is the major causative agent of mastitis in cows **(Rabello et al., 2007)**. Bacterial contamination of milk usually occurs during the milking process and it depends on the sanitary condition of the environment, the equipment used for milking and the milking personnel. It could also occur from microorganisms that enter the udder through the teat canal opening **(Kalsoom et al., 2004)**. Therefore, milk and dairy products may pose a risk to consumers. Antibiotic resistant *S. aureus* isolates pose a major challenge for both veterinary and human medicine because they have a negative impact on treatment of infections **(Brouillette et al., 2005)**. Currently, it is detected adverse increasing trend worldwide prevalence of methicillin-resistant strains of staphylococci, especially *Staphylococcus aureus* (MRSA). The incidence of MRSA strains were found not only in humans but also in animals, including the food production ones **(Karpíšková et al., 2009)**. The aim of this study was to confirm the presence of the bacteria *S. aureus* in the samples of raw milk and milk filters, occurrence of antibiotic resistance with a special focus on MRSA strains.

MATERIAL AND METHODOLOGY

Samples of raw milk and milk filters were collected in the period from 2012 till 2014, from 50 dairy farms in the Czech Republic. From 261 samples collected 164 samples were of raw milk and 97 samples of filters. The milk samples of the amount of 250 ml were taken in the sterile bottles and transported at 4 ±1 °C to the laboratory where they were immediately processed. The milk filters were collected into sterile PE packet and transported at 4 ±1 °C to the laboratory too where they were immediately processed.

Detection of *Staphylococcus aureus* was performed as follows: 25 ml of milk was diluted with 225 ml of buffered peptone water (Oxoid, UK) and milk filters were diluted with 225 ml also of buffered peptone water (Oxoid, UK) and homogenized. After enrichment at 37 °C overnight samples were cultivated on Baird - Parker agar (Oxoid, UK) supplemented with egg yolk-tellurite emulsion. From each plate, both the typical and atypical colonies were examined by plasmacoagulase test and confirmation of suspected *S. aureus* strains was carried out by polymerase chain reaction (PCR) method of a species specific fragment SA442 and *mec*A gene which encodes the resistance to methicillin **(Martineau et al., 1998, Bosgelmez-Tmaz et al., 2006)**.

Antibiotic resistance of *S. aureus* was tested by the standard disk diffusion method on Mueller - Hinton agar (Oxoid, UK) according to the CLSI methodology (2012a).

Strains were tested for resistance to 14 therapeutically significant antimicrobial agents: oxacillin (OX) (1 μg), tetracycline (TE) (30 μg), erythromycin (E) (15 μg), chloramphenicol (C) (30 μg), co-trimoxazole (SXT) (25 μg), amoxicilin/clavulanic acid (AMC) (20/10 μg) and clindamycin (DA) (2 μg), gentamicin (CN) (10 μg), ciprofloxacin (CIP) (15 μg), vancomycin (VA) (30 μg), teicoplanin (TEC) (30 μg), rifampicin (RD) (5 μg), cefoxitin (FOX) (30 μg) and cefotaxime (CTX) (30 μg) (Oxoid, UK).

Based on the inhibition zone size isolates were evaluated as sensitive, intermediately resistant or resistant, according to the criteria specified in CLSI (2012b).

More attention was dedicated on the occurrence of methicillin - resistant *S. aureus* strains, which were resistant to penicillins and other beta-lactam antibiotics **(David et al., 2010)**.

RESULTS AND DISCUSSION

In this study we described the isolation and antibiotic susceptibility characterization of *S. aureus* from 50 dairy farms in the Czech Republic. From 261 samples collected 164 (62.8%) samples were of raw milk and 97 (37.2%) samples of filters. The bacterium *Staphylococcus aureus* was detected on 25 farms (50%). *S. aureus* was detected from 0% to 66.7% of the samples. The results reported in our study were similarly high when compared to the study by **Gündoğan et al. (2006)**. According to his results, *S. aureus* was isolated from all raw milk samples.

According to our results, *S. aureus* was detected in 37 samples of raw milk (22.6%) and 21 samples of milk filters (21.6%). From these samples we isolated 62 *Staphylococcus aureus* strains, 41 isolates bacteria *S. aureus* from raw milk (66.1%) and 21 isolates *S. aureus* from milk filters (33.9%). **Gran et al., (2003)** reported similar results, and showed that *S. aureus* was found in 49 out of 60 milk samples (82%).

Similar results were obtained in a study by **Karpíšková et al. (2011)**, where the detection of zoonotic foodborne pathogens in raw milk was described. In their work a total of 56.6% of raw cow´s milk taken from vending machines were positive for the presence of *S. aureus* in the Czech Republic. This bacterial species is one of the major etiological agents causing mastitis in dairy cattle **(Cretenet et al., 2011)**.

Data of the *Staphylococcus aureus* occurrence are shown in the Table 1.

Samples	No.	No. of *S. aureus* strains	%
Raw milk	164	37	14.2
Milk filters	97	21	8.1
Total	261	58	

Table 1 Number and percentage of *S. aureus* strains isolated from raw milk and milk filters

A further objective of the study was to characterize and compare the antibiotic resistance profiles of bacteria *S. aureus* isolated from the investigated samples. *S. aureus* strains were once nearly uniformly susceptible to semi-synthetic penicillinase-resistant β-lactams (e.g. methicillin, oxacillin), the most commonly used class of antibiotics for skin infection. These strains were termed methicillin resistant *Staphylococcus aureus*, or MRSA, a term that implied cross-resistance to all β-lactams including all penicillins and cephalosporins **(Larsen et al., 2000)**. *Staphylococcus aureus* has an impressive ability to adapt to environmental conditions and it can fast become resistant to almost all antibiotics **(McCallum et al., 2010)**. In the last few decades a large increase in bacterial dissemination of antibiotic resistance was reported, which becomes a major health challenge **(Goñi et al., 2004)**. The spread of antibiotic resistance among *S. aureus* strains is very important, especially in the treatment of staphylococcal infections **(Ito et al., 2003)**. Development of resistance to a particular antibiotic depends on the level of exposure to antimicrobial agents **(Rychlík et al., 2006)**.

Teicoplanin and vancomycin were the drugs to which a large proportion of the isolates from bulk tank milk and filters were sensitive (Figure 1).

As showed in Figure 1, the largest amount of *S. aureus* isolates obtained from milk filters were resistant to oxacillin, tetracycline, erythromycin, clindamycin, rifampicin and cefotaxime about 3.3%, followed by amoxicilin/clavulanic acid (1.6%). The largest amount of *S. aureus* isolates isolated from raw milk were resistant to amoxicillin / clavulanic acid about 11.3%, followed by oxacilin, tetracykline, erythromycin and cefotaxime (6.5%), clindamycin and rifampicin (3.3%) and vancomycin and teicoplanin (1.6%).

The antimicrobial resistance profile of the tested *S. aureus* strains to different antibacterial agents revealed that 17.8% (n = 11) of the strains were resistant to at least one antibiotic.

The antimicrobial resistance showed differences between the farms. These isolates had identical resistance patterns to oxacillin, tetracycline and cefotaxime. These results are comparable with studies by **Juhász-Kaszanyitzky et al. (2007)** where the MRSA isolates from cows were highly resistant to ampicillin, cephalexin, erythromycin and tetracycline and susceptible to enrofloxacin, gentamicin and potentiated sulphonamides. After positive findings of MRSA, repeated sample collections were carried out as described above.

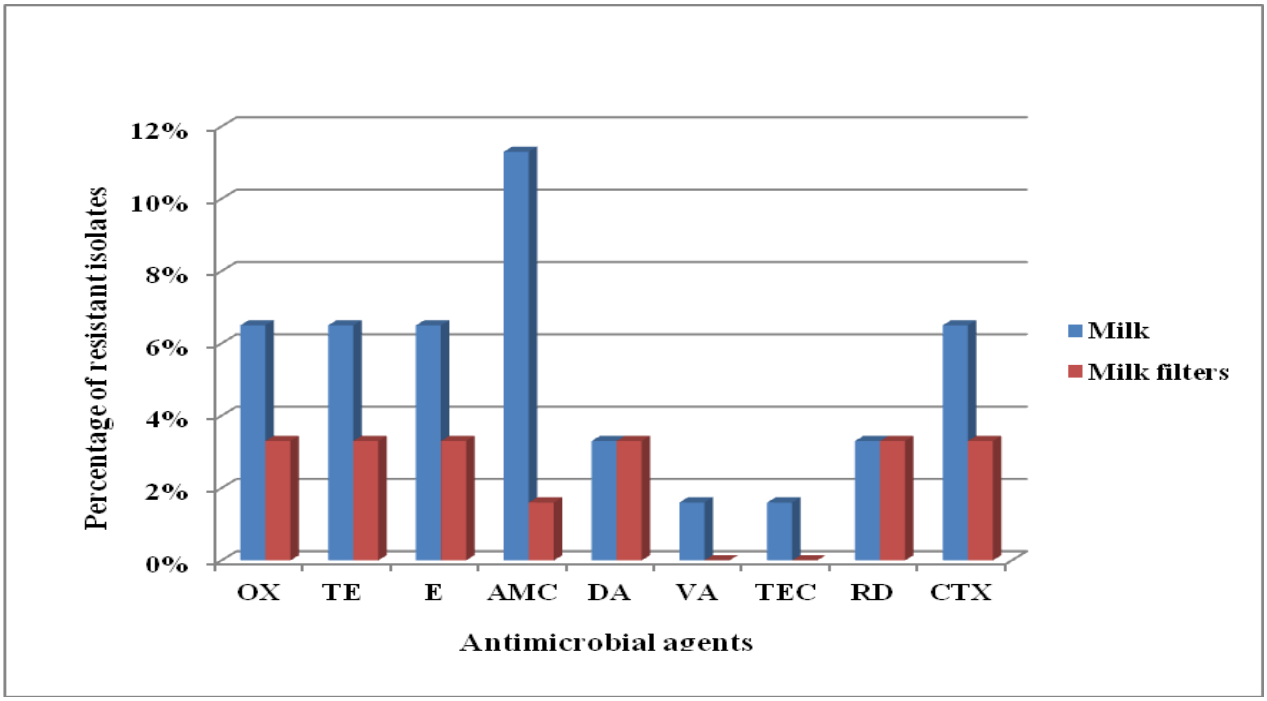

Figure 1 Antimicrobial resistance of *Staphylococcus aureus* in bulk tank milk and milk filters

Data of the *Staphylococcus aureus* resistance are shown in the Figure 1.

The altered transpeptidase called penicillin-binding protein (PBP2a) with low affinity for beta-lactams **(Ibrahem et al., 2005)** is determined by *mec*A gene encoding the resistance to oxacilin.

The emergence of resistant pathogens to commonly used antibiotics is worldwide fear of 21 century. One of the most important bacteria in this regard is *Staphylococcus aureus*, in particular methicillin-resistant strains. According to the results obtained by the PCR method for the methicillin - resistant *S. aureus* (MRSA), the *mec*A gene was present in 6 strains (9.7%), 4 isolates obtained from milk samples (6.5%) and 2 isolates from milk filters (3.2%). The detection of MRSA in milk samples was irregular. All the isolates from the study area were resistant to oxacillin and were also resistant to tetracycline, erythromycin, amoxicilin/clavulanic acid and cefotaxime. In other studies carried out in cows, MRSA were most frequently isolated from milk of animals showing signs of subclinical mastitis **(Lee, 2003)**.

In addition, from all samples that were positive for the presence of *mec*A gene, were collected from 4 farms at different localities. These results are comparable with studies made by **Karpíšková et al. (2008)**, where the prevalence of MRSA was monitored in ruminants. There were 9 MRSA isolates (2%) detected in 444 samples (including raw milk, environmental and animal samples) collected from 12 farms in the Czech Republic.

Čížek et al. (2008) in their study showed that from 45 isolates of *S. aureus* bacteria obtained from 192 milk filters collected from Moravian farms were none of MRSA strains.

According to our results and findings as far as the occurrence of MRSA strains it is concerned that these strains are presented in primary food production in the Czech Republic, but their frequency is still relatively low.

CONCLUSION

Results obtained in this study confirm the occurrence of *Staphylococcus aureus* in raw milk and milk filters. The occurrence of strains resistant to antimicrobials and MRSA in the environment of primary food production posses a potential risk of its spreading into the environment and colonization of personnels that are within the close contact with food during processing. MRSA strains appear rarely in the food of animal origin in the Czech Republic, however, there is a minimal potential risk that these strains can be transfered to humans via food chain.

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SENSORY QUALITY, COLOUR AND OXIDATIVE STABILITY OF CURED COOKED HAM WITH PROPOLIS EXTRACT

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ABSTRACT

The effect of 0.06% propolis ethanol extract on the sensory quality, colour and oxidative stability of cured cooked ham was evaluated. Half of the experimentally processed hams treated with 0.06% ethanolic extract of propolis with ascorbic acid (EEP), only with ascorbic acid (AA) and control samples (C) were sliced and vacuum packaged. Samples were kept at 4 °C 21 days (sliced) respectively 20, 50 and 100 days (unsliced). The results revealed that all samples were characterized without any significant colour discrepancies. In general, the thiobarbituric acid value (mg malondialdehyde/kg) increased gradually in all samples examined, with a significantly lower ($P < 0.05$) level for treated samples than for controls. The significantly lowest ($P < 0.05$) sensory parameters in comparison to unsliced hams were observed in sliced hams packaged in vacuum. Sliced hams with EEP were characterized with significantly lowest ($P < 0.05$) intensity of characteristic aroma. Undesirable taste was detected in control sliced hams after storage period. Significantly ($P < 0.05$) more desirable taste of sliced hams was observed in those with only ascorbic acid in comparison with EEP. In our study was demonstrated that 0.06% ethanol extract of propolis positive affected oxidation stability and not negative affected others technological (pH, colour) and sensory characteristics of poultry meat product - cured cooked ham.

Keywords: chicken meat; propolis extract; oxidation; colour; sensory quality

INTRODUCTION

High consumption of poultry meat leads to concern that the products marketed should be safe, have a low spoilage rate and show the right composition, packaging, colour, taste and appearance **(Rio et al., 2007)**. The oxidation of lipids in meat products is a key problem that reduces shelf life of frozen meats, fermented processed meat such as dry sausages, and cured raw ham **(Ladikos and Lougovois, 1990)**. A reduction of oxidation processes in meat and meat products can also come from the application of natural substances like propolis.

Propolis is an adhesive, dark yellow to brown coloured balsam that smells like resin. It is collected from buds, leaves and similar parts of trees and plants mixed with wax, sugar and plant exudates collected by bees from certain plant sources. More than 300 constituents have been identified in different propolis samples **(Valle, 2000; Banskota et al., 2001; Shalmany and Shivazad, 2006)**. Propolis is rich in biochemical constituents, including mostly a mixture of polyphenols, flavonoid aglycones, phenolic acid and their esters, phenolic aldehydes and ketones, terpenes, sterols, vitamins, amino acids etc. **(Walker and Crane, 1987)**. These components possess antimicrobial, antifugal and antioxidant properties **(Lu et al., 2005; Trusheva et al., 2006)**.

It is worth mentioning that propolis can be used as a water or ethanolic extract; both extracts can reduce the total volatile basic nitrogen content in meat products and can thus serve as a good preservative and contribute to promote human health, because they are produced naturally **(Han et al., 2001)**. However, water - extracted propolis has a weaker antibacterial, antioxidant and antifungal action than ethanolic extract **(Garedew et al., 2004)**. In recent years, propolis has been taken for health reasons but has had limited use in meat processing and food preservation **(Ali et al., 2010)**.

Our study was designed to evaluate the effect of propolis added to cured cooked ham. The sensory, colour and antioxidant stability of sliced and unsliced hams packaged in vacuum were determined after 21 days (sliced) resp. 20, 50 and 100 days (unsliced) after refrigerated storage at 4 °C.

MATERIAL AND METHODOLOGY

Preparation of propolis:

Propolis extract was prepared from minced propolis (50 g) in the conditions of the 96% ethanol in the 100 ml flask. After ten days of storage at room temperature the extract was filtered through Whatman no. 1. The resulting filtrate was evaporated and lyophilized. The ethanol and aqueous solutions as a solvent were utilized for resuspending and preparing 0.06% ethanol - water soluble propolis extract (EEP).

Preparation of cooked ham:

Chicken meat (12 kg of breasts and thighs) was minced with the 2 cm blade, cured (2.0% salt and 0.01% nitrite) and cooling 24 hours at 4 °C. The next day, minced meat was divided into three equal parts: non-treated meat was then tumbled with 10% of water (C), second part of meat was tumbled with 10% of water plus 0.5 g/kg ascorbic acid (AA) and third part was treated with 0.06% w/w ethanol-extracted propolis with 0.5 g/kg ascorbic acid

(EEP 0.06%). Each part were separately filled into polyamide casings and heat treated in water bath until the temperature in the core reached the value 70 °C for 10 min. Half of ham samples from each of the group were sliced and vacuum packaged. Samples were kept at 4 °C 21 days (sliced) respectively 20, 50 and 100 days (unsliced).

Determination of antioxidant activity:

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) test following the recommendations of **Grau et al. (2000)** and measured by spectrophotometric method at 532 nm (Jenway UV/VIS - 7305, UK). TBARS values were calculated from a standard curve of malondialdehyde (MDA) and expressed as mg MDA/kg sample.

Determination of pH value:

The pH value of cooked hams was measured using a Gryf 209 (Gryf HB, Czech Republic) apparatus during whole period of storage.

Determination of colour:

Colour spaces L*, a*, b* of cooked hams were determined by CM 2600D spectrophotometer (Konica Minolta, Germany) after homogenization according to **Hunt and Manciny (2002)**. Colour on the surface of homogenized hams was measured with SCE (Specular Component Excluded).

Sensory evaluation of cooked hams:

Samples of cooked hams were evaluated by a 6 member semi-trained panel of laboratory co-workers. Panelists evaluated, colour, aroma, juiciness and taste on 8 point hedonic scale where 1 (the worst) and 8 (the best) were the extremes of each characteristic.

RESULTS AND DISCUSSION

The pH value of unsliced hams of experimental and control groups fluctuated from 6.02 to 6.13. Significantly higher values of pH ($P < 0.05$) were determined in sliced hams (6.17 to 6.23). Final pH value of hams was near to pH values of both chicken thighs and chicken breasts. These values correspond with ultimate values of pH determined in chicken muscles by **Šulcerová et al. (2011)**. In both groups (sliced and unsliced) were not detected significant differences of pH value between experimental and control hams, so propolis had no negative effect to cooked ham acidity.

All samples were characterized without any significant colour discrepancies. It was found that propolis in combination with ascorbic acid not significantly improve intensity of red colour in sliced hams after 21 days of storage. Intensity of red colour (a*) in unsliced hams decreased ($P > 0.05$) but lightness (L*) was improved after 50 and 100 days of storage. Intensity of yellow colour (b*) was the highest in unsliced hams with ascorbic acid, but differences during storage were not significant (Figure 1).

Lipid oxidation is one of the main limiting factors for the quality and acceptability of this type of ham. The antioxidant activity has been measured in the past using a TBA assay in model meat systems with addition of essential oils, showing a potential for protecting meat from oxidation **(Ruberto and Baratta, 1999)**. The rate and extent of oxidative deterioration can be reduced by various means, such as curing to preserve the meat tissues, vacuum packaging to remove the oxygen source, or adding of antioxidants to scavenge the oxidants **(Wong et al., 1995)**.

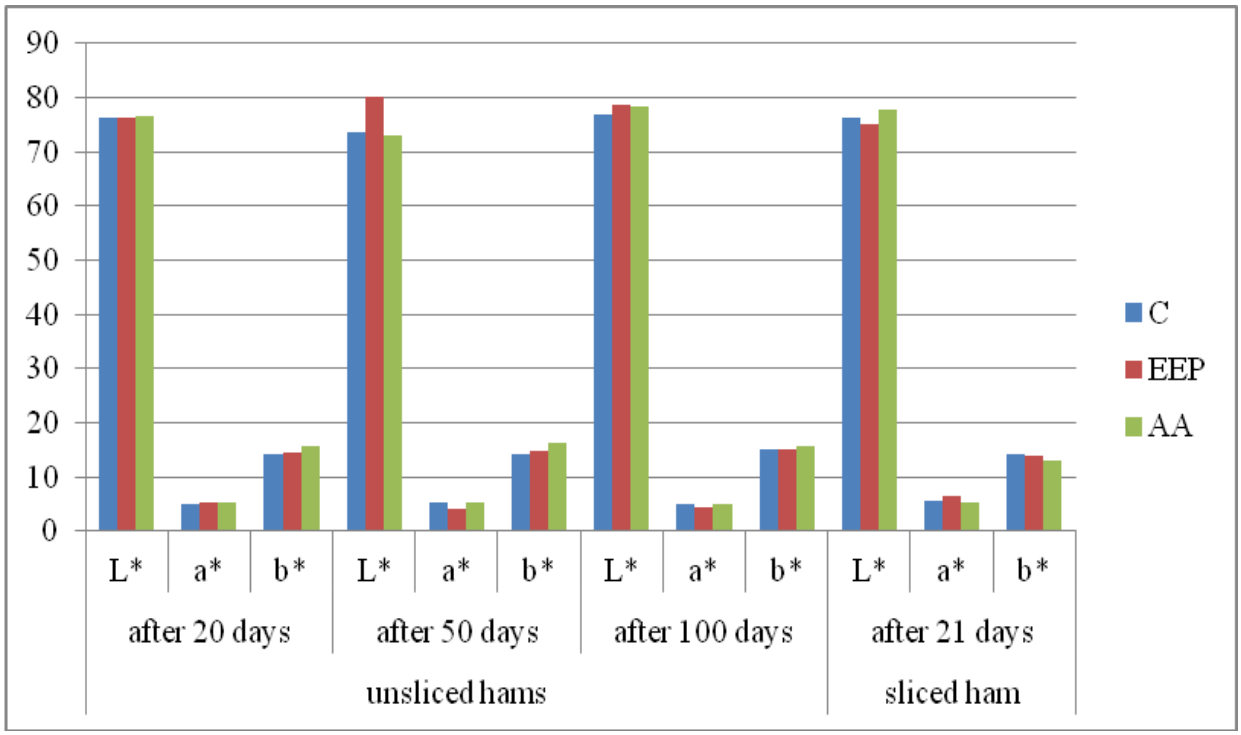

Figure 1 Effect of propolis extract on the ham colour during chilling storage (4°C)

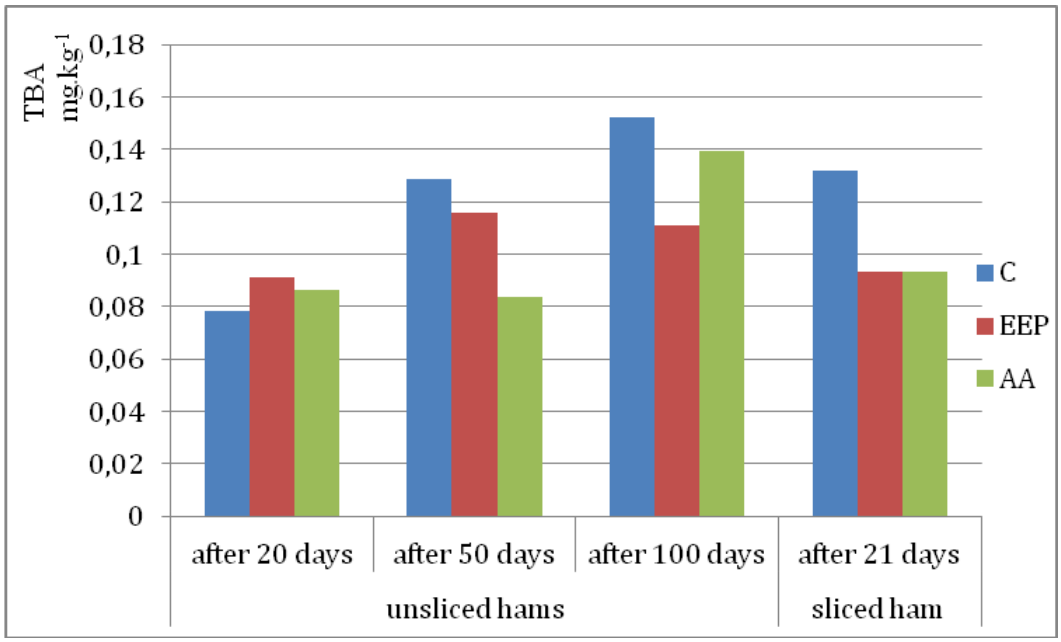

Figure 2 Effect of propolis extract on the amount of malondialdehyde during chilling storage (4°C)

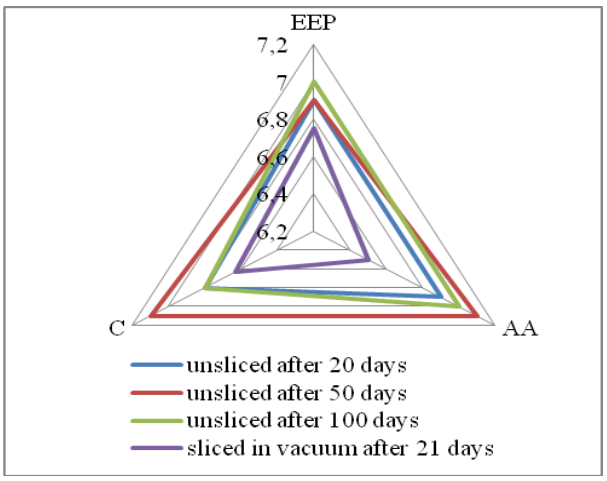

Figure 3 Effect of propolis on ham colour determined by sensory evaluation

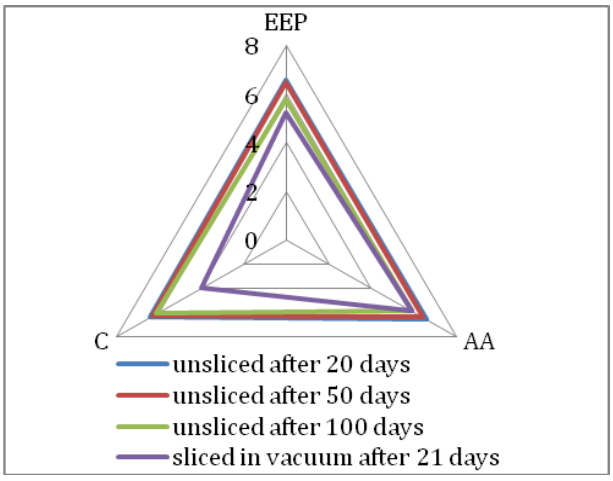

Figure 5 Effect of propolis on ham taste determined by sensory evaluation

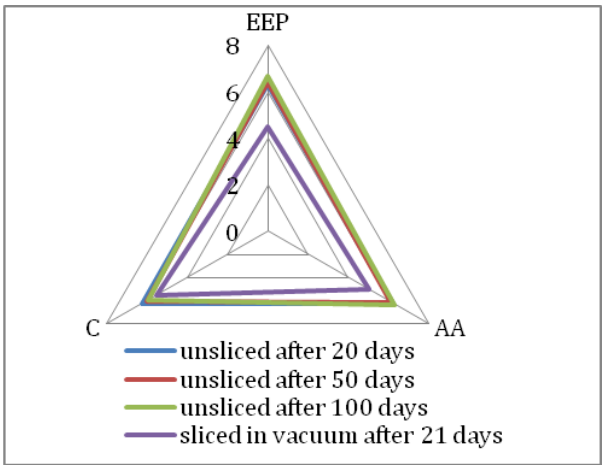

Figure 4 Effect of propolis on ham aroma determined by sensory evaluation

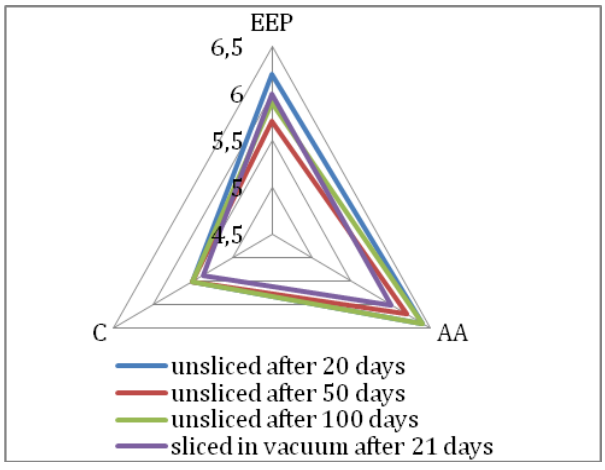

Figure 6 Effect of propolis on ham juiciness determined by sensory evaluation

The application of antioxidants is one of the simplest ways of reducing lipid oxidation. Antioxidants minimise lipid peroxidation, act as oxygen scavengers, react with free radicals and chelate catalytic metals and consequently retard oxidative deterioration **(Shahidi and Wanasundara, 1992)**.

It was found that the TBA value increase during storage in unsliced and also in sliced hams mainly in control group (Figure 2). In the unsliced hams with ascorbic acid addition was determined higher oxidative stability than in the hams with the addition of EEP. However, the highest oxidation stability in unsliced hams was found after 100 days of storage in hams with EEP. In sliced vacuum packaged hams significantly lower ($P < 0.05$) TBA values were found in hams treated with EEP and AA in compare to control. In comparison with unsliced hams, after 20 days higher amount of TBA was found in sliced hams packaged in vacuum. The strong antioxidative and antibacterial activity of honey, propolis, pollen and royal jelly after to different kind of meat addition confirmed the work of **Koo et al. (2000)**.

Propolis has a very characteristic and strong odour; the addition of its extract in formulations could confer its color and particularly its odour, to the product, and affect the acceptance of the product by the consumer **(Gonçalves et al., 2011)**. However, **Yang et al. (2010)** reported that concentrations of aroma-active components in propolis were closely related to the regions of propolis origin and components with high concentrations did not always play important roles in odour contribution.

Storage period had not significant effect ($P > 0.05$) on colour and aroma sensory parameters of unsliced hams (Figure 3 and 4,). Intensity of typical taste (Figure 5) of unsliced hams with EEP not significantly decreased after 100 days of storage. Significantly increased ($P < 0.05$) juiciness (Figure 6) was observed in ham only with ascorbic acid in compare with EEP and C. However, overall acceptability of unsliced hams with EEP during 100 days of storage was not changed. The significantly lowest ($P < 0.05$) sensory parameters in comparison to unsliced hams were observed in sliced hams packaged in vacuum. Sliced hams with EEP were characterized with significantly lowest ($P < 0.05$) intensity of characteristic aroma.

Undesirable taste was detected in control sliced hams. Significantly ($P < 0.05$) more desirable taste of sliced hams was observed in those with only ascorbic acid in comparison with EEP.

CONCLUSION

Addition of 0.06% propolis extract not substitute addition of ascorbic acid, but the results clearly confirm that the quality of the hams with the addition of EEP is due to their oxidative stability, color and sensory parameters significantly higher than without the addition of antioxidants. Also, the additions of natural antioxidant - propolis in this concentration to the hams enrich the food chain of human with natural flavonoids and polyphenols.

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BONE ADAPTATION TO SIMULTANEOUS CADMIUM AND DIAZINON TOXICITY IN ADULT MALE RATS

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ABSTRACT

Food contamination from natural or anthropogenic sources poses severe risks to health of human and animals. Bone is a metabolically active organ, which can be affected by various toxic substances, such as cadmium (Cd) and diazinon (DZN), leading to disruption in bone metabolic processes. The present study was designed to investigate the effect of simultaneous peroral administration to Cd and DZN on femoral compact bone structure in adult male rats. A total of twenty 1-month-old male Wistar rats were randomized into two experimental groups. In the first group (EG), young males were dosed with a combination of 30 mg $CdCl_2$/L and 40 mg DZN/L in drinking water, for 90 days. Ten 1-month-old males without Cd-DZN intoxication served as a control group (CG). After 90 days of daily peroral exposure, evaluations of femoral bone macro- and micro-structure were performed in each group. We found no significant differences in body weight, femoral weight, femoral length and cortical bone thickness between both groups (EG and CG). However, rats from the group EG displayed different microstructure in the middle part of the *substantia compacta* where primary vascular radial bone tissue appeared. In some cases, vascular expansion was so enormous that canals were also present near the periost. On the other hand, they occurred only near endosteal surfaces in rats from the control group. Moreover, a smaller number of primary and secondary osteons was identified in Cd-DZN-exposed rats. This fact signalizes reduced mechanical properties of their bones. Anyway, our results suggest an adaptive response of compact bone tissue to Cd-DZN-induced toxicity in adult male rats in order to prevent osteonecrosis.

Keywords: bone; osteotoxicology; cadmium; diazinon; rats

INTRODUCTION

Most foods contain natural or synthetic chemicals that could represent a toxic hazard for the consumers (**Nassredine and Parent-Massin, 2002**).

Cadmium (Cd) is a heavy metal that is widely present in the environment as pollutant **(Moulis and Thévenod, 2010)**. It still attracts the attention of researchers and the public because its level in food products often exceeds the maximum allowable limits **(Toman et al., 2011)**. According to **Järup and Akesson (2009)**, the diet is the major source (~ 99%) of Cd exposure in the general non-smoking population. Upon absorbed, Cd irreversibly accumulates in the human body, in particularly in kidneys and other vital organs such as lungs and liver **(Bernard, 2008)**. In addition to its cumulative properties, it is also highly toxic metal which can disrupt a number of biological systems, usually at doses that are much lower than most toxic metals **(Järup et al., 1998)**. One of the target organs for Cd toxicity is also bone **(WHO, 1992)**. Exposure to Cd has been linked to bone loss, low bone mass and osteoporosis, and even to an increased incidence of bone fractures **(Wilson et al., 1996; Wang et al., 2003)**. The results obtained by **Brzóska and Moniuszko-Jakoniuk (2005a)** have shown that chronic, even low-level exposure to Cd disturbs bone metabolism during skeletal development and maturity by affecting bone turnover most probably through a direct influence on bone formation and resorption, and indirectly via disorders in Ca metabolism. Besides interfering with Ca, Cd also alters the metabolism of other metals essential for bone health, mainly zinc (Zn), iron (Fe) and copper (Cu); **Noël (et al., 2004)**.

Diazinon (DZN) is a contact organophosphate (OP) pesticide, which is extensively used in agriculture **(Salehi et al., 2009)**. Like other organophosphates (OPs), the main toxic action of DZN is inhibition of acetylcholinesterase (AChE) activity, which results in accumulation of acetylcholine (ACh) and associated neurotoxicity **(Oruc and Usta, 2007)**. Diazinon toxicity varies widely within and among species, and is modified by organism age, sex, body size, climatic conditions, pesticide formulation, chemistry of the environment, and other factors **(Montz, 1983)**. According to **Garg et al. (2004)**, a potential target of pesticide toxicity is the skeletal system. Marked impairment in the development of the backbone in ducklings due to OPs toxicity has been observed in the study by **Ludle et al. (1979)**. Higher amounts of DZN caused additional defects in quail and chicken including folding of the spinal cord, shortening of the neck **(Wyttenbach and Hwang, 1984)**, fusing and twisting of vertebrae, abnormal development of ribs and breastbone **(Meneely and Wyttenbach, 1989)**, curled claws, reduced

growth of leg and wing bones **(Cho and Lee, 1990)**, and reduced bone calcification **(Cho and Lee, 1991)**. In addition, OPs cause a significant reduction in bone mass and density in individuals following chronic low-level intoxication **(Compston et al., 1999)**. Results by **Lari et al. (2011)** indicate that DZN exposure is associated with decrease in trabecular and cortical bone density and might be one of the causes for worldwide increasing prevalence of osteoporosis.

The aim of the current study was to investigate the effect of co-administration to Cd and DZN as food contaminants on femoral bone structure in adult male rats.

MATERIAL AND METHODOLOGY

Our experiment was conducted on twenty 1-month-old male Wistar rats obtained from the accredited experimental laboratory (number SK PC 50004) of the Slovak University of Agriculture in Nitra. The animals were housed individually in plastic cages under constant temperature (20-22 °C), humidity (55 ±10%), and 12/12 h cycle of light and darkness with the provision of food (feed mixture M3, Machal, Bonargo, Czech Republic) and water *ad libitum*. Clinically healthy rats (free of typical rodent pathogens) were randomly divided into two groups, of 10 animals each. In the first group (EG), young males were dosed with a daily intake of 30 mg $CdCl_2$/L in combination with 40 mg DZN/L in drinking water for 90 days. The second group without Cd and DZN supplementation served as a control (group CG). The water consumption was daily monitored during the whole experiment. The xenobiotics used in our experiment were chosen on the basis of their possible occurrence in the human and animal food (**Toman et al., 2011**). Indeed, correlation coefficients found between Cd and DZN in men (0.70) and women (0.69) indicate high probability of exposure to both compounds (**Toman et al., 2012**). The doses of Cd and DZN (chosen based on studied literature and our previous experiments with tested dose-response effects) were high enough to reach a toxicity level but also safe enough to prevent animal mortality (non-lethal doses). All procedures were approved by the Animal Experimental Committee of the Slovak Republic. At the end of the experiment, all animals were killed, weighed, and both their femurs were used for macroscopic and microscopic analyses. After cleaning all soft tissues, right femurs were weighed on analytical scales with an accuracy of 0.01g and the length was measured with a sliding instrument. The unpaired Student′ s T-test was used for establishing statistical significance between both experimental groups. The significance level was accepted at $P < 0.05$. For histological investigation, each right femur was sectioned at the midshaft of its diaphysis. The obtained segments were placed in HistoChoice fixative (Amresco, USA). Specimens were then dehydrated in ascending grades of ethanol and embedded in epoxy resin Biodur (Günter von Hagens, Heidelberg, Germany) according to **Martiniaková et al. (2007)**. Transverse thin sections (70-80 μm) were prepared with a sawing microtome (Leitz 1600, Leica, Wetzlar, Germany) and affixed to glass slides by Eukitt (Merck, Darmstadt, Germany) as previously described (**Martiniaková et al., 2008**). The qualitative histological characteristics of the compact bone were determined according to the internationally accepted classification systems of **Enlow and Brown (1956)** and **Ricqlés et al. (1991)**, who classified bone into three main categories: primary vascular tissue, non-vascular tissue and Haversian bone tissue. Various patterns of vascularization can occur in primary vascular bone: longitudinal, radial, reticular, plexiform, laminar, lepidosteoid, acellular, fibriform and protohaversian. There are three subcategories identified in Haversian bone tissue: irregular, endosteal and dense.

RESULTS

Body weight, femoral weight, femoral length and cortical bone thickness did not differ significantly between both experimental groups (Tab. 1).

Femoral diaphyses of rats from the control group had the following microstructure in common. The internal layer surrounding the medullary cavity (i.e. endosteal border) was formed by non-vascular bone tissue in all views of the thin sections. The bone tissue contained cellular lamellae and osteocytes. Primary and/or secondary osteons were not present. Additionally, there were also identified some areas of primary vascular radial bone tissue in anterior, posterior and lateral views. This type of bone tissue was created by branching or non-branching vascular canals radiating from the bone marrow cavity. Some primary and secondary osteons were also found especially in the anterior and posterior views near the endosteal surfaces.

Table 1 Body weight, femoral weight, femoral length and cortical bone thickness in adult male rats co-administered to 30 mg $CdCl_2$/L and 40 mg DZN/L in drinking water (group EG) and control rats (group CG)

Rat's group	N	Body weight (g)	Femoral weight (g)	Femoral length (cm)	Cortical bone thickness (mm)
EG	10	427.78±19.22	1.03±0.07	3.98±0.09	0.573±0.066
CG	10	405±52.65	1.05±0.17	3.94±0.09	0.575±0.048
T-test		NS	NS	NS	NS

N: number of rats, NS: non-significant changes

In the middle part of the compact bone, a few primary and secondary osteons were identified.

However, dense Haversian bone tissue characterized by dense concentration of secondary osteons was not observed. Finally, the periosteal border of analysed bones was again composed of non-vascular bone tissue, mainly in the anterior and posterior views (Fig. 1).

The rats simultaneously exposed to Cd and DZN displayed a similar microstructure to rats from the control group, except for the middle part of the compact bone where primary vascular radial bone tissue was observed. Vascular canals expanded into the central area of the bones from endosteal surfaces. The canal expansion was in some cases so enormous that the canals also occurred near periosteal surfaces. As a result of this process, a smaller number of primary and secondary osteons was identified in the Cd-DZN-intoxicated rats (Fig. 2). But no clinical manifestations of osteoporosis (i.e. resorption lacunae or osteoporotic fractures) were revealed in these rats.

DISCUSSION

Our results showed non-significant effect of simultaneous peroral application of Cd and DZN on body weight, femoral weight and femoral length in adult male rats. Correspondingly, no demonstrable changes in body weight **(Brzóska and Moniuszko-Jakoniuk, 2005b; 2008; Martiniaková et al., 2013)** and femoral length **(Brzóska et al., 2007; 2008; 2010; Martiniaková et al., 2013)** have also been reported in adult male rats after their peroral exposure to 5, 30 or 50 mg Cd/L in drinking water. Also, subchronic intoxication with a sole dose of DZN (the same level as it was used in our study) did not induce significant alterations in body weight of rats **(Cabaj, 2012)**. Weight of femoral bone in rats perorally receiving mixture of Cd and DZN were similar to those from the control group; however, in our previous study **(Martiniaková et al., 2013)** we have found that Cd administered in single dose had a positive impact on femoral weight in adult male rats. On the basis of these findings we can conclude that beneficial effect of Cd on femoral weight in adult male rats is in interaction with DZN suppressed.

The thickness of cortical bone is generally accepted as an important parameter in the assessment of cortical bone quality and strength. According to **Garn et al. (1991)**, cortical thickness of femoral shaft is a good measuring site for evaluation of bone mass. Our research demonstrates no significant differences in cortical bone thickness between rats co-administered to Cd and DZN, and those of the control group. In the study by **Comelekoglu et al. (2007)**, cortical thickness in the femoral diaphysis was also unchanged in adult female rats after common low intraperitoneal administration of Cd for 18 weeks. On the other hand, cortical bone thickness in rats from our control group was higher in comparison with the value published by **Comelekoglu et al. (2007)** for 4 month-old female rats (0.45 ±0.0057 mm). This discrepancy may be influenced by the different gender and strain of the animals in the two experiments. It is well known that earlier completion of longitudinal growth and earlier inhibition of periosteal apposition produces a smaller bone in females **(Seeman, 2008)**.

The results of the qualitative histological analysis of femurs from the control rats correspond with previous works **(Enlow and Brown, 1958; Martiniaková et al., 2005; Reim et al., 2008; Martiniaková et al., 2009)**. The basic structural pattern of compact bone tissue was non-vascular. In addition, there were some areas of primary vascular radial and/or irregular Haversian bone tissues. However, there was no evidence of true Haversian intracortical bone remodeling. It is generally known that aged rats and mice lack true Haversian cortical bone remodeling but not cancellous bone remodeling **(Erben et**

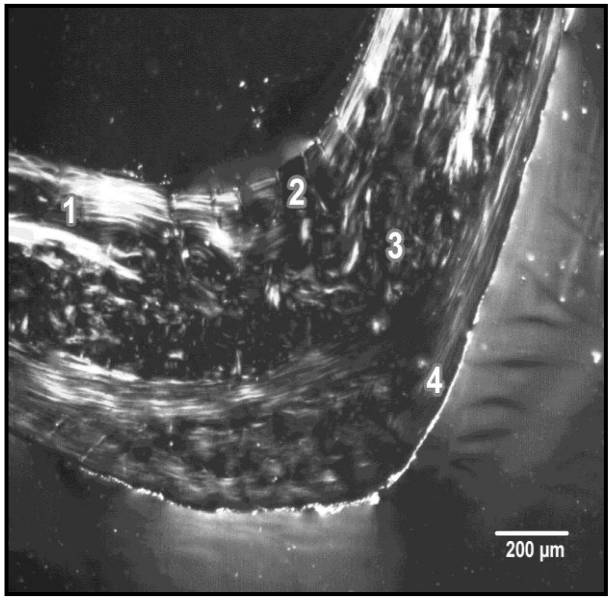

Fig. 1 Microscopic structure of compact bone in rats from the control group:
1 non-vascular bone tissue; 2 vascular canals radiating from marrow cavity; 3 primary and secondary osteons in middle part of compact bone; 4 non-vascular bone tissue.

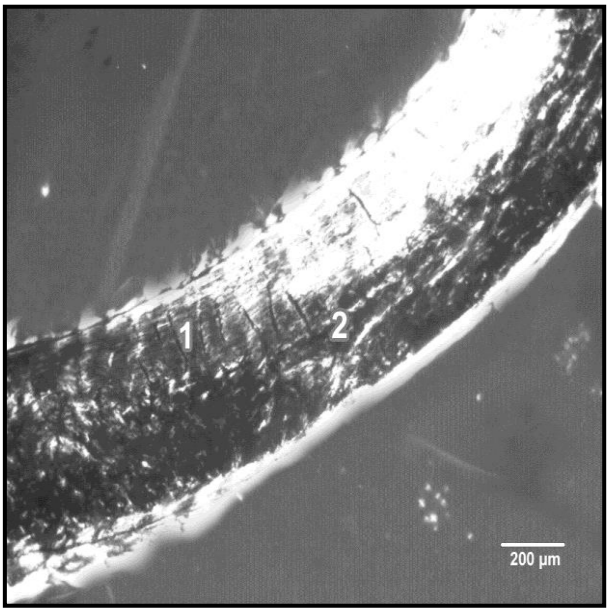

Fig. 2 Microscopic structure of compact bone in Cd-DZN-exposed rats:
1 enormous vascular canals radiating from marrow cavity; 2 smaller number of primary and secondary osteons in middle part of compact bone.

al., 1996; **Reim et al., 2008)**. Therefore, some secondary osteons can be observed in the long bones (near the endosteal border). In our study, the newly formed remodeling units within compact bone originated from the endocortical surface and extended deep into the underlying compact bone. The same findings have also been documented in the study of **Reim et al. (2008)** in 13 month-old male rats.

Prolonged intake of Cd and DZN mixture resulted in induction of demonstrable changes in the middle part of compact bone where vascular canals expanded from endosteal border and led to the formation of primary vascular radial bone tissue. In some cases, vascular canals were also present near periosteal surfaces. The final result of this process was a smaller number of primary and secondary osteons indicating the reduced bone mechanical properties. In general, bone is dynamic tissue that is continuously remodeled to remove microfractures, to adapt to changing mechanical strains and metabolic demands **(Hofstetter, 2007; Chen et al., 2009)**. Disappearance of the Haversian canal system, which was replaced by a large quantity of degenerated, necrotic, and restorative tissues have been demonstrated in the study by **Li et al. (1997)** for ovariectomized rats after a long-term Cd administration. Also, Cd-induced apoptosis of bone cells was documented in many studies **(Coonse et al., 2007; Lévesque et al., 2008; Chen et al., 2009; Smith et al., 2009; Arbon et al., 2012; Brama et al., 2012)**. Furthermore, decreased number of active osteons in broiler chicks was found after exposure to OP pesticides **(Garg et al., 2004)**. In general, DZN exerts its toxicity through inhibition of AChE. According to **Genever et al. (1999)** and **Inkson et al. (2004)**, this enzyme is also expressed by osteoblasts suggesting a role for AChE (i.e. bone matrix protein) in bone tissue. Thus, the expression of high levels of AChE in bone-forming osteoblasts and their progenitors supports a toxic effect of AChE inhibitors (including DZN) on these cells **(Genever et al., 1999; Grisaru et al., 1999; Inkson et al., 2004; Hoogduijn et al., 2006)**. Based on all mentioned aspects we propose that the formation of primary vascular bone tissue, mainly in the central area of the femur, could be explained as an adaptive response of bone to Cd-DZN toxicity, in order to protect the tissue against death of cells and subsequent necrosis. Interestingly, changes in qualitative histological characteristics of compact bone tissue were in these rats less pronounced than in those exposed to only Cd in sole dose **(Martiniaková et al., 2013)**. Indeed, Cd-intoxicated rats displayed a presence of resorption lacunae near endosteal surfaces indicating an early stage of osteoporosis while in rats co-intoxicated with Cd and DZN these structures were absent. This finding suggests that the significant effect of Cd on rat bone microstructure is in combination with DZN partially eliminated. We speculate that the fact could be associated with a molecule of AChE expressing also in bone tissue. According to **Compston et al. (1999)**, this molecule may have a role in the regulation of cell-matrix interactions and in the coupling of bone resorption to bone formation. In addition, the demonstration of Cbfa - 1 and other osteogenic factor-binding motifs on the AChE gene promoter offers further support for a physiological role of AChE in bone formation **(Grisaru et al., 1999)**. Hence, the presence of AChE in bone matrix provides a possible mechanism for OP-induced effects in bone **(Genever et al, 1999)**. The fact was confirmed in the study by **Compston et al. (1999)**, who have found that chronic OP exposure significantly decreased bone formation in agricultural workers. Reduced bone mineral density (BMD) was also observed in rats after application of 15 and 30 mg DZN /kg for 4 weeks **(Lari et al., 2011)**. In respect to all mentioned findings and available scientific papers we propose that absence of resorption lacunae in Cd-DZN-intoxicated rats can be attributed to an opposite impact of Cd and DZN on AChE activity. Indeed, there is evidence that while DZN inhibits AChE, Cd is able to stimulate it. The experiment by **Carageorgiou et al. (2004)** has shown that effect of Cd on brain AChE activity in rats is dose- and exposure duration-dependent. Results revealed that short-term treatment of rats with Cd induces a dose-dependent decrease of brain AChE activity, while long-term Cd administration stimulates it. In accordance with this finding, **Toman et al. (2012)** observed decreasing in AChE activity in rats 36 hours exposed to Cd. On the other hand, Cd administered in drinking water for 3 weeks led to considerable increased the enzyme function in rats **(Srinivasan and Ramprasath, 2011)**. Therefore, we consider that opposite (competitive) effect of Cd and DZN on AChE activity resulted in elimination of DZN adverse impact on function of AChE in bone metabolism that was in microstructural level reflected by absence of resorption lacunae in Cd-DZN treated rats.

CONCLUSION

Our study demonstrates that simultaneous peroral exposure to Cd and DZN did not influence macroscopic structure of femoral bone in adult male rats. However, it has significant impact on bone microstructure in these animals. Co-administration to Cd and DZN affected mainly the middle part of rats' bones where primary vascular radial bone tissue was identified as a result of adaptive response to xenobiotic-induced osteonecrosis. On the other hand, the vascular canal expansion into central area of *substantia compacta* led to a smaller number of primary and secondary osteons signalizing weakened mechanical properties of the bones. Moreover, our study showed that Cd in combination with DZN had less expressive effect on bone microstructure in male rats than Cd in a sole dose.

Food chain contamination is one of the important pathways for the entry of toxic pollutants such as Cd and DZN into the human or animal body. Therefore, monitoring of xenobiotic presence in foods is the first step to prevent their toxic effects on human health including bones.

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MICROBIOLOGICAL QUALITY OF EGG LIQUID PRODUCTS

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ABSTRACT

Egg liquid products are most commonly used as semi-products in different branches of food industry and catering industry. The main goal of this work was to assess the microbiological quality of egg liquid products (liquid pasteurized egg white, pasteurized egg yolk with sugar 33%, pasteurized whole egg-blend) sampled in a period from April until February, and evaluate whether parameters such as the total aerobic count (TAC), coliform bacteria (KFB), enterococci, staphylococci, moulds and yeast changed significantly during this period of time. Microbiological analysis detected the highest ($P < 0.05$) total aerobic count in egg yolk (2.8 log CFU.ml^{-1}). The incidence of coliform bacteria was also highest ($P < 0.05$) in egg yolk (1.7 log CFU.ml^{-1}) but very low in egg white and whole egg. The highest count ($P < 0.05$) of enterococci was detected in samples of egg white (1.0 log CFU.ml^{-1}). The counts of staphylococci, moulds and yeast were particularly high ($P < 0.05$) in egg yolk (1.2 log CFU.ml^{-1} for staphylococci and 1.1 log CFU.ml^{-1} for moulds and yeast). Statistical analysis showed ($P > 0.05$) no correlation between respective seasons and microbial counts for any of the egg substances investigated. It follows from our results that all analysed egg products comply with the TAC limit defined by Council Directive (EC) 89/437/EEC on hygiene and healthy problems affecting the production of egg products, however, all egg products analysed in our study would fail to meet the required criteria for other groups of microorganisms.

Keywords: egg liquid substances; total aerobic count; coliform bacteria; enterococci; staphylococci; moulds and yeast

INTRODUCTION

Demand for shell eggs has recently declined whereas egg liquid products have attracted increasing interest in food industry. Egg liquid products are most commonly used as semi-products in different branches of food industry and catering industry **(Simeonovová, 2013)** since they provide a rich source of high-quality proteins, vitamins and trace elements **(Patrignani et al., 2013; Rossi et al., 2010)**. The term 'egg products' means liquid whole eggs (blend), egg whites and enriched sweetened or salted egg yolks or blends **(Chmielevski et al., 2013)**. Only fresh, refrigerated, preserved, sorted or unsorted eggs are subjected to egg breaking and processing. Such eggs may not comply with the requirements for size and shape specified for the shell eggs intended for consumers but they are wholesome and of good quality **(Steinhauserová and Simeonovová, 2003)**. Microbial contamination on the shell eggs is one of the major factors to indicate egg quality, affecting the level of exogenous microbial contamination in the egg contents. It poses a fundamental problem in the production of eggs intended for consumers **(Dev et al., 2013; Englmaierová and Tůmová, 2007)**, particularly with regard to the total aerobic count and contamination with Gram-negative bacteria from the family *Enterobacteriaceae* **(Németh et al., 2011; Reu et al., 2005)**. Egg liquid products are responsible for a high number of foodborne illnesses **(Latimer et al., 2008)** every year. Psychrophilic microorganisms and moulds are most common species to grow on the contents of the frozen eggs. The growth of moulds arises mainly due to insufficient air exchange and high humidity. The most common moulds detected in an egg include *Cladosporium herbarum, Penicillium cyclopium, Alternaria humicola, Trichoderma viride* and *Aspergillus niger*. The optimum temperature for the growth of these species varies in a range of 18 – 28 °C, with relative humidity being at least 80% **(Hejlová, 2001)**.

MATERIAL AND METHODOLOGY

Analysis was performed with the samples of egg liquid products used for the production of baked goods and pastry in a bakery located in the surroundings of Brno. This included liquid pasteurized egg white, pasteurized egg yolk with sugar 33% and pasteurized whole egg. Respective samples of egg liquid products were transported in sterile bottles in a cool box into the laboratory. 3 representative samples from each egg substance were collected from a package unit (5 kg). Analyses were performed in the microbiological laboratory of the Institute for Food Technology, MENDELU in Brno, during one year (April – February). A total of 24 samples were analysed from each egg liquid products (overall 72 samples). The samples were storing at temperature 5°C and relative humidity of 70 – 80% by recommendation of producer.

Parameters such as the total aerobic count (TAC), coliform bacteria (KFB), coagulase-positive staphylococci, enterococci and moulds and yeast were determined in all samples.

The following microbiological parameters were determined in egg products:

The total aerobic count (TAC). Culture on the growth medium Plate Count Agar (PCA, NOACK, France) according to CSN EN ISO 4833 at 30°C for 72 hours.

Coliform bacteria. Culture on the growth medium Violet Red Agar (VRBL, NOACK, France) according to CSN ISO 4832 at 37°C for 24 - 48 hours.

Coagulase-positive staphylococci: Culture on Baird-Parker Agar (NOACK, France) according to CSN EN ISO 6888, incubation at 37°C for 24 – 48 hrs. Confirmation was performed by adding rabbit fibrinogen (Fibrinogen Plasma Trypsin Inhibitor Supplement, Oxoid).

Enterococci. Culture on the growth medium Compass-enterococcus agar **(**NOACK, France) at 37°C for 24 – 48 hrs.

Moulds and yeast. Culture on the growth medium Chloramphenicol glucose agar (GKCH) according to CSN ISO 21527-1 at 25°C for 5 days.

Samples were collected and processed according to CSN ISO 7218 and CSN EN ISO 6887-1.

The following methods were used for statistical evaluation: the calculation of basic statistical parameters (mean, standard deviation, standard deviation of the mean) and the simple sorting method of analysis of variance. Evaluation was performed using the programme STATISTICA CZ, version 9.

RESULTS AND DISCUSSION

Liquid pasteurized egg white (Fig. 1)

The highest value of the total aerobic count was found for Sampling 4 (October) 3.1 log CFU.ml^{-1} whereas the lowest value (1.6 log CFU.ml^{-1}) was detected in November (Sampling 5).

Coliform bacteria were detected only once (in October, 1.3 log CFU.ml^{-1}). Coagulase-positive staphylococci were found in egg white in two samplings (June and October) 2.5 log CFU.ml^{-1}. Enterococci were detected at four occasions, the highest count was found in May (2.8 log CFU.ml^{-1}), whereas in April, November, January, and February no enterococci were detected in pasteurized egg white. Moulds and yeast were detected in pasteurized white twice, in June and October. The highest value detected was 1.3 log CFU.ml^{-1}. No yeast and moulds were detected in other samplings.

Pasteurized egg yolk with sugar 33% (Fig. 2)

The highest value of the total aerobic count was found in pasteurized egg yolk with sugar 33% in June 4.3 log CFU.ml^{-1}, whereas in January, no colonies on dishes were recorded.

The highest count of coliform bacteria was found in October (4.1 log CFU.ml^{-1}). Three samplings show no coliform bacteria at all. The highest value of coagulase-positive staphylococci was found in February (3.5 log CFU.g^{-1}). Findings at three other samplings were negative. Enterococci, resp. moulds and yeast were detected in samples three, resp. four times with the highest count being found in May (2.7 log CFU.ml^{-1} and 3.1 log CFU.ml^{-1}, respectively).

Pasteurized whole egg (Fig. 3)

The highest value of TAC in pasteurized whole egg was detected in October (2.8 log CFU.ml^{-1}), whereas in May (Sampling 2) and January (Sampling 7) the TAC was < 10. Coliform bacteria were only found at Sampling 1 (April), 1.0 log CFU.ml^{-1}. Coagulase-positive staphylococci were detected in June, October and December; with the highest value being 2.3 log CFU.ml^{-1}. The highest value for enterococci was 1.5 log CFU.ml^{-1}. No enterococci were present in pasteurized whole egg blend in five sampling events. Moulds and yeast were only detected in January at an amount of 1 log CFU.ml^{-1}.

It follows from the comparison of microbial quality of all three egg liquid products that the highest ($P < 0.05$) levels of the total aerobic count, coliform bacteria, coagulase-positive staphylococci and moulds and yeast were found in pasteurized egg yolk with sugar 33% (n=24). The highest ($P < 0.05$) count of enterococci was found in egg white (1.0 log CFU.ml^{-1}; n=24). Statistical evaluation showed no correlation ($P > 0.05$) between the period of sampling and the count of particular microorganisms, for any of the egg liquid products.

Regulation (EC) 2073 (2005) on microbiological criteria

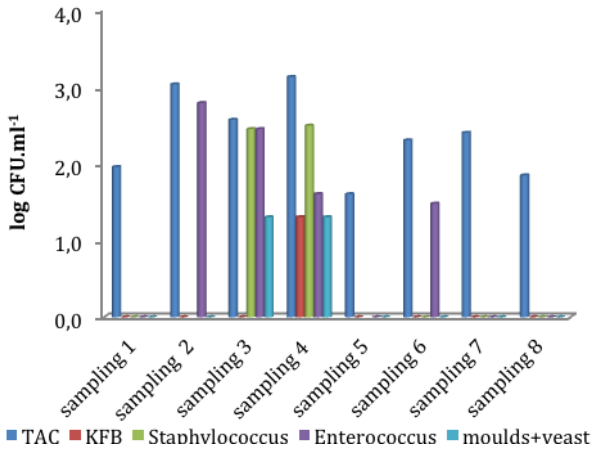

Fig. 1 The count of microorganisms (log CFU.ml^{-1}) in samples of liquid pasteurized egg white collected in 8 time points (April - February)

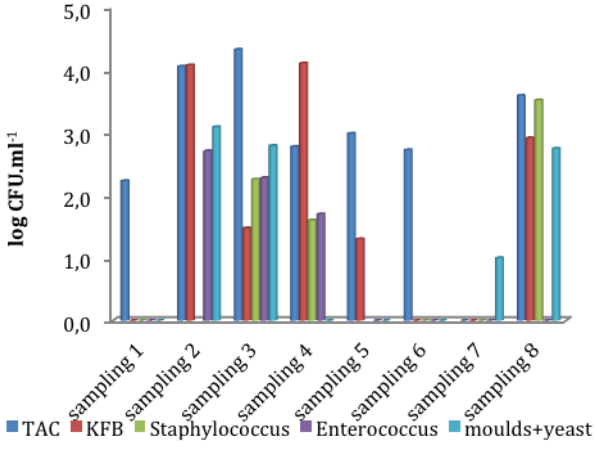

Fig. 2 The count of microorganisms (log CFU.ml^{-1}) in samples of pasteurized sweetened egg yolk, collected in 8 time points (April – February)

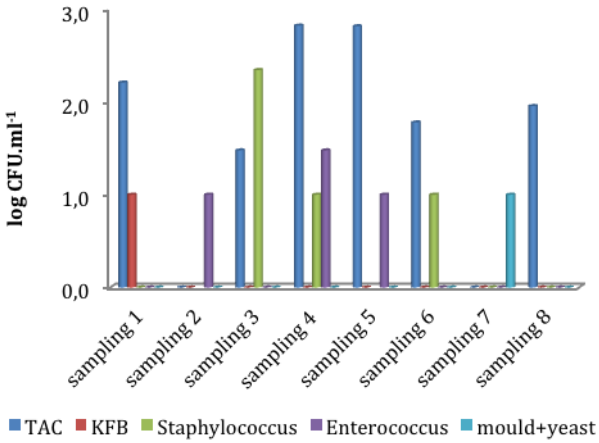

Fig. 3 The count of microorganisms (log CFU.ml^{-1}) in samples of pasteurized egg blend collected in 8 time points (April – February)

for foodstuffs does not specify the limits for the total aerobic count, the count of enterococci, coliform bacteria, staphylococci, moulds and yeast. **Regulation No. 287 (1999)** concerning veterinary requirements of animal products that is no longer in force defined the limit for the total aerobic count, which was 5.10^4 CFU per ml of a liquid egg product. According to this regulation, coliform bacteria, staphylococci, moulds and yeast should not be present in 1 ml of a particular pasteurized substance. The TAC values found in our analysis of egg liquid products would therefore meet the respective legal limit. However, egg liquid products analysed would fail to meet the counts of yeast, moulds, coliform bacteria and staphylococci. **Directive 89/437/EEC (1989)** on hygiene and health problems affecting the production and the placing on the market of egg products only specifies the limit for the total aerobic count 10^5 CFU.ml^{-1}, *Staphylococcus aureus* should not be present in 1 ml of any egg product. Our samples would therefore only comply with the TAC limit set in this directive. **Németh et al., (2011)** reported the TAC of 4.10^4 in the whole egg and 1.10^4 CFU.ml^{-1} in egg yolk after pasteurization. The TAC in the pasteurized egg white in the above mentioned experiment was <10. Unlike **Németh et al., (2011),** we found on the basis of our analysis that the mean total aerobic count after pasteurization was lower for whole egg (5.10^1 CFU.ml^{-1} , i.e. 1.64 log CFU.ml^{-1}) and egg yolk (6.8.10^2 CFU.ml^{-1}, i.e. 2.84 log CFU.ml^{-1}) and higher for egg white (2.5.10^2 CFU.ml^{-1}, i.e. 2.35 log CFU.ml^{-1}). **Mukhopadhyay et al., (2010)** reported that the value of the TAC in liquid egg products after pasteurization usually exceeds 10^3 CFU.ml^{-1}. **Rossi et al. (2010)** found in their experiment that the TAC was 1.10^2 CFU ml^{-1} in whole egg blend after pasteurization, which is in a good agreement with our findings (1.6 log CFU.ml^{-1}; n=24).

Muchová (2004) assumes that the counts of microorganisms in egg liquid products depend on several factors such as the quality of eggs, the sanitation of an egg breaker and continuous inspection to ensure the cleanliness of egg liquid products separated from shell eggs in breaker tanks. According to the guideline Good Hygiene and Manufacture Practice for Egg and Egg products **(Míková and Zvárová, 2011),** properly pasteurized egg substances contain less than 10^3 CFU per g or ml of an egg substance. Egg yolk is more likely to become contaminated by microbes since it does not contain natural inhibitors of microbial growth such as lysozyme **(Görner a Valík, 2004)** or lecithin **(Németh et al., 2011)**. This is in a good agreement with our results. The increased number of microorganisms can be found in products where undesirable bacterial growth occurred due to insufficient cooling or extended storage **(Görner a Valík, 2004)**. Although coliform bacteria are unable to survive heat treatment during pasteurization, they pose a serious hygienic risk since they usually constitute accompanying microflora of pathogenic microorganisms. The incidence of coliform microorganisms in egg substances found in our study indicates insufficient heat treatment during pasteurization or subsequent contamination due to poor hygiene and sanitation practices. As reported by **Voldřich and Šotolová (2009)**, staphylococci can normally be found on the skin, skin glands, or the upper airway in man. Food processors are the major source of contamination. Bacterial transfer from devices and external environment should also be considered **(Komprda, 2004)**. The increased occurrence of enterococci in non-fermented foodstuffs indicates insufficient heat treatment and bacterial contamination on surfaces that have not been properly cleaned and decontaminated. Enterococci can grow in foodstuffs with increased salt levels and decreased water activity **(Görner and Valík, 2004)**. **Hejlová (2001)** have found the highest incidence rate of enterococci as heat-resistant bacteria in liquid egg products. This is in a good agreement with our findings where the highest count of enterococci was detected in egg white (according to the regulation, egg white is pasteurized at a lower temperature compared to other egg substances). As a result, these products must be processed in sanitary facilities under continuous inspection and pasteurized before distributed for consumption **(Unluturk et al., 2008)**.

CONCLUSION

Microbiological analysis revealed the highest (P <0.05) total aerobic count 2.8 log CFU.ml^{-1} and the highest (P <0.05) incidence rate of coliform bacteria (1.7 log CFU.ml^{-1}) in egg yolk. In contrast, egg white and whole egg contained coliform bacteria only at very low counts. The highest (P <0.05) number of enterococci was found in egg white (1.0 log CFU.ml^{-1}). Staphylococci, moulds and yeast were present at highest levels (P <0.05) in egg yolk (1.2 log CFU.ml^{-1} and 1.1 log CFU.ml^{-1}, respectively). Statistical analysis (P >0.05) showed no correlation between the season and the counts of monitored microorganisms for any of the egg liquid products.

It follows from our results that all analysed egg products comply with the TAC limit defined by Council Directive (EC) 89/437/EEC on hygiene and healthy problems affecting the production of egg products, and correspond with other studies that deal with liquid egg substances. However, all egg products analysed in our study would fail to meet the required criteria for other groups of microorganisms. Bacterial contamination in egg products caused by staphylococci, KFB, enterococci, moulds and

yeast probably results from insufficient heat treatment during pasteurization or subsequent contamination caused by poor hygiene and sanitation practices or storage at unsuitable conditions. Thermal pasteurization still represents the most available and best understood technique. Alternative pasteurization methods including ultrasonic wave treatment, high electric field pulses, high hydrostatic pressure or ultrapasteurization combined with aseptic packaging have been explored to extend the shelf life and minimize disadvantages of thermal processing of liquid egg products. Of course these methods does not cause substantial changes in the structure of liquid egg products by causing coagulation and denaturation of proteins **(Unluturk et al., 2008)**.

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CHEMICAL COMPOSITION OF FRUITS OF A FEIJOA (F. SELLOWIANA) IN THE CONDITIONS OF SUBTROPICS OF RUSSIA

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ABSTRACT

The feijoa is culture quite widespread on the Black Sea coast of Russia. Difficulties are connected with absence in Russia of grades. All gardens have only the mix of grades, which is grown up from seeds. At institute are going of work on creation of productive varieties, with good quality parameters. Data on chemical and biochemical composition of fruits of high-yielding forms of a feijoa are provided in article. After carried-out selection work we selected a large number of perspective forms of a feijoa (more than 50). However not all of them was repeated by the results on productivity and were excluded from works on further studying. For today after rejection there were no more than two tens perspective forms. They differ from each other on productivity, terms of maturing and quality of fruits. On efficiency the D-1 forms (Dagomys), 0-01 (Country) and 10-22 are allocated - productivity of fruits (on the average in 7 years) made respectively 33.3, 20.1 and 12.4 kg from a bush. The plants are sorted out according to the productivity, ripening terms and fruit quality. It is defined that early ripe grades are characterized by higher activity of oxidizing enzymes (263.1 $mL.O_2.g^{-1}$). In a zone of damp subtropics the grades containing increased quantity of carotinoids are steadier (0.31 $mg.g^{-1}$). The contents in fruits of a feijoa of such substances, as vitamin P, P-active and pectin substances, ascorbic acid, macro and microelements are revealed. Fruits of a feijoa are differed the increased accumulation of sugars, at some forms the content of sucrose prevails over amount of monosaccharide. High accumulation of vitamin C (41.89 - 78.68 $mg.dL^{-1}$) is noted. But we don't confirm the high content of iodine in fruits. Fruits of a feijoa can be considered as potential raw materials for production of canned products with a functional purpose.

Keywords: feijoa; form; vitamin; sugar; catalase; pigment; sugar-acid coefficient

INTRODUCTION

Among the subtropical orchard grows on the Black Sea coast of Russia, a feijoa is conceded to grow only in the area with persimmon. The homeland of a feijoa is the subtropical zone of South America. For the first time plants of a feijoa were described by Otto Carle Berg in 1854 under the name *Orthotemon Sellowianus*. Till then was this sort only identified by R. Brown as a part of *Gentiaceae* family. Berg replaced the name of the sort *Orthotemon* with Feijoa, in honor of the well-known naturalist Joani de Silva Feijoa who became further the director of San Sebastyan`s museum of natural history (Brazil). Subsequently this name (feijoa) was accepted everywhere. Into Russia the culture was introduced for the first time in 1900, and in a subtropical zone of Krasnodar region in 1930 is delivered. In total in damp subtropical areas under this culture about 300 hectares are occupied now. The feijoa is ranked high in decorative gardening that was promoted by its beautiful silvery leaves and unusual bright crimson-red flowers with white-pink fleshy petals. Feijoa perfectly fits in a humid climate of Sochi where are grown 70 – 80-year old plants, which are one of, oldest in country. Usually it grows a bush with 4-6 trunks and approximately 2.5 meters high. The crown expands in width up to 3 meters. In the conditions of subtropics of Russia vegetation begins at a temperature of 10 °C with 200 – 220 days of vegetation period. Unlike metioned above citrus feijoas is rather frost-resistant and maintains temperature fall to -10 °C. It is unpretentious to soil conditions and grows on soils of various types. Near Sochi - feijoa is grown up by nearly 70 thousand gardeners on farmer sites, in the agricultural organizations. Now, all existing plantings of a feijoa in a subtropical zone of Krasnodar region represent mix of hybrid forms which in the biological and morphological relation are characterized by huge variety, differing from each other not only on productivity and frequency of fructification, but also by the size of fruits and their taste.

In the conditions of damp subtropics of Russia there are special conditions for cultivation of many subtropical cultures that influences the biochemical structure of their fruits. The Black Sea coast of Krasnodar region is the most northern border of subtropics, which begins from the southern border of the Tuapse area in the north to frontier with Abkhazia (river of Psou) in the south. Damp and rather warm climate of this zone is caused by existence of the mountain ridges reaching around Sochi 3000 - 3500 m, playing a role of a barrier to penetration of cold winds. At the same time they detain on the coast warmly and moisture, creating high humidity of air and abundance of a

precipitation. So, the quantity them in Sochi make 1534 mm, in Tuapse - 1264 mm. The vegetative period here is shorter (185 - 200 days), it is less and the sum of temperatures (3000 - 3300 °C against 4100 °C in Anaseuli, Georgia). In Krasnodar region during the droughty period the maximum daily temperatures in the soil in the horizon 20cm reach 32°C. In the territory of Krasnodar region January is coldest month. However negative average monthly temperatures here happen seldom. One of important conditions for studied cultures is repeatability of temperature minima and their duration. Rather severe winters on the Black Sea coast of Krasnodar region repeat on the average each 12 - 15 years. At the same time, according to **Gutiyev et al. (1977)**, in Sochi winters with snow cover are quite rare, it low-power and sticks on the average to 8 - 10 days in a year. And studying of biochemical structure of fruit plants in such unique conditions is actually. Difficulties are connected with absence of grades in Russia. All gardens have only the mix of grades, which are grown up from seeds. Inspection of a plantation of institute (area of 1. 2 hectares) showed that among of 739 bushes of a feijoa, which were planted in 1978, 1.7% is high-yielding plant, 50.3% - low-yielding and 48% of plants have single fruits. Similar results were made for others a plantation of the feijoas, which are available around Sochi (state farms at Matsesta, Adler, etc.). There is carrying out a work on creation of productive varieties, with good quality parameters at the institute.

Early researches on studying of a chemical composition of feijoa fruits were conducted by the staff of laboratory of biotechnology, physiology and biochemistry of plants in the 80th years of the XX. century **(Koval et al., 1984; Voronzov et al., 1950)** that became a basis for allocation of the most perspective forms. However these works weren't completed.

In recent years at institute a large number of forms (more than 50), which differ in efficiency, were selected. Among them is perspective the forms of D-1 (Dagomys), 0-01 (Dachnaja) and 10-22 which on the productivity (on the average in 7 years) are made respectively 33.3, 20.1 and 12.4 kg. The form of D-1 differs not only in the general efficiency, but also in the mass of a fruit reaching in separate years (differences were 150 - 160 g). All forms, which allocated on terms of ripening, were divided on early-ripening (ripening in 2-3 decade of September - 1 decade of October), mid-season (2-3 decades of October - 1 decade of November) and late-ripening forms (November - the beginning of December). The more earlier are forms 8-10 (September) and 0-01 (Dachnaja). Fruits which are characterized by maturing in the first decade of September are preferred by reason of more simply realization of harvesting, absence of autumn rains and frosts, production has a trade appearance and better transportationmiss.

MATERIAL AND METHODOLOGY

Objects of researches were 18 of the most perspective forms of the feijoa, differed on terms of ripening, productivity and mass of fruits. Definition of biochemical indicators was carried out by the conventional techniques. Control is the grade of Superba, which is considered as the best grade. Fruits of this grade can reach 200g. Adult plants maintains to -12 frost degrees. Feijoa fruits used for experiments were in the period of a technical maturity (10-20 November). Studied biochemical parameters: dry matter - by drying samples to constant weight at t = 100 °C **(Gunar, 1972)**; content of sugars (glucose, sucrose, fructose) by methods of Bertrand **(Voznesensriy et al., 1962)**; content of ascorbic acid (vitamin C) - iodine's method **(Pochinok, 1976)**, total acidity - titration with 10 N NaOH **(Pochinok, 1976)**. For physiological researches were selected leaves at a stage of a physiological maturity. Objects of physiological researches were only grades of a feijoa of Superb (control), D-1 (Dagomys) and 8-10 (September). We studied enzymes from oxidation-reduction group (catalase) and the contents of photosynthetic pigments. Activity of catalase enzyme was studied by the gasometrical method made by **Gunar, (1972)**, content of chlorofills and carotinoids - of method by **Shlyk, (1971)** in extract for 100% acetone.

Statistical analyze of results of researches - methods of the correlation and cluster analysis, applying a package of the statistical programs Statgraphics Centurion XV.

RESULTS AND DISCUSSION

The biochemical structure of fruits is formed under the influence of the sheet device. It is known that the metabolism in leaves is made with the participation of a number of enzymes. According to some authors **(Chirkova, 2002; Golodriga et al., 2002**) activity of a catalase depends on vegetation phases a little, thus, positive correlation between activity of a catalase and duration of the vegetative period is noted. By us it is shown that at an early ripe grade of 8-10 (September) activity of enzyme is less, than at late-ripening grades of D-1 (Dagomys) and Superb.

Except ferment`s systems and the pigment`s system of a leaf participates in processes of assimilation of biochemical components. The content of chlorophyll *a* and *b* would be an indirect indicator of photosynthetic activity of plants. It is established that content of a chlorophyll at a grade of 8-10 (September) is significantly higher, than at other studied grades that defines more active synthetic processes at this grade. Unlike chlorophyll carotinoids not only characterize photosynthetic activity, but also take part in protection of a plant against influence of abiotic stressor. We defined that grades of 8-10 (September) and Superb are steadier against adverse factors of a zone of damp subtropics of Russia, namely – a drought. The content of carotinoids in leaves of these grades is significantly higher, than at a grade of D-1 (Dagomys). Therefore, we can recommend these grades for cultivation in our climatic conditions of subtropics of Russia.

Results of long-term monitoring of biochemical structure of fruits of the allocated forms of a feijoa are presented in Table 2. In feijoa fruits at forms 8-20, 10-6 and 8-10 (September) had the greatest accumulation of dry matter. Fruits of forms 4-3 and 8-10 (September) differ in increased accumulation of sugars, and, at forms 4-3, 6-3, 8-10 (September), 10-11 and 10-21 content of sucrose prevails over amount of monosaccharide. Parameters of the general acidity are in studied fruits ranging from 0.94%

(form 11-8) to 2.47% (form 8-20). The ratio of content of sugars and the general acidity is important because defines optimum flavoring features of fruits and is classified as sugar-acid coefficient. The most harmonious combination of sugar and acids is noted at forms 4.3, 6-3, 12-5, 11-8 and 8-10 (September) as this indicator in their fruits is 1.3 - 1.7 times more than control (sugar-acid coefficient is equal to 4.0 relative units).

Also the value of fruits of a feijoa is connected with the content of vitamin C, which is ranging from 41.89 to 78.68 mg.dL^{-1}. that is equally to the contents of this important component in a citrus, wild strawberry or cabbage. And in 10 forms among investigated the amount of vitamin C is more than 50 mg.dL^{-1}.

Except the biochemical analysis of forms of the feijoas, which are grown up on a plantation of institute, we investigated the general chemical composition of fruits. The contents in fruits of a feijoa of such valuable substances, as vitamin P, P-active and pectin substances, ascorbic acid, macro and microelements are important (Table 2). The analysis showed existence in fruits of a large number of phenolic connections, among them - catechins and leucoanthocyan, that impacting to fruits knitting relish, but they are containing mostly in a peel.

Fruits of a feijoa are attractive to the consumer not so much thanks to specific taste and aroma of fruits, but because of very high content of a digestible form of iodine **(Melkadze, 2007)**. According to **Sergeyev (1934)** content of iodine in fruits are about 3 mg.dL^{-1}.that much more surpasses the content of iodine in foodstuff rich with iodine both vegetable, and an animal origin. However the author notes that these plants grew in a seaside zone, the soil about them were in addition covered with seaweed and probably these factors led to increase the content of iodine in fruits. At the same time, in the fruits, which have been grown up in the Batumi botanical garden, researchers didn't establish existence of a large amount of iodine. An

Table 1 Physiology and biochemistry characteristic of leaves of perspective grades of a feijoa

Forms	Activity of a catalase ml O_2.g^{-1}	C a+b mg.g^{-1}	C car. mg.g^{-1}
Superb (control)	362.0±13.5	1.00±0.07	0.31±0.02
D-1 (Dagomys)	298.6±50.3	1.16±0.09	0.26±0.02
8-10 (September)	263.1±28.0	1.29±0.01	0.31±0.08
The smallest distinction at 95 % level	90.79	0.15	0.08

Table 2 Biochemical composed of a feijoa

Forms	Dry matter, %	Ascorbic acids, mg.dL^{-1}	Acidity, %	Sucrose, %	Monosaccharide,%		Sugar-acid coefficient
					fructose	glucose	
Superb (control)	16	48.12	1.75	3.03	2.8	1.17	4.0
0-01 (Dachnaja)	12	50.51	2.30	2.69	2.16	1.11	2.6
D-1 (Dagomys)	17	42.62	1.87	4.02	2.69	1.47	4.3
8-10 (September)	22	50.46	1.41	4.41	2.3	1.47	5.8
3-3	17	48.58	1.99	3.34	3.02	0.83	3.6
4-3	23	53.13	1.75	5.9	3.39	0.41	5.5
6-3	17	48.92	1.54	4.28	2.94	0.8	5.2
6-24	16	51.04	2.26	2.9	2.8	0.75	2.9
8-20	20	59.58	2.47	2.91	1.4	1.14	2.2
8-24	19	49.62	1.84	2.15	2.22	0.99	2.9
9-3	19	54.74	1.92	3.72	4.32	0.16	4.3
10-6	22	43.21	1.63	3.36	2.12	1.06	4.0
10-11	17	54.21	1.39	4.21	3.6	0.07	5.7
10-21	17	50.51	1.88	4.02	2.97	0.23	3.8
10-22	12	49.72	2.1	3.23	2.83	0.34	3.0
11-8	16	41.89	0.94	3.23	1.87	1.33	6.8
12-5	15	75.68	1.43	3.17	2.87	1.13	5.0
12-8	18	52.19	1.7	3.53	2.23	0.95	3.9

Table 3 Chemical composed of a feijoa (at 100 g)

Parameters	A peel	A fruit pulp
Vitamins (mg.dL^{-1})		
Ascorbic acids	47.2	37,1
β-carotene	0.66	0,32
P-active substances (mg.dL^{-1})		
Catechine	153.0	81,0
Leucoanthocyan	109.8	69,6
Nutrient (mg.dL^{-1})		
Potassium	100.0	
Sodium	13.8	
Calcium	17.5	
Magnesia	8.2	
Iron	0.5	
Iodine (mkg %)	0.34	

increase of the content of this element in fruits contactsonly with proximity to the sea **(Voronzov et al., 1950)**.

Earlier biochemical analyses of fruits of the feijoas which have been grown up on a plantation of all-union scientific research institute of floriculture and subtropical cultures which is located in 500-700 meters from the Black Sea, showed that the accumulation of iodine in a feijoa is small **(Omarov et al., 2003)**. Our last researches confirmed these data. The content of iodine in feijoa fruits is a small and makes about 0.34 mkg % (Table 3).

CONCLUSION

Thus, researches showed that the forms allocated by us are differed in high productivity and high quality of fruits. As a result of physiological researches is defined perspective for cultivation in climatic conditions of subtropics of Russia of the grade (Superb and Dagomys), characterized by the raised contents in leaves of carotinoids that defines their resistance to a drought. As a result of biochemical researches the chemical composition of forms of a feijoa, which are growing at the institute, is established that shaped features cause them. And, feijoa fruits, possessing high biological value thanks to presence of β-carotene, vitamin C, R-active agents and a complex of valuable macro elements, can be considered as potential raw materials for food of guests, inhabitants of the resort and production of canned food of a functional purpose. Chemical composition of a feijoa forms macro - and micronutrients. The content of solids varies from 12% (form 10-22) to 23% (form 4-3). Generally for fruits of a feijoa the content of the sugar presented by fructose and glucose is characteristic low (no more than 4,28%). In the conditions of damp subtropics of Russia fruits of a feijoa differs by the low acidity, which is in limits 0,94% (form 11-8) - 2,47% (form 8-20). Availability of pectin gives a feijoa preventive effect caused by good solidification of a product for production of configure and jam. In a feijoa are the complex of phenolic connections (catechins and leucoanthocyan) which cause P - vitamin activity and gives the fruit an astringent taste In studied group of forms of a feijoa the amount of catechins varies from 81 mg (in pulp) to 153 mg/100g (peel). The contents of leucoanthocyan are 1.2 times lower than the content of catechins.

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SPECIES IDENTIFICATION OF ENTEROCOCCI BY BIOCHEMICAL TEST AND MOLECULAR-GENETIC METHODS

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ABSTRACT

The aim of this study was comparison different methods of species identification of enterococci. One hundred and fifty three suspected colonies were isolated from milk and dairy products (cheeses from cow´s, ewe´s and goat´s milk). On the bases of their growth on BEA agar, microscopic characteristic, results of Gram staining, catalase test and PYRAtest was thirty four isolates assigned to the genus *Enterococcus*. These isolates were identified by commercial biochemical test EN-COCCUS. 52.9% of them were included in species *E. faecalis*, 29.4% in *E. faecium*, 14.7% in *E. durans* and 2.9% in *E. group III*. This group includes 3 species: *E. durans, E. hirae, E. faecalis asaccharolytic var*. Then 16S rRNA sequencing nucleotide of all isolates was realized. Results of sequencing were compared with NCBI database. Only 14.7% of isolates were in 100% accordance. One from them was species *E. durans* and others were designated as *E. faecium.* For 20.6% of detected isolates was in accordance with more reference strains. Other isolates were identical with reference strain on 99%. For verification of all results species-specific PCR was used and 52.9% isolates were identified as species *E. faecalis*, 32.4% as *E. faecium* and 14.7% as *E. durans*. Strains belonging to the species *E. faecalis* were identified the most reliable by all used methods.

Keywords: enterococci; identification; EN-COCCUS test; PCR method

INTRODUCTION

Enterococci are frequently associated with many foods from animal (dairy and meat products) and vegetable origins **(Krebs-Artimová et al., 2013)**. The reason for the prevalence of enterococci in dairy products has long been considered as a result of unhygienic conditions during the production and processing of milk **(Fabianová et al., 2010)**. However, their presence in foods has often been shown to be unrelated with direct faecal contamination **(Krebs-Artimová et al., 2013)**. Enterococci are a bacterial group that is commonly found in a high population in a large number of traditional cheeses produced with raw or pasteurized milk **(Nieto-Arribas et al., 2011)**.

One possibly negative aspect of enterococci in cheeses is their ability to produce biogenic amines **(Valenzuela et al., 2008)**. On another side, enterococci belong to the probiotic microorganisms that are able to produce bacteriocins **(Čanigová et al., 2012)** and are component of starter culture **(Ducková et al., 2012)**. Several strains share interesting biotechnological traits and they have a positive effect on cheese flavour development, by means of citrate metabolism, proteolytic and lipolytic activity **(Serio et al., 2010)**. In spite of all this, the clinical research underlines that the safety of dairy products containing enterococci is an issue and the industry must carefully address before proceeding to their application for production of products **(Jamaly et al., 2010)**.

A lot of ways isolation, identification and confirmation of enterococci can be used. There are over 100 modifications of selective media for the isolation of enterococci from various specimens. Especially the BEA medium seems to be the best suited for selective enumeration since it still demonstrates sufficient selective properties, even in combination with other LAB bacteria (lactobacilli and pediococci) and bifidobacteria **(Domig et al., 2003)**. For genus identification phenotypic and biologic methods have conventionally been used. Biochemical methods and genotyping techniques have been recommended for taxonomical characterization.

The objective of the present study was to evaluate accuracy of identification molecular and biochemical methods for enterococci isolated from raw cow milk and dairy products.

MATERIAL AND METHODOLOGY

Isolation of enterococci.

Presence of enterococci in samples of raw cow milk from milk machines and dairy products (cheeses from cow´s, goat´s and ewe´s milk) was determined by cultivation at 37 ±1 °C on medium Slanetz-Bartley (HiMedia Laboratories, India) during 48 ±2 hours (**STN 56 0100, 1970**). Suspected colonies (n = 153) were isolated on selective medium containing bile, aesculin and azide - BEA agar (HiMedia Laboratories, India) during 24 ±2 hours at 37 ±1 °C. Bacteria of genus *Enterococcus* created creamy, pale gray or dark gray colonies with strong hydrolysis of aesculin on this medium.

Genus identification

The genus *Enterococcus* was confirmed by microscopic characteristic of colonies, Gram staining, production of catalase and pyrolidonylarylamidase enzyme. These microorganisms were Gram-positive, catalasa-negative

and PYRAtest positive (Lachema, Czech Republic) cocci that often occur in pairs or short chains.

Species identification

For species identification of enterococci isolates (n = 34) by means of EN-COCCUS test (Lachema, Czech Republic) the bacteria suspension from overnight culture was adjusted to equal the 2 McFarland standard with Densi-La-Meter (Pliva, Lachema Brno). This commercial method is based on biochemical reaction of arginine, sorbose, arabinose, mannitol, sorbitol, melibiose, raffinose and melezitose. Colored reactions were evaluated after 24 ±2 hours cultivation at 37 ±1 °C according to EN-COCCUS key (Analytic Profile Index).

Results of EN-COCCUS test were confirmed by 16S rRNA sequencing and species-specific PCR methods. DNA of enterococci was isolated by peqGOLD Bacterial DNA Kit (Peqlab, Germany) and concentration and purity was detected on spectrophotometer NanoDrop 2 000c spectrophotometer (Thermo Scientific, Germany). DNA sequencing was performed by primer amplification according to **di Cello et al. (1997)** and following purification of PCR product by Extra Mini Kit (5 prime, Germany). The nucleotide sequences were accomplished by commercial firm Eurofins (Austria). Results were evaluated by database NCBI (URL 1).

The PCR method for the species identification of enterococci isolates were performed using specific primers (Table 1). Mixture (25 µL) for *E. durans* and *E. hirae* identification was composed: 2.5 µl of PCR buffer 10 x concentrated (Finnzymes, Finland), 0.5 µl of dNTP with concentration 10 mM (Carl Roth, Austria), 0.5 µL of DynaZyme II (Finnzymes, Finland) with concentration 2 $U.\mu L^{-1}$, 18.5 µL of sterile water, 0.5 µL of each primer (25 $pmol.\mu L^{-1}$) and 1 µL of DNA template. Same mixture was used for identification of *E. faecalis* and *E. faecium*.

Optimal PCR program for each primer was described by **Arias et al. (2006)** and **Dutka-Malen et al. (1995)**.

Analysis of PCR products was performed on agarosa gel (2%) at 80 V and 200 mA during 50 minutes, staining by ethidium bromide and visualisation in UV light (ChemiDocTM XRS + System with Image Lab™, Software, Bio-rad Laboratories, USA).

RESULTS AND DISCUSSION

Of the 153 isolates from milk and dairy products were 34 included in *Enterococcus* genus.

Results of enterococci identification by three methods are summarized in Table 2.

On the basis on EN-COCCUS test 52.9% (n = 18) were identified as *E. faecalis*, 29.4% (n = 10) in *E. faecium*, 17.7% (n = 5) in *E. durans* and 2.9% (n = 1) in *E. group III* (*E. durans, E. hirae, E. faecalis asaccharolytic var.*). In samples from raw cow milk (n = 46) **Fabianová et al. (2010)** identified 56.5% *E. faecalis*, 19.5% *E. group III*, 15% *E. faecium*, 7% *E. mundtii* and 2% *E. casseliflavus* by same method. Also **Kročko et al. (2011)** determined *E. faecalis* (51.5%) as prevalence species followed *E. durans / E. hirae* (12%), *E. faecium* (11%), *E. mundtii* (2%), *E. casseliflavus* (1%) and *Enterococcus* spp. (22.5%) from total amount of enterococci (n = 101) isolated from cow milk. According to **Račková (2012)** is this method less accurate for identification of species *E. faecium*. **Brtkova et al. (2010)** stated that EN-COCCUS test are not able to recognize some enterococci, especially unusual species. EN-COCCUS test is able to identify only 19 enterococci species. For example lactose-negative strain of *E. faecalis* can be misidentified as *E. solitarius*. The problem is with ability of some strain of *E. faecium* to utilize sorbitol and identification is prolonged for next 1-2 days. Other problem with EN-COCCUS test is associated with individual personal experiences and manual reading of the results.

From this reason some authors use combination of biochemical and PCR method for enterococci identification. **Jurkovič et al. (2006)** found some discrepancies between results of enterococci identification from Bryndza cheese samples, obtained by commercial biochemical test and PCR method. Seven enterococci strains identified by commercial biochemical test were identified as *E. faecium* and by PCR method as *E. faecalis*. Three strains of *E. casseliflavus* were determined by PCR method as *E. faecium* (two strains) and *E. faecalis* (one strain).

Table 1 Primers used for species identification of enterococcal isolates

Species	Primer	Sequence (5´→ 3´)	Size of PCR product (bp)	References
E. durans	Mur-2ed/F	AAC AGC TTA CTT GAC TGG ACG C	177	**Arias et al., 2006**
	Mur-2ed/R	GTA TTG GCG CTA CTA CCC GTA TC		
E. hirae	MurG-F	GGC ATA TTT ATC CAG CAC TAG	521	**Arias et al., 2006**
	MurG-R	CTC TGG ATC AAG TCC ATA AGT GG		
E. faecium	Dut-F1	GCA AGG CTT CTT AGA GA	550	**Dutka-Malen et al., 1995**
	Dut-F2	CAT CGT GTA AGC TAA CTT C		
E. faecalis	Dut-E1	ATC AAG TAC AGT TAG TCT	941	**Dutka-Malen et al., 1995**
	Dut-E2	ACG ATT CAA AFC TAA CTG		

Table 2 Comparison biochemical and molecular genetic methods used for identification of enterococci

Isolate number	EN-COCCUS test	PCR methods	16S rRNA sequenation	
			Reference strain	Similarity
29	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. thailandicus (FP48-3)	99 %
96	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
98	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
99	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
100	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
101	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
108	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
110	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
114	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
118	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
125	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
126	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
127	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
128	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
129	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
131	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %
132	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. thailandicus (FP48-3)	99 %
			E. faecium (LMG 11423)	99 %
			E. mundtii (ATCC 43186)	99 %
			E. villorum (88-5474)	99 %
133	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %
134	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. thailandicus (FP48-3)	99 %
			E. faecium (LMG 11423)	99 %
			E. mundtii (ATCC 43186)	99 %
135	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
137	*E. group III*	*E. durans*	*E. durans (98D)*	100 %
138	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. faecium (LMG 11423)	99 %
			E. thailandicus (FP48-3)	99 %
			E. mundtii (ATCC 43186)	99 %
139	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %
140	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. faecium (LMG 11423)	99 %
141	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %

Continuation of the Table 2.

Isolate number	EN-COCCUS test	PCR methods	16S rRNA sequenation	
			Reference strain	Similarity
142	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. thailandicus (FP48-3)	99 %
			E. faecium (LMG 11423)	99 %
			E. mundtii (ATCC 43186)	99 %
143	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. faecium (LMG 11423)	99 %
			E. thailandicus (FP48-3)	99 %
			E. mundtii (ATCC 43186)	99 %
146	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
147	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
148	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
149	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
150	*E. durans*	*E. faecium*	*E. faecium (LMG 11423)*	99 %
152	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
153	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %

Using of species-specific PCR was 52.9% isolates identified as *E. faecalis*, 32.4% as *E. faecium* and 14.7% as *E. durans*. One isolate previously identified by EN-COCCUS test as *E. durans* was identified by PCR method as *E. faecium*. Isolate no. 137 determined by EN-COCCUS test as *E. group III* was specified by PCR method as *E. durans*.

Also another authors **(Citak et al., 2006, Nieto-Arribas et al., 2011)** the most frequently identified *E. faecalis* (54.2%, 81.8%, respectively) from cow milk and cheeses by PCR method. However, from fresh cheeses **Pesavento et al. (2014)** identified mostly *E. faecium* (63.1%) followed *E. faecalis* (23.7%), *E. avium* (10.5%) and *E. durans* (2.63%).

According our results we can conclude that method of identification by 16S rRNA sequencing is not exact. Only 14.7% of isolates (no. 131, no. 133, no. 137, no. 139, no. 141) were in 100% accordance with reference strain. One of them was *E. durans* and four isolates were *E. faecium*. 20.6% of detected isolates was in accordance with more reference strains occurred in NCBI database. For example isolate no. 132 had similar nucleotide sequences with 7 reference strains and isolates no. 134, no. 138, no. 142, no. 143 with 6 reference strains and is not possible to exactly chosen only one species. On another side, strains that were detected by PCR methods were everytime confirmed. It may be explained by the 16S rRNA sequencing was performed in only one direction. If it was used two-sided sequencing, it would be possible to detect anomalies that may arise in the one direction sequencing and it would be more reliable in comparing sequences studied strains with reference strains in the database NCBI. In study of **Fei et al. (2006)** was found that one tested strain was phylogenetically closely related to *E. mundtii* (100% sequence similarity), *E. hirae* (99%) and *E. durans*, *E. faecium*, *E. azikeevi*, *E.villorum* (98%). They also stated that presently the acceptable standard is that if the similarity of strain under investigation and a reference strain sequences is 99-100%, they are regarded as belonging to the same species while if similarity is 97-98%, they are regarded as belonging to the same genus. According to this standard tested strain can belong to the species *E. mundtii*. **Nikolic et al. (2008)** used this method as a supplement rep-PCR, because two isolates of enterococci had same profile. By nucleotide sequence was shown that these isolates belong to the species *E. faecalis* with 99% similar to a reference strain. Results of other authors confirm that method 16S rRNA sequencing is suitable only as supplementary method for identification of enterococci.

The species E. faecalis was identified the most reliable by all three used methods. Suitable method for the identification of this species can be EN-COCCUS test. Problem was with identification of E. faecium and E. durans by commercial biochemical method and 16S rRNA sequencing. Therefore for the thorough identification of another species of enterococci (except E. faecalis) we recommend to use not only EN-COCCUS test but also PCR method.

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CHARACTERISATION OF LACTOBACILLI FROM EWE´S AND GOAT´S MILK FOR THEIR FURTHER PROCESSING RE-UTILISATION

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ABSTRACT

Raw ewe's and goat's milk is a good source for isolation of wild lactobacilli which are able to bring unique processing properties in development of dairy products - cheeses or fermented dairy products. 34 strains of lactobacilli were isolated, purified and identified from fermented ewe's and goat's dairy products. These products were processed without thermal treatment and without using of any commercial starters. After preliminary selection, the final collection of 5 strains was established. The strains were identified as: *Lbc. plantarum* (2), *Lbc. paraplantarum* (1), *Lbc. paracasei* (1) and *Lbc. johnsonii* (1). Except two strains, all were able to coagulate milk. After hydrolysis of lactose in milk, two strains were able to form sensorial attractive coagulate too. All of the strains were homofermentative, they produced lactic acid but they did not produce CO_2. Their ability to produce diacetyl was low. They did not show strong proteolytic activity. All strains grew at 30 °C and 37 °C, however *Lbc. johnsonii* much slower at 30 °C than the others. Except *Lbc. johnsonii*, all strains tolerated 2% concentration of NaCl and even in presence of 5% concentration of NaCl their growth was inhibited only moderately. All of characterized strains can be provisionally used as starter or starter adjuncts in dairy technology, during production of cheeses or fermented milk products from pasteurised milk. These results will be used in further processing studies of isolated strains and will be supplemented with other properties e.g. safety, probiotic and antimicrobial properties.

Keywords: ewe's milk; goat's milk; lactobacilli; isolation; technological properties

INTRODUCTION

Addition of lactic acid bacteria (LAB) in cheesemaking process has both advantages and disadvantages. It is associated mostly with heat treatment before their application. The advantages include: safety, uniformity of production thus longer period of expiration. The disadvantage of this attitude is decreasing variability of "wild" lactic acid bacteria for customers. Several corporations that produce wide range of starters and starter adjuncts manage the market. However, they are much narrow than those that are found in spontaneous fermented products. Therefore, it is essential to maintain the adequate balance between both of approaches. Isolation of LAB from typical dairy products for certain region and their technological re-utilisation subsequently is a suitable alternative **(Ayada et al., 2004)**.

Ewe's milk is a substance that is used for manufacture of traditional and EU protected dairy speciality in Slovakia, usually without addition of secondary starters. These include: „Ovčí hrudkový syr - salašnícky" (TSG) (sheep lump cheese, typically produced in chalets) **(OA EC 2010/C20/9)** and Slovak Bryndza (PGI) as its final product **(OA EC 2007/C 232/10)**, or žinčica. These products are ideal for isolation of „wild" LAB. Studies performed with bryndza reported several strains of bacteria, yeasts and filamentous fungi **(Berta et al., 2009; Sulo et al., 2009)**. Most of them have potential utilization as starters or starter adjuncts.

Goat's milk has its own specificity. Because of the risk of encephalitis, it is recommended to consume goat's products only from pasteurized milk **(Zavadska et al., 2013)**. From the other point of view, raw goat's milk is an excellent source of wild microflora, because goats graze wide range of vegetation.

Lactobacilli are significant part of LAB, which are used in cheesemaking process and fermented dairy products. LAB cause rapid decrease of pH through production of organic acids, especially lactic acid. They are also important for production of exopolysaccharides, aromatic substances, bacteriocines and other antimicrobially active compounds. It contributes to improve quality, health safety and sensory properties of dairy products **(Leroy and De Vuyst, 2004)**.

The aim of this study is to isolate of lactobacilli from raw ewe's and goat's milk, followed by characterization of their processing properties: ability to grow in milk, production of organic acids and sensory attractive coagulate, CO_2, diacetyl, optimal growth temperature, resistance to different concentrations of NaCl and proteolysis. These properties are essential for characterization of collection of process-wise suitable lactobacilli from regional specialities that are typical for Slovakia.

MATERIAL AND METHODOLOGY

Presumptive lactobacilli were isolated from products based on raw ewe's and goat's milk by using de Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany), after cultivation at 37 °C for 24 h to 72 h. Typical colonies were purified several times and then they were identified by matrix-assisted laser desorption ionization-time-of flight mass spectrometry (MALDI-Tof MS). Bacterial identification MALDI-Tof MS was performed on a Microflex LT instrument (Bruker Daltonik, Leipzig, Germany) as described **Bessede et al., (2011)**.

MRS medium or raw cow's milk sterilised by autoclaving was used in tests that deal with bacterial growth and production of acids. Lactose was hydrolysed by commercial lactase Maxilact (DSM, Heerlen, The Netherlands) according to the recommendation of the enzyme producer.

Reference HPLC assays of DL-lactic acid and lactose were run on a DeltaChrom liquid chromatograph (Watrex, Bratislava, Slovakia) equipped with Applied Biosystems 759A Absorbance Detector and simultaneously connected to WellChrom K-2301 refractive index detector (Knauer, Berlin, Germany). The analytical conditions were as follows: a column, Polymer IEX in H^+ form, 250 mm × 8 mm, 5 μm (Watrex, Bratislava, Slovakia); a guard column, Polymer IEX in H^+ form, 10 mm × 4 mm, 8 μm (Watrex, Bratislava, Slovakia); a column thermostat: DeltaChrom™ Temperature Control Unit (50.0 ±0.1) °C; a mobile phase, H_2SO_4, 1 mM in demineralized water; flow rate, 1.0 $mL.min^{-1}$; data were collected and processed by Clarity chromatography station (DataApex, Prague, Czech Republic). The samples were clarified prior to analysis by Carrez I and Carrez II and then they were diluted in mobile phase and filtered through 0.2 μm Chromafil AO filters (Macherey-Nagel, Düren, Germany).

Enzymatic activities were measured by API ZYM assay (bioMérieux, Marcy l'Etoile, France). It was performed according to the manual of the producer.

Effect of NaCl on growth of the strains was evaluated by measuring of the optical density at 600 nm of MRS media with various concentrations of NaCl cultivated at 37 °C. The media were inoculated with 1% overnight cultures. Specific growth rate (μ_m) and lag phase (λ) were calculated from Gompertz function using software TableCurve 2D for Windows (Jandel Scientific, California, USA) and Origin 8.1 (Microcal Software, Northampton, USA) **(Zwietering et al., 1990)**.

Production of CO_2 was estimated by observation of gas in Durham tubes, during cultivation of the strains in MRS medium at 37 °C for 24 h.

Production of diacetyl was observed by visual check in milk which was fermented by tested strains at 30 °C and 37 °C for 24 h. 1 mL of fermented milk was than mixed with 0.5 mL 16% KOH (Merck, Darmstadt, Germany) and 0.5 mL 1% α-naphthol (Merck, Darmstadt, Germany) and was incubated at 30 °C for 10 minutes. Colour of the mixture was observed. Pink colour of the mixture indicated production of diacetyl.

Proteolytic activity was estimated by measuring of clear zones around 1.3 cm holes filled with 100 μL concentrated tested strains, in M 17 agar medium (Merck, Darmstadt, Germany) with addition of 10% of skimmed milk, cultivated at 37 °C for 24 h.

RESULTS AND DISCUSSION

Presumptive lactobacilli were isolated from several milk and dairy products. The products were made from unpasteurised (except „Žinčica") and only spontaneously fermented sheep and goat milk. After preliminary selection on the elective MRS medium, the strains were identified by MALDI-Tof MS. The results are shown in the Table 1. Besides phenotype identification by MALDI-Tof MS, also further genotype identification of strains is necessary. Therefore classification of strains by 16S rRNA PCR is in the progress nowadays.

The enzymatic activities of strains were characterised by API-ZYM. They are discussed later, mostly from technological point of view. Of course many enzymes are significant also for safety criteria **(Delgado et al., 2008)**, but this will be mentioned in a following study.

All strains grew at both temperatures 30 °C and 37 °C, however *Lbc. johnsonii* KB2-1 much slower at 30 °C (Figure 1). The 37 °C is the optimal temperature for growth of lactobacilli, although their metabolic activity can be higher at different temperatures **(Hujanen and Linko, 1996).**

The most important processing properties of lactic acid bacteria, which can be used as starters, is their ability to precipitate milk to the sensory pleasant coagulate in adequate time and temperature. With exception of two strains (*Lbc. casei* 21L10 and *Lbc. paraplantarum/plantarum* 25/1 L) all strains were able to coagulate milk at 37 °C. The explanation is that these two strains showed (according to API ZYM) very low lactase activity. After addition of external lactase, they fermented glucose and galactose well. Production of lactic acid after 48 hours fermentation on sterile cow milk is given in Table 3. The achieved amounts of lactic acid were a bit lower as described **(Zimanová, 2011)**, which resulted in good, typical acid-milk taste and smell of coagulates, without any significant foreign flavour. Of course addition of lactase enhanced sweet taste of coagulates.

Diacetyl flavour was not observed, since *Lbc. casei* 21L10 did not produce it and the rest of the strains formed only negligible amounts.

The strains produced only lactic acids, no CO_2. Therefore, they can be considered as homofermentative. These species of lactobacilli are usually facultative heterofermenters (*Lbc. plantarum*, *Lbc. paraplantarum* and *Lbc. casei*) or obligatory homofermenters (*Lbc. johnsonii*) **(Stiles and Holzapfel, 1997)**.

The tested strains (except *Lbc. johnsonii* KB2-1) were not very sensitive to NaCl in model MRS medium. Although the concentration of 5% NaCl slowed their growth at 37 °C, typical concentration of 2% NaCl in hard and semi-hard cheeses did not inhibit them. Specific growth rates, lag phases according to strains and NaCl concentrations are summarized in Table 4. Similar observations were also published by **Zimanova, 2011**, where some strains were even stimulated at the presence of 2% NaCl.

Table 1 Identification of lactobacilli by MALDI-Tof MS

Strain	Identification 1st match	Identification 2nd match
17L1	*Lactobacillus plantarum*	*Lactobacillus plantarum*
18L2	*Lactobacillus plantarum*	*Lactobacillus plantarum*
21L10	*Lactobacillus paracasei*	*Lactobacillus paracasei*
KB2-1	*Lactobacillus johnsonii*	*Lactobacillus gasseri**
25/1L	*Lactobacillus paraplantarum*	*Lactobacillus plantarum*

* Score bellow 2, it means worse probability of identification

Table 2 Enzymatic characterisation of lactobacilli by API ZYM (strain identification see Table 1)

Activity* / strain	17L1	18L2	21L10	KB2-1	25/1L
Control	0	0	0	0	0
Alkaline phosphatase	0	0	1	1	1
Esterase (C4)	1	1	2	1	2
Esterase Lipase (C8)	1	1	2	2	2
Lipase (C14)	1	0	1	1	1
Leucine arylamidase	5	3	5	5	2
Valine arylamidase	5	3	5	1	1
Cystine arylamidase	1	0	1	1	1
Trypsin	1	1	0	1	1
α-chymotrypsin	1	0	1	1	1
Acid phosphatase	2	2	4	1	2
Naphthol_AS-BI-phosphohydrolase	1	2	3	3	1
α-galctosidase	1	0	0	3	1
β-galactosidase	5	5	1	4	1
β-glucuronidase	0	0	0	0	0
α-glucosidase	4	3	1	2	2
β-glucosidase	2	2	1	3	1
N-acetyl-β-glucosaminidase	2	2	1	3	1
α-mannosidase	0	0	0	0	1
α-fucosidase	2	1	1	0	1

*Values (0-2) are negative, (3-5) are positive

Table 3 Production of lactic acid by fermentation on sterile cow milk at 37 °C (strain identification see Table 1)

Strain	0 (h)		24 (h)		36 (h)		48 (h)	
	Lactic acid ($g.100g^{-1}$)	pH	Lactic acid ($g.100g^{-1}$)	pH	Lactic acid ($g.100g^{-1}$)	pH	Lactic acid ($g.100g^{-1}$)	pH
17L1	0.00	6.59	0.16	5.57	0.31	5.18	0.39	4.84
18L2	0.00	6.59	0.22	5.25	0.36	4.96	0.42	4.76
21L10	0.00	6.59	0.01	6.07	0.03	6.10	0.08	5.84
21L10*	0.00	6.41	0.54	4.53	0.73	4.20	0.82	3.97
KB2-1	0.00	6.59	0.23	5.17	0.34	4.94	0.42	4.90
25/1L	0.00	6.59	0.06	5.95	0.09	5.80	0.17	5.54
25/1L*	0.00	6.41	0.30	5.06	0.50	4.69	0.64	4,56

* After hydrolysis of lactose in milk

Table 4 Growth of lactobacilli in MRS medium with various concentration of NaCl at 37 °C (strain identification see Table 1)

Strain	0% NaCl		2% NaCl		5% NaCl	
	$\mu_m(h^{-1})$*	λ (h)**	$\mu_m(h^{-1})$*	λ (h)**	$\mu_m(h^{-1})$*	λ (h)**
17L1	0.358	5.2	0.305	6.2	0.107	10.0
18L2	0.311	5.2	0.323	6.0	0.059	7.4
21L10	0.228	3.4	0.237	4.2	0.100	6.9
KB2-1	0.298	6.7	0.148	9.2	No growth	No growth
25/1L	0.315	7.4	0.297	8.6	0.158	13.2

* Specific growth rate, ** Lag phase

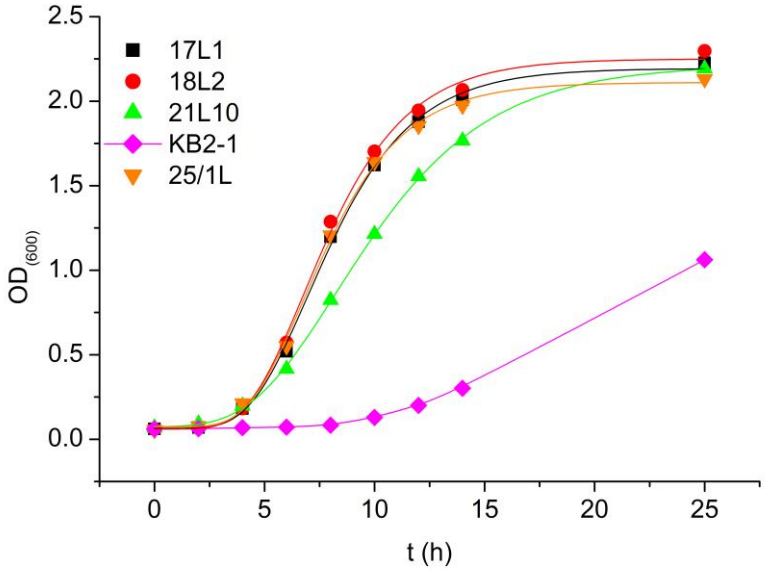

Figure 1 Growth of lactobacilli strains at 30°C in MRS medium (strain identification see Table 1)

The screening for the proteolytic activity showed only negligible proteolysis of the strains. The diameters of clear zones were from 1.4cm to 1.7cm.

Based on API ZYM result, (see Table 2) activities of trypsin and α-chymotrypsin were very low. However all strains, except *Lbc. paraplantarum/plantarum* 25/lL, exhibited positive leucine arylamidase activity and the strains *Lbc. plantarum* 17L1, *Lbc. plantarum* 18L2 and *Lbc. paracasei* 21L10 also exhibited positive valine arylamidase activity. Cysitine arylamidase was negative. Proteolytic activity should be confirmed during ripening of model cheese production, using a suitable method e.g. OPA (reaction of amino groups from amino acids with o-phthaldialdehyde).

CONCLUSION

Five strains of lactobacilli isolated from goat´s and ewe´s milk showed different technological properties.

All the strains (with exception of *Lbc. johnsonii* KB2-1) grew well at typical temperatures used in dairy technology: 30 °C and 37 °C.

Lbc. johnsonii KB2-1 is suitable for production of fermented dairy drinks because his growth was most affected by addition of NaCl. The rest of the strains tolerated NaCl up to concentration of 5%. Therefore, they are also suitable for cheesemaking when higher salt concentration is expected.

Lactase activity of two strains – *Lbc. casei* 21L10 and *Lbc. paraplantarum/plantarum* 25/1L was a bit lower. This handicap can be eliminated either by external addition of lactase (in production of fermented dairy drinks) or by appropriate co-cultivation with other starters like lactococci (in process of cheesemaking).

No one of the strains produced CO_2. From among organic acids, they only produced lactic acid.

If typical diacetyl flavour is requested, it will be necessary to use beside these cultures also other strains – e.g. leuconostocci.

Lower proteolytic activity of all strains can be an advantage during cheesemaking because sensory problematic bitter peptides may not be formed.

Created collection of five strains of lactobacilli can be used as starters or starter's adjuncts in manufacture of dairy products made from pasteurised milk.

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THE EFFECT OF EWES RELOCATION ON MILK COMPOSITION AND MILK FLOW KINETICS

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ABSTRACT

The investigation of an influence of ewes relocation and milking in other parlour (treatment) on milk flow kinetics, milkability and milk composition was the aim of this study. In total 34 ewes of two breeds and crosbreeds Tsigai (14 heads) and Improved Valachian (20 heads) with Lacaune were tested. Two weeks after lamb weaning the ewes were milked in parallel milking parlour (1x16 stalls) under shelter. On the last evening milking (first experimental milking, EB) before relocation of flock to another parlour, and during next three continuous evening milkings (E0 - second, E1 - third and E2 - fourth milking of exp.) after relocation the milk flow kinetics were measured using electronic collection jar. On day E0 after morning milking the flock was moved on a pasture and milked in other parlour (1x24-stalls). During E0 we recorded a significant decrease of total milk yield in comparison with EB (0.527 ± 0.04 and 0.647 ± 0.04 L). Significant differences were also recorded in machine milk yield, machine stripping, milking time and in maximum milk flow rate. During E0 there was a higher number of nonbimodal and lower numbers of bimodal flow types. The response of ewe to E0 depended on its response to EB. Ewes with bimodal flow at EB responded more negatively to E0 than ewes with nonbimodal or plateau flow. During E2 there were significantly increased protein content and solids not fat in milk. Thus the treatment significantly influenced the milkability of ewes in a negative way, but more clear response was found out in ewes with bimodal flow response to machine milking before treatment. We could assume that relocation to other milking conditions caused only short-term changes in milk flow kinetic and milk yield.

Keywords: milk flow kinetic; relocation; adaptation of ewes

INTRODUCTION

For fast and complete milk removal, in of spite larger volume of cistern in ewes, the milk ejection reflex occurrence is also needed **(Labusiere, 1981)** as it is well presented for dairy cows **(Tančin and Bruckmaier, 2001)**. In ewes of lower milk yield the milk ejection is often observed as a second emission of milk during milking causing a bimodal milk flow kinetics **(Mačuhová et al., 2008, 2012; Rovai et al., 2008; Tančin et al., 2011)** due to oxytocin release in response to udder stimulation by machine and its effect to expulse of alveolar milk to cistern **(Bruckmaier et al., 1997)**. However, under the normal milking conditions there are many ewes of main Slovak breeds (around 40%) with only one emission, which indicates no response to udder stimulation **(Tančin et al., 2011)**.

Under the various milking conditions and manipulation with ewes the milk ejection reflex could be disturbed causing inhibition of second emission occurrence **(Kulinová et al., 2012)**. Inhibition of second emission causes lower milk and fat yield **(Antonič et al., 2013)**. It was also confirmed that despite of larger cisternal volume in ewes, milk ejection reflex and also complete milk removal from udder is very important to keep milk production due to the effect of milk born feedback mechanisms **(Silanikove et al., 2010)**.

The ewe's relocation to new milking conditions is one of the possible stress factors for milk removal. The change of surroundings and milking conditions caused milk flow failures in dairy ewes **(Marnet et al., 1998)** and cows **(Mačuhová et al., 2002; Tančin et al., 2004)**. Because of high individual variability of milk ejection occurrence in response to machine milking under normal conditions **(Tančin et al., 2011)** we could expect different response of ewes to milking under stress conditions. The aim of the work was to study the effect of relocation and milking in other parlour on milk flow kinetic, milkability parameters and milk composition. The importance of differed milk flow kinetic of ewes under the control milking conditions to mentioned stress load was evaluated as well.

MATERIAL AND METHODOLOGY

Experimental conditions and design

Experiment was performed on 34 experimental ewes of two breeds and crossbreeds Tsigai (14 heads, two of them pure Tsigai, and others were crosbreed with Lacaune from 25-75) and Improved Valachian (20 heads, three of them pure IV, and others were crosbreed with Lacaune from 25-75). Ewes were randomly selected from the flock and used for experimental purposes two weeks after lambs weaning in April. During these two weeks the flock was milked in parallel milking parlour (1x16 stalls) under shelter with following parameters: pulsation rate

120 cycles per min., milking vacuum 52 kPa, pulsation ratio 50:50. On the last evening milking, (first experimental milking, evening before relocation - **EB**) before relocation of flock to another milking parlour, the milk flow kinetics of experimental ewes were measured using electronic collection jar (described below). After the following morning milking the flock was moved on a pasture and milked in another milking parlour (1x24-stalls) with 12 standard milking units and with following parameters: pulsation rate 180 cycles per min., milking vacuum 40 kPa and pulsation ratio 50:50. Milk flow kinetic of experimental ewes was recorded during the next three continuous evening milkings after relocation **(E0 - second, E1 - third** and **E2 - fourth milking of experiment)**. Thus data from four continuous evening milkings (one before and three after relocation) were available for statistical evaluation.

During the whole experiment the ewes were fed by concentrate 0.5 kg and 0,2 kg oat grain at milking parlour and before relocation they received oat silage (6 kg) and hay (0,5 kg) and after relocation they were on meadow pasture (7 kg).

On the basis of milk flow kinetics during EB the ewes were divided into three groups for statistical evaluation. The first group was represented by animals with bimodal (2 emissions) milk flow curve (**BG,** n=12), in the second group were animals with non-bimodal (1 emission) milk flow curve (**NG,** n=10) and the last one was represented by animals with plateau (steady) milk flow curve (**PLG,** n=12) to test the importance of differed milk flow kinetics of ewes under the control milking conditions to mentioned stress - relocation and milking in another parlour.

Milk flow recording and parameters

Milk flow kinetics was recorded individually by using four electronic jars (1.5 L each) collecting total milk produced at the milking. Within each jar there was a 2-wire compact magnetostrictive level transmitter (Nivotrack, Nivelco Ipari Elektronika Rt, Budapest, Hungary) connected with the computer. Milk level in the jar was continuously measured by a transmitter recording signal on the computer every second. Measured changes of height level of milk were transformed into values, from which were detected parameters of milkability (TMY - total milk yield, MMY - machine milk yield, MS - machine stripping, MT - milking time, LT - latency time, MMF - maximum milk flow rate) and types of milk flow (B - bimodal, two emissions, N - nonbimodal, one emision, PL - plateau, steady flow, PLL - plateau with low peak flow) were evaluated according to **Bruckmaier et al., (1997); Mačuhová et al. (2008)** and **Rovai et al., (2008)**. During experiment the samples of milk were taken for analysis of basic milk components (fat, protein, lactose, dry matter, solids not-fat) with MilkoScan FT120 (Foss, Hillerød, Denmark) and log SCC with Somacount 150. There were 136 samples of milk for analysis collected in total.

Statistical analysis

Data from four evening milkings were evaluated statistically using mixed model (Mixed procedure; **SAS/STAT 9.1,** 2002-2003). This model was applied to study the influence of the sources of variation in studied traits (milk production and milk emission/milkability).

$y = X\beta + Zu + e$

where:

y = vector of studied traits: TMY (L), MMY (L), MS (L), MT (s), LT (s), maximum milk flow (L/min), fat (%), fat in dry matter (%), protein (%), lactose (%), solids not fat (%), dry matter (%), somatic cells count (log)

β = vector of unknown fixed effects: flow type (bimodal, non-bimodal, plateau), day of milking (four evening milkings described above),

u = vector of random effect of ewe, $u \sim N(0, I\sigma_w^2)$

e = residual vector, $e \sim N(0, I\ \sigma_e^2)$

X, Z = incidence matrices for fixed effects and random ewe effect.

RESULTS AND DISCUSSION

Relocation and milking in another parlour significantly influenced the TMY between EB (evening before) and E0 (evening after morning ewes relocation) what pointed to stress from changed milking conditions (Table 1). Next two milkings showed adaptation to milking conditions. A statistically significant increase was also in E1 compared to E0 for MMY (Table 1). Statistically significant differences were also recorded in MT. Thus relocation of ewes and their milking in another place significantly influenced milkability. The effect of ewes' relocation on TMY was also confirmed by others authors in ewes or cows **(Sevi et al., 2001; Mačuhová et al., 2002)**.

MS and MMF were also significantly influenced by treatment but changes did not corresponded to the changes of above mentioned parameters. The volume of milk obtained by machine stripping and maximal milk flow are significantly positively influenced by the vacuum level **(Sinapis et al., 2000, 2006)**. Thus higher MS and MMF were caused by higher vacuum level at EB milking. Therefore we are aware of possible effect of changed milking parameters on the response of ewes to the relocation and milking in another parlour.

As it was mentioned above TMY was significantly lower in E0 in comparison to EB and E2 (Figure 1). This physiological response of ewes to the change of milking place supports also high occurrence of N milk flow type in E0 milking (Figure 1). High occurrence of N milk flow type could indicate inhibition of oxytocin release as a consequence of stress effect. The higher occurrence of N type of milk flow during stress response in ewes was found by other authors as well **(Marnet et al., 1998; Kulinová et al., 2012)** or in cows during milking in unfamiliar surroundings **(Mačuhová et al., 2002)**. Relatively higher frequency of N milk flow type during EB milking could be explained by higher vacuum level at parallel milking parlour under shelter causing faster milk flow from cistern.

Table 1 The effect of change of milking conditions and milking parlour (treatment) on parameters of milkability

Milking (n=34)					
Factor	**EB**	**E0**	**E1**	**E2**	**P**
TMY, (L)	0.647 ±0.04^{A}	0.527 ±0.04^{Ba}	0.576 ±0.04^{B}	0.593 ±0.04^{b}	0.000
MMY, (L)	0.341 ±0.03	0.272 ±0.03^{A}	0.374 ±0.03^{B}	0.384 ±0.03^{B}	0.000
MS, (L)	0.305 ±0.03^{A}	0.256 ±0.03^{a}	0.198 ±0.03^{Bb}	0.209 ±0.03^{B}	0.000
MT, (s)	60 ±7^{a}	59 ±7^{a}	87 ±7^{b}	70 ±7	0.008
LT, (s)	18 ±5	19 ±5	27 ±5	21 ±5	0.456
MMF, (L)	1.412 ±0.12^{A}	1.133 ±0.13	0.818 ±0.12^{B}	0.909 ±0.12^{B}	0.000

Total milk yield, machine milk yield, machine stripping, milking time, latency time, maximal milk flow, Evening milking before treatment (EB), on day of treatment (E0) and next two milkings (E1, E2).
$^{A, B}$ Means differ at *P <0.001, P <0.01,* $^{a, b}$ Means differ at *P <0.05*

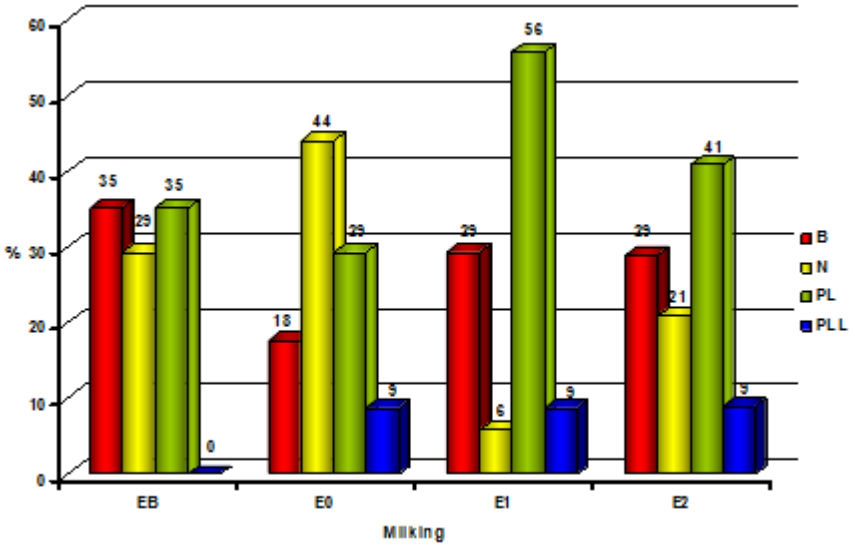

Figure 1 Frequency of distribution of milk flow types influenced by change of milking conditions and milking parlour

From more detail point of view the response of ewes to treatment was different on the basis of their sorting into groups. Within all groups, which were divided according to milk flow type (BG, NG, PLG) during first experimental milking (EB), frequency of milk flow types was changed as shown on Figure 2 A, B, C. The highest change of milk flow kinetic was observed in BG (Figure 2 - A) and the lowest change in B (Figure 2 - B) in response treatments.

Bruckmaier et al., (1993) and **Mačuhová et al. (2002)** observed strong disruption of oxytocin released in machine milking of cows in unfamiliar surroundings. Adaptation of primiparous ewes to machine milking was accompanied by reduced or inhibited oxytocin release **(Negrao and Marnet, 2003)**. Therefore we expected that ewes with B milk flow at EB milking will response more clearly due to inhibition of oxytocin release and thus changing of milk flow from B to N type. This was clearly confirmed in ewes in BG. It seams that ewes with B milk flow type could be considered as suitable animals because they represent the ewes with physiological response to machine milking and also with pathophysiological response to the change of milking conditions. Therefore these animals could be used forthe study the effect of milking management on milkability of ewes **(Mačuhová et al., 2012)**.

Table 2 The effect of change of milking conditions and milking parlour (treatment) on base milk components

Milking (n=34)					
Factor	**EB**	**E0**	**E1**	**E2**	**P**
fat (%)	7.22 ± 0.21	-	7.49 ± 0.21	7.44 ± 0.21	0.237
protein (%)	5.33 ± 0.1^{A}	-	5.34 ± 0.1^{a}	5.51 ± 0.1^{Bb}	0.002
lactose (%)	4.91 ± 0.05	-	4.93 ± 0.05	4.89 ± 0.05	0.623
SNF (%)	11.08 ± 0.10^{a}	-	11.12 ± 0.10	11.26 ± 0.10^{b}	0.043
DM (%)	18.04 ± 0.24	-	18.32 ± 0.24	18.44 ± 0.25	0.106
log SCC	5.37 ± 0.11	-	5.53 ± 0.11	5.46 ± 0.11	0.346

SNF - Solid non fat, DM - dry matter, SCC - somatic cell counts
^{A,B} Means differ at $P < 0.01^{a,b}$, Means differ at $P < 0.05$

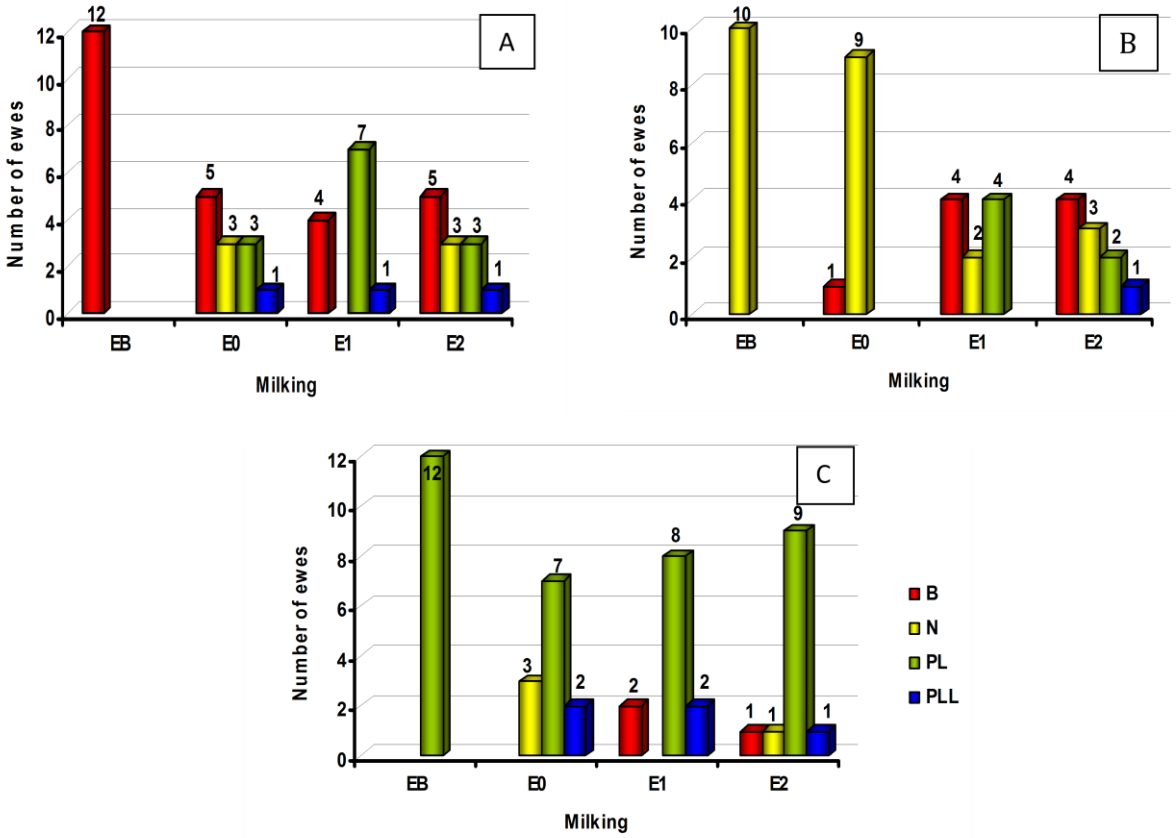

Figure 2A, B, C Frequency of distribution of milk flow types in groups differed by milk flow type during first experimental milking (EB) influenced by change of milking conditions and milking parlour (EO, E1, E2)

In milk composition, no significant differences were found between machine milkings for fat content, lactose content, dry matter and log SCC (Table 2). There was a significant increase of protein content in E1 and E2 compared to EB and increase of SNF in E2 compared to EB. In E0 we did not evaluate milk composition but when we take into account the fact that N milk flow types were high we could assume that percentage of fat content could be lower as compared with EB milking as reported **Antonič et al. (2013)**. The protein content is not influenced by milk distribution in the udder **(Tančin et al., 2007; Antonič et al., 2013)** though our aim was not to study nutrition effect the increasing protein level in milk after relocation could be ascribed to the intake of more protein nutrition (pasture, concentrate) as before relocation (corn silage, hay and concentrate). Feeding significantly influences the milk components in ewes **(Pulina et al., 2006)**.

CONCLUSION

The responses of ewes to stress from relocation and adaptation to other milking conditions were studied in ewes of Tsigai and Improved Valachian breed. Treatment significantly negatively influenced the milkability of ewes

but a more clear response was found in ewes with bimodal milk flow response to machine milking before treatment. From obtained results the response of ewes was partially influenced by technical parameters of the milking machine. However ewes have soon adapted to other milking conditions.

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MICROBIOLOGICAL EVALUATION OF POULTRY SAUSAGES STORED AT DIFFERENT TEMPERATURES

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ABSTRACT

The aim of our study was to evaluate the microbiological quality of poultry sausages, which were stored at different temperatures (4 °C, 15 °C). Total bacterial count, coliform bacteria, yeasts and filamentous microscopic fungi were detected in poultry sausages. Microbiological quality was evaluated using the horizontal method for the determination number of microorganisms. Total bacterial count in sausages stored at 4 °C ranged from 1×10^1 CFU.g^{-1} in sample 1 (after opening) to 4.35×10^4 CFU.g^{-1} in sample 1 (7th day of storage). Total bacterial count in sausages stored at 15 °C ranged from 3.25×10^3 CFU.g^{-1} in sample 1 (after opening) to 3.12×10^6 CFU.g^{-1} in sample 1 to 3.12×10^6 CFU.g^{-1} in sample 1 (7th day of storage). Coliform bacteria in sausages stored at 4 °C ranged from 1×10^1 CFU.g^{-1} to 3.15×10^5 CFU.g^{-1}. Coliform bacteria in sausages stored at 15 °C ranged from 1.54×10^3 CFU.g^{-1} to 1.40×10^6 CFU.g^{-1}. Yeasts and microscopic filamentous fungi in sausages stored at 4 °C ranged from 2.75×10^4 CFU.g^{-1} to 1.40×10^6 CFU.g^{-1}. Yeasts and microscopic filamentous fungi in sausages stored at 15 °C ranged from 1.30×10^4 CFU.g^{-1} to 1.44×10^6 CFU.g^{-1}. Total bacterial count, coliform bacteria, yeast and microscopic fungi were not in accordance with Codex Alimentarius of Slovak Republic on 3rd day in samples stored at 15 °C.

Keywords: quality; poultry sausage; meat product; shelf life; microbiological quality

INTRODUCTION

Meat production is one of the major activities in Europe. The main type of meat produced is pork (48.7 %) followed by poultry (23.6 %) and bovine (23.3 %). Meat and meat products present an ideal substrate supporting the growth of several spoilage and pathogenic bacteria **(Mataragas et al., 2006)**.

Kozelová et al. (2011) investigated consumer's opinion about quality of meat and meat products of Slovak and foreign production on the Slovak markets. Quality of foreign products is highly appreciated by 15% of respondents; higher quality was highlighted by 36% of respondents, 30% of respondents highlighted the quality as lower, 19% of respondents labelled the quality of those products as very low.

In the last decade, chicken-based meat products have become increasingly popular worldwide due to their high nutritional quality and low cost and are available as either fresh or precooked (i.e. fried) chicken and/or microbiological products, which after subsequent packaging are usually stored under refrigeration **(Barbut, 2002)**. Additionally, frozen chicken-based meat products also available on the market include specialties such as: nuggets, meatballs, hamburgers, frankfurters, etc.

Pathogenic non-spore-forming/spore-forming bacteria and viruses constitute a large proportion of all foodborne illness **(EFSA, 2007)**. The presence of these microorganisms in raw pork and poultry is the result of their contamination from the live animal, equipment, employees and environment **(Gianfranceschi et al., 2003, Gudbjornsdottir et al., 2004, Reij and Den Aantrekker, 2004 and Gibbons et al., 2006)**.

Poultry meat spoils after 4-5 days under refrigerated conditions **(Morshedy and Sallam, 2009)**, limiting trade in fresh product and causing considerable financial loss to the poultry industry **(Jimenez et al., 1997 and Patsias et al., 2006)**. Shelf-life is the period of time a product may be stored without becoming unfit for human consumption. The sensory shelf life is defined by organoleptic parameters and the product may be considered as spoilt when discolouration, off-odours and/or slime develop **(Nychas et al., 2008)**. The microbial shelf-life of poultry may be defined by the Total bacterial count (TBC) and the product is generally spoilt when bacterial counts reach 10^7-10^8 CFU.g^{-1}. The time to spoilage, and therefore shelf-life, depends on the initial carcasses counts. Psychrotrophic (cold tolerant) total viable count are used as an indicator of shelf-life for poultry **(Nychas et al., 2008)** while mesophilic (organisms that grow between 20 and 45 °C) total viable count, *Enterobacteriaceae*, *Pseudomonas* spp., lactic acid bacteria and yeast/moulds are used in the poultry industry as indicators of processing hygiene and microbiological quality **(Alonso-Calleja et al., 2004 and Álvarez-Astorga et al., 2002)**.

Minimising of microbial contamination on meat, including poultry, is dependent on the strict application of good farming practices (GFP) and hygienic processing. The latter is documented in the prerequisite (GMP/GHP) programme and hazard analysis and critical control point (HACCP) plans. HACCP includes critical control points (CCP), where an intervention may be used to prevent,

reduce or eliminate microbial contamination **(Loretz et al., 2010)**.

The aim of this study was to evaluate the microbiological quality of poultry sausages on the first day of storage, after three days of storage, and after seven days of storage of the products at different temperatures (4 °C and 15 °C). In poultry sausages microbiological parameters: total bacterial count, coliform bacteria, yeasts and filamentous microscopic fungi were observed.

MATERIAL AND METHODOLOGY

Microbiological quality of poultry sausages was evaluated. These products are categorized of soft meat products.

Microbiological evaluation consisted of three parts:
- determination of total bacterial count,
- determination of coliform bacteria,
- determination of yeasts and filamentous microscopic fungi.

There were evaluated 10 samples of poultry sausage, two analyzes were performed. Evaluation of poultry sausages were performed as follows:
- two samples were evaluated immediately after opening (first day of storage),
- two samples were evaluated after three days of storage at temperature 4 °C,
- two samples were evaluated after three days of storage at temperature 15 °C,
- two samples were evaluated after seven days of storage at temperature 4 °C,
- two samples were evaluated after three days of storage at temperature 15 °C.

Characteristic of coliform bacteria

Coliforms are commonly used bacterial indicators of sanitary quality of water and foods. They are rod-shaped Gram-negative non-spore forming bacteria which ferment lactose into acid and gas at 35-37 °C. Coliforms are common inhabitants of the gut of the warm-blooded animals, but they can be found in the environment, on vegetation and in soil. Their presence indicates the potential presence of pathogenic organisms. *Escherichia coli* is a facultative mixed-acid fermenting member of the coliform group being capable of fermenting lactose at 44 °C. Presence of *E. coli* is considered as an almost sure sign of fecal contamination **(Harwood et al., 2002)**.

Characteristic of total bacteria count

Detection of microbial contamination, particularly total bacterial count, sterility testing and selective determination of microorganisms, are common microbiological tests used on a large scale on food, environmental, medical and biological samples. Total bacterial count includes determination of mesophilic aerobic and facultative anaerobic microorganisms **(Baylis, 2003)**.

Characteristic of microscopic fungi

Microscopic fungi include yeasts and microscopic filamentous fungi (moulds) are very important organisms. They are employed in the production of pharmaceuticals, enzymes, organic acids and food, and some of them are associated with several diseases affecting humans and other animals **(Domingues et al., 2005)**.

Determination of total bacterial count, coliform bacteria, yeasts and filamentous microscopic fungi

The total bacterial count (TBC), coliform bacteria (CB), yeasts (Y) and microscopic filamentous fungi (MF) were determined. Plate diluting method was applied for quantitative CFU (Colony Forming Units) counts determination of respective groups of microorganisms in 1 g of meat products. Homogenized samples of meat components were prepared in advance by sequential diluting based on decimal dilution system application. Basic dilution (10^{-1}) was prepared as follows: 5 g of meat product was added to the bank containing 45 mL of distilled water. The cells were separated from substrate in shaking machine (30 minutes). Petri dishes of gelatinous nutritive substrate were inoculated with 1 mL of meat samples (TBC, CB, Y, MF) in three replications. For microorganism cultivation three types of cultivating mediums were used, to segregate individual microorganism groups. Plate count agar (E. coli) was used for CFU segregation of TBC (incubation 48-72 h at 30 °C, aerobic cultivation method). Dilutions of 10^{-3} and 10^{-4} were used to determine of TBC. Violet red bile agar (E. coli) was used for CFU segregation of CB (incubation 24 h at 37 °C, aerobic cultivation method). Dilutions of 10^{-1} and 10^{-2} were used to determine of CB. Chloramfenicol yeast glucose agar (E. coli) was used for CFU segregation of Y and MF (incubation 5-7 days at 25 °C, aerobic cultivation method). Dilutions of 10^{-1} and 10^{-2} were used to determine of Y and MF.

RESULTS AND DISCUSSION

The total bacterial count (TBC) in poultry sausages after opening of the products was 5.70×10^3 CFU.g^{-1} (sample 1) and 1×10^1 CFU.g^{-1} (sample 2). TBC on 3rd day of storage at 4 °C was 3.65×10^3 CFU.g^{-1} (sample 1) and 2.10×10^3 CFU.g^{-1} (sample 2).

TBC in poultry sausages on 7th day of storage at 4 °C was 4.35×10^4 CFU.g^{-1} (sample 1) and $2.56.10^4$ CFU.g^{-1} (sample 2) (Tab. 1). The Codex Alimentarius of Slovak republic (CA SR) indicates TBC (10^5), number of coliform bacteria (5.10^2) and microscopic fungi ($<10^1$). TBC in poultry sausages stored at 4 °C were in accordance with **CA SR (2006)**.

TBC in sausages on 3rd day of storage at 15 °C was 3.25×10^3 CFU.g^{-1} in sample 1 and 4.45×10^3 in sample 2. TBC in poultry sausages on 7th day of storage at 15 °C was 3.12×10^6 CFU.g^{-1} in sample 1 and 4.05×10^5 CFU.g^{-1} in sample 2 (Tab. 2). TBC in sausages on 3rd day of storage at 15 °C were in accordance with CA SR. Samples examined after seven days of storage at 15 °C were not in accordance with **CA SR (2006)**.

Al-Dughaym and Altabari (2010) found that the total bacterial count (TBC) in chicken nuggets were 2.7×10^4 and 3.0×10^6 CFU.g^{-1}. The *Staphylococcus aureus* counts were less than 10^2 CFU.g^{-1} and *Escherichia coli* was isolated from chicken nuggets in incidence of 60%, while *Salmonella* sp. was not detected.

Coliform bacteria (CB) in sausages after opening of product was lower than 1×10^1 CFU.g^{-1} in sample 1 and 1.0×10^1 CFU.g^{-1} in sample 2. CB on 3rd day of storage at 4 °C was lower than 1×10^1 CFU.g^{-1} in sample 1 and 2.20×10^2 CFU.g^{-1} in sample 2. CB in poultry sausages on 7th day of storage at 4 °C was lower than 1×10^2 CFU.g^{-1} in sample 1 and 3.15×10^5 CFU.g^{-1} in sample 2 (Tab. 3). The number of coliform bacteria in meat products on 7th day of storage at 4 °C were not in accordance with **CA SR (2006)**.

Olsen et al. (2000) found that the most common pathogens per meat category were (% mean values): a) bovine, *E. coli* (40.4); *Salmonella* spp. (26.9); and *Clostridium perfringens* (21.2), b) pork, *Salmonella* spp. (40); *Yersinia enterocolitica* (20); *Clostridium perfringens* (10); and *Staphylococcus aureus* (10), c) chicken, *Salmonella* spp. (60); *Staphylococcus aureus* (10); *Shigella* spp. (10); *Bacillus cereus* (10); and virus (10), and d) turkey, *Salmonella* spp. (50); *Staphylococcus aureus* (33.3); and *Clostridium perfringens* (16.7). Most common places of exposure were home and restaurants (malpractices/mishandling during food preparation).

CB in poultry sausages on 3rd day of storage at 15 °C was 1.95×10^3 CFU.g^{-1} in sample 1 and 1.54×10^3 in sample 2. CB in meat products on 7th day of storage at 15 °C was 3.05×10^4 CFU.g^{-1}in sample 1 and 1.40×10^6 CFU.g^{-1} in sample 2 (Tab. 4). The number of coliform bacteria in meat products at 15 °C were not in accordance with **CA SR (2006)**.

Enterobacteriaceae, a hygiene indicator **(Zeitoun et al., 1994)**, were also part of the microbiota of ground chicken meat. The population of *Enterobacteriaceae* (3.4 log CFU.g^{-1}) is indicative of adequate hygiene conditions of production in the poultry plant.

According to **Adams and Moss (1997)** *Enterobacteriaceae* can grow under vacuum packaging and high-pH values in meat and produce high levels of H_2S giving meat objectionable odours.

Pérez-Rodrígues et al. (2010) determined number of coliforms in cooked meat products from different establishments. Coliforms were found in 65% of analyzed samples, and counts were significantly lower than the other groups of microorganisms. The average value was 1.88 log CFU.g^{-1}, though it was obtained a maximum value of 4.90 log CFU.g^{-1}. *E. coli* was detected in 8 samples (<10 CFU.g^{-1}).

Yeasts and microscopic filamentous fungi (YaMF) in sausages after opening of product was lower than 1×10^1 CFU.g^{-1} in sample 1 and 2. YaMF on 3rd day of storage at 4 °C was lower than 1×10^1 CFU.g^{-1} in sample 1 and also in sample 2. YaMF in poultry sausages on 7th day of storage at 4 °C was 2.75×10^4 CFU.g^{-1} in sample 1 and 2.31×10^5 CFU.g^{-1} in sample 2 (Tab. 5). Number of yeasts and microscopic fungi in both samples on 7th day of storage at 4 °C were not in accordance with **CA SR (2006)**.

YaMF after three days of storage at 15 °C was 1.44×10^4 CFU.g^{-1} in sample 1 and 1.30×10^4 CFU.g^{-1} in sample 2. YaMF in poultry sausages on 7th day of storage at 15 °C was 3.24×10^4 CFU.g^{-1} in sample 1 and 1.44×10^6 CFU.g^{-1} in sample 2 (Tab. 6). Samples investigated on 3rd and 7th day of storage at tepmerature 15 °C were not in accordance with **CA SR (2006)** in number of microscopic filamentous fungi.

A total of 52 samples of ethnic meat products were collected and analyzed by **Rai et al. (2010)**. In all traditionally prepared meat products, lactic acid bacteria (LAB) were found at 10^6-10^8 CFU.g^{-1}. Yeasts were also recovered in all samples at 10^4-10^6 CFU.g^{-1}. The counts of bacilli were $<10^3$ CFU.g^{-1}. Filamentous fungi were also detected in a few samples at less than 10^3 CFU.g^{-1}. The occurrence of *Micrococcaceae* was found at 10^4-10^7 CFU.g^{-1}. The total viable count in the samples, collected from different places of the Himalayas, varied between 10^5 and 10^9 CFU.g^{-1}.

Table 1 Total bacterial count in sausages stored at 4 °C

Sample	TBC (CFU.g^{-1})		
	After opening	3rd day	7th day
1	5.70 x 10^3	3.65 x 10^3	4.35 x 10^4
2	1 x 10^1	2.10 x 10^3	2.56 x 10^4

Table 2 Total bacterial count in sausages stored at 15 °C

Sample	TBC (CFU.g^{-1})	
	3 rd day	7 th day
1	3.25 x 10^3	3.12 x 10^6
2	4.45 x 10^3	4.05 x 10^5

Table 3 Coliform bacteria in sausages stored at 4 °C

Sample	CB (CFU.g^{-1})		
	After opening	3 rd day	7 th day
1	<1 x 10^1	< 1 x 10^1	< 1 x 10^2
2	1.0 x 10^1	2.20 x 10^2	3.15 x 10^5

Table 4 Coliform bacteria in sausages stored at 15 °C

Sample	CB (CFU.g^{-1})	
	3 rd day	7 th day
1	1.95 x 10^3	3.05 x 10^4
2	1.54 x 10^3	1.40 x 10^6

Table 5 Yeasts and microscopic filamentous fungi in sausages stored at 4 °C

Sample	YaMF (CFU.g^{-1})		
	After opening	3rd day	7th day
1	<10	<10	2.75 x 10^4
2	<10	<10	2.31 x 10^5

Table 6 Yeasts and microscopic filamentous fungi in sausages stored at 15 °C

Sample	CB (CFU.g^{-1})	
	3 rd day	7 th day
1	1.44 x 10^4	3.24 x 10^4
2	1.30 x 10^4	1.44 x 10^6

CONCLUSION

The microbiological quality of poultry sausages stored under various temperature conditions was evaluated in this study. Quality of meat products is affected by the quality of raw meat, storage temperature and handling conditions. Current challenges and concerns related to consumption of meat products may be divided into those associated with microbial pathogens and into other meat safety issues. Major challenges related to microbial pathogens include foodborne illness outbreaks, associated product recalls, regulatory compliance, and issues related to microbiological control.

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ANALYSIS OF GENE EXPRESSION IN RABBIT MUSCLE

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ABSTRACT

Increasing consumer knowledge of the link between diet and health has raised the demand for high quality food. Meat and meat products may be considered as irreplaceable in human nutrition. Breeding livestock to higher content of lean meat and the use of modern hybrids entails problems with the quality of meat. Analysing of livestock genomes could get us a great deal of important information, which may significantly affect the improvement process. Domestic animals are invaluable resources for study of the molecular architecture of complex traits. Although the mapping of quantitative trait loci (QTL) responsible for economically important traits in domestic animals has achieved remarkable results in recent decades, not all of the genetic variation in the complex traits has been captured because of the low density of markers used in QTL mapping studies. The genome wide association study (GWAS), which utilizes high-density single-nucleotide polymorphism (SNP), provides a new way to tackle this issue. New technologies now allow producing microarrays containing thousands of hybridization probes on a single membrane or other solid support. We used microarray analysis to study gene expression in rabbit muscle during different developmental age stages. The outputs from GeneSpring GX sotware are presented in this work. After the evaluation of gene expression in rabbits, will be selected genes of interest in relation to meat quality parameters and will be further analyzed by the available methods of molecular biology and genetics.

Keywords: rabbit meat**;** meat quality**;** microarray; gene expression; candidate gene

INTRODUCTION

Increasing consumer knowledge of the link between diet and health has raised the awareness and demand for high quality food. Meat and meat products may be considered as irreplaceable in human nutrition, because they are important sources of protein, fats, essential amino acids, minerals (iron, selenium), vitamins (A, B12 and folic acid) and other nutrients **(Biesalski, 2005)**. Meat quality is characterized by a summary of all the factors which guarantee the nutritional, sensoric, hygienic and technological parameters of meat. Breeding livestock to higher content of lean meat and the use of modern hybrids also entails problems with the quality of meat. Consequently we encounter with the occurrence of defective physico-chemical, technological and sensory parameters of meat. Efforts of geneticists, breeders and processors are therefore now more directed at improving the meat quality and meat products **(Brestenský et al., 2002)**. Analysing of livestock genomes could get us a great deal of important information, which may significantly affect the improvement process. For example, mapping the genomes of cattle revealed a number of genes responsible for influencing the quality and quantity of meat **(Gábor et al., 2012)**. Knowledge of the sequence of the entire genome can immediately start studying the expression of any gene in the organism. Nowadays it is possible to synthesized oligonucleotide hybridization probes complementary to a transcript segment of any gene and we can use PCR method to produce a million copies of each gene in the genome. It also allows monitoring changes in gene expression of the entire genome in time during development, or in response to some changes in the environment. New technologies now allow producing microarrays containing thousands of hybridization probes on a single membrane or other solid support. Gene chips can carry more than 10000 oligonucleotide probes on a silicon membrane size of a few square centimeters **(Snustad & Simmons, 2009)**. Using conventional methods of molecular biology and genetics and the application of the latest technologies in the food industry, including microarray, it is possible to obtain accurate results with a specific meaningful values at a much shorter time.

Domestic animals are invaluable resources for study of the molecular architecture of complex traits. Although the mapping of quantitative trait loci (QTL) responsible for economically important traits in domestic animals has achieved remarkable results in recent decades, not all of the genetic variation in the complex traits has been captured because of the low density of markers used in QTL mapping studies. The genome wide association study (GWAS), which utilizes high-density single-nucleotide polymorphism (SNP), provides a new way to tackle this issue **(Zhang et al., 2012a)**. These studies help scientists widely to reveal the connection between individual SNPs and disorders that are passed down from one generation to another according to Mendel's laws of heredity **(Norrgard, 2008)**. Depending on the position and flanking sequences in the gene, SNPs may have different functional effects on protein sequence, transcriptional regulation, RNA splicing or miRNA binding. Decades various ongoing studies provide a wealth of information about

genes, chromosomal regions and pathways that may be associated with certain types of disorders and diseases. GWAS offer an agnostic approach to research associations between SNPs and disorders, and the results of these studies offer a countless data for studies of new generation **(Xu & Taylor, 2009)**. Currently, there are many information obtained through GWAS for a lot of livestock. Found information for well-studied species, can be used for comparison with other less explored.

Sporer, et al., 2011 developed and validated a turkey skeletal muscle-specific microarray as a tool for functional genomics studies and identified gene pathways and uncovered novel genes important in turkey muscle growth and development. In future experiments they will focus further on several of these candidate genes and the expression and mechanism of action of their protein products. Skeletal muscle growth and development from embryo to adult consists of a series of carefully regulated changes in gene expression. Understanding these developmental changes in agriculturally important species is essential to the production of high quality meat products. For example, consumer demand for lean, inexpensive meat products has driven the turkey industry to unprecedented production through intensive genetic selection. However, achievements of increased body weight and muscle mass have been countered by an increased incidence of myopathies and meat quality defects. The goals of study were to utilize this microarray to elucidate functional pathways of genes responsible for key events in turkey skeletal muscle development and to compare differences in gene expression between two genetic lines of turkeys. A total of 3474 genes were differentially expressed between at least two developmental stages across both genetic lines. Of these, 2544 genes were significantly affected for the RBC2 birds, whereas 2248 were significantly affected for the F birds. A greater number of genes were up-regulated in the earlier developmental stage of direct comparisons as compared to those that were down-regulated, and this observation was more pronounced in the RBC2 line than the F line.

Rabbit (*Oryctolagus cuniculus*) is very significant and globally widespread species of animal used as a laboratory model for research anatomy and physiology of the human diseases. It also serves as a model for studies of larger production domesticated species. Moreover, it is in Central Europe and certain parts of Asia reared as a meat animal. Beneficial properties of rabbit meat are widely recognized and have recently been examined thoroughly **(Paredi et al., 2012)**.

The main area of livestock research deals with the study of muscle fibers. MSTN and MYOG are considered candidate genes in relation to the growth and development of muscles. Myostatin (MSTN) is a secreted growth factor that is expressed predominantly in skeletal muscle, which negatively regulates the growth of skeletal muscle, whereas myogenin (MYOG) is involved in many important processes such as differentiation of myofibrillar cells. Therefore, it is expected that these genes may affect the growth of rabbit muscle. So far, however, their impact has not been clearly confirmed **(Kuang et al., 2012)**. Important endocrine factor growth hormone (GH) regulates the metabolism of growth and development. During the biological process, the first step is binding the GH to growth hormone receptor (GHR), followed by activation of the JAK-STAT pathway and expression of IGF1 (Insulin-like growth factor 1) and the other target genes. In addition, the GHR is a member of the superfamily of cytokine / hematopoietin receptors and consists of three functional domains, extracellular (ligand-binding) domain, a transmembrane domain and cytoplasmic domain (signal-transferring). GH binding to a receptor causes GHR dimerization and initiates signaling cascades through the cytoplasmic domain. GHR gene polymorphisms may affect the binding capacity of GH, which is considered as a candidate gene affecting growth and development carcasses in livestock. In rabbits, however, has not yet been extensively studied **(Zhang et al., 2012b)**. Among the candidate molecules involved in the regulation of energy homeostasis gets a special interest the melanocortin 4 receptor (MC4R), it is doubled G-protein receptor. Not only disruption of the gene encoding this receptor results in obesity in mice, but the interaction between leptin and MC4R signaling pathways highlights the correlation between body weight and food intake. Moreover, the mutations in the MC4R gene in swine associated with growth and fat thickness in relation to the feed intake. The cDNA encoding bovine melanocortin 4 receptor (MC4R) has been cloned and sequenced. Comparing with human, porcine and rat homologues showed 87, 85 and 89% identity at the DNA level, respectively more than 90% identity at the protein level **(Haegeman et al., 2001)**. In different breeds of rabbits were found SNPs in the coding region of MC4R gene. At position 237 bp were exchanged adenine for guanine. Allele A prevailed over the G allele in all studied meat breed. Analysis for determination the effect of genotype on rabbit utility confirmed the significant relationship between genotype AG, body weight and feed conversion. The results showed that the MC4R gene could be a candidate gene for the mass and slaughter yield in rabbits **(Jianq et al., 2008)b**.

MATERIAL AND METHODOLOGY

Analyzed animals were rabbits (males) inbred line M91, aged 6-weeks followed by 2, 3, and 5 months, in order to study the variation during ontogenetic developement. Breeding was carried out under the statute and zootechnical conditions of the experimental breeding rabbits at ÚMHZ CVŽV Nitra. The experiment was carried out 3 times.

Samples of muscle tissue from hind leg were collected immediately after slaughter of the animal using a sterile scalpel and under strict sterile conditions were immediately placed into pre-chilled tubes containing an equivalent amount of a stabilizing solution of RNA in tissue RNA Later ™ (Sigma).

RNA isolation:

Samples of muscle tissue were transferred to new plastic tubes in RNase-free conditions and immediately frozen in liquid nitrogen for 30 minutes. Then rapidly removed and homogenized in a small amount of liquid nitrogen in sterile friction plates using a mortar. About 30 mg of the homogenate was used for the isolation of RNA using a commercial kit Gene Jet™ RNA Purification Kit

(Fermentas) as described in the manual of the relevant kit. The concentration and quality of isolated RNA was measured by UV-VIS spectrophotometer NanoDrop ™.

RNA amplification and labeling:

Pure RNA was then amplified and labeled using the Two-Color RNA Spike-In Kit (Agilent) as described in the kit manual. After labeling, the samples were purified by converting Clean up protocol of a commercial kit Gene Jet™ RNA Purification Kit (Fermentas). Subsequently, the samples were again measured on a NanoDrop™ using module for microarray. Using this module it is possible to determine the amount of dye (pmol) built on micrograms of DNA / RNA.

Hybridization:

Labeled samples were applied to the Rabbit Gene Expression Microarray chip (Agilent), which were covered with a cover glass to separate the fields on the chip. Prepared chip was placed in a hybridization oven with carousel where the slide was revolving to ensure a constant flow of the sample through that field. Hybridization was carried out for 17 hours at 65 °C. The next step included washing in Wash solutions to remove unhybridized cRNA.

Scanning the microarray chip and data processing: Microarray slides were scanned using microarray scanner (Agilent) with two lasers: SHG-YAG laser (532 nm) and helium-neon laser (633 nm) at a resolution of 10 μm. TIFF images were visualized and processed using Feature Extraction Software 10.7.3.1 and text files were further analyzed by GeneSpring GX software.

RESULTS AND DISCUSSION

In addition to analyzing the different stages alone were also carried out two-color comparative experiments whose outputs (from GeneSpring GX) are presented in this work. They were compared with each other age stages in the order of 1. (6 weeks) versus 2. (2 months), 2. (2 months) versus 3. (3 months), 3. (3 months) versus 5. (5 months) and 5. (5 months) versus 1. (6 weeks). The samples were always labeled as green versus red: Cy3 versus Cy5 plot (Raw). Expression value is in this case referenced to a red sample.

One of the next steps in evaluating gene expression tends to be inclusion of genes into metabolic pathways and analysis of gene ontology. GeneSpring GX software provides these options but not for the rabbit genome. Therefore are included in this work only comparative analyzes, which allow easier manipulation with such a large amount of data (an average of 10000 genes). Summary of resulting data is shown in table 1. After evaluating the differences in expression between stages were selected only genes with expression changes greater than 2.

Comparison of gene expression in 1. and 2. age stage

In the analysis of differential gene expression in muscle tissue at 1. age stage (six weeks) and 2. age stage (2 months) were 7870 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 960 and down regulated genes with significant (multiple) expression changes were 635.

Comparison of gene expression in 2. and 3. age stage

In the analysis of differential gene expression in muscle tissue at 2. age stage (2 months) and 3. age stage (3 months) were 13802 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 380 and down regulated genes with significant (multiple) expression changes were 679.

Comparison of gene expression in 3. and 5. age stage

In the analysis of differential gene expression in muscle tissue at 3. age stage (3 months) and 5. age stage (5 months) were 8993 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 685 and down regulated genes with significant (multiple) expression changes were 1338.

Comparison of gene expression in 5. and 1. age stage

In the analysis of differential gene expression in muscle tissue at 5. age stage (5 months) and 1. age stage (6 weeks) were 9921 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 861 and down regulated genes with significant (multiple) expression changes were 988.

Table 1 Overview of gene expression in different age stages.

Age stage	Genes with expression changes >2 (total)	Significantly up regulated genes	Significantly down regulated genes
1. versus 2.	7870	960	635
2. versus 3.	13 802	380	679
3. versus 5.	8993	685	1338
5. versus 1.	9921	861	988

CONCLUSION

Based on these results we determine the application outputs focused on quality and safety of rabbit meat. We have found that there has been a change in gene expression in rabbit muscle, depending on their age. Genes have been identified, and further study will be analyzed individually using real time PCR. We are expecting to receive comprehensive information about gene expression in muscle cells of rabbits during critical stages of ontogenesis of muscle tissue. After the evaluation of gene expression in rabbits, will be selected genes of interest in relation to meat quality parameters and will be further analyzed by the available methods of molecular biology and genetics. Evaluating the gene expression could help us obtain knowledge and information about the energy metabolism of myocytes, which could then be used to determine the significant associations to the rabbit meat quality parameters.

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INFLUENCE OF MAGNETIC FIELD ON GERMINATION, GROWTH AND PRODUCTION OF TOMATO

Jaroslav Jedlička, Oleg Paulen, Štefan Ailer

ABSTRACT

In the study with tomatoes, there was ivestigated the impact of extremely low frequency electromagnetic fields on seed germination of tomato (*Solanum lycopersicum* L.) after treatment before sowing, as well as the growth of young plants that were exposed to low frequency electromagnetic field before planting in the field when growing transplants under cover. In the experiments conducted in two consecutive years (2012 and 2013), we followed the length of seed germination period of tomato variety „Pavlína“, growth of young plants expressed in plant height and root length, and the fruit size. Magnetization of seeds and young plants was carried out in laboratory conditions, plant growth was evaluated under cover prior to planting, and fruit traits were followed in field conditions where plants were grown on experimental plots (80.0 m^2). The plants were grown in accordance with the standards of an agricultural practice for tomato. At the generative phase, the fruits were collected at regular intervals, and their number and weight were evaluated. Low frequency electromagnetic fields acting at the three inductance levels (20, 40 and 60 mT) and exposure of 20 minutes a day with frequency of 50 Hz, significantly positively influenced the germination, plant growth and fruit size of the studied tomato variety.

Keywords: tomato; electromagnetic field; germination; growth; production

INTRODUCTION

Tomato crop is demanding, requiring thorough approach to the growing technology. It requires a warm location, ample moisture, especially at the time of fruit ripening. Irrigation water should not be applied onto leaves and fruits in order to prevent fruit cracking and spreading of fungal diseases due to wet leaves **(Uher et al., 2009)**.

In the experiment we investigated the effect of low-frequency static magnetic fields on germination, growth and fruits of tomato variety „Pavlína“ which is medium early variety, with determinant growth and robust stature, suitable for the cultivation in southern regions of Slovakia. The fruits are firm, sweet, and suitable for direct consumption and have a shelf life of 2-3 weeks.

Electromagnetic stimulation of seed as well as stimulation of plant growth are among environmentally sound pre-sowing treatment techniques, and should be assigned to the non-invasive, environmentally clean and friendly technologies **(Aguilar et al., 2009, Nimm and Madhu, 2009)**, suitable for organic farming **(Aladjadjiyan, 2010, Bilal et al., 2012)** and for weakening of seed dormancy **(Carbonell et al., 2004, Ratushnyak et al., 2008, Pittman, 1977, and Alikamanoglu and Sen, 2011)**.

The magnetic field affects living systems in interaction of its size and nature of exposure to biological material. It influences cells, tissues, and the other organs and organ systems different ways **(Belyavskaya, 2004, Ottová - Leitmanová, 1993 Toroptsev and Taranov, 1982)**. Studies in cells and organisms of plants confirmed that magnetic fields affected the metabolism of cells **(Belyavskaya et al., 1992, Dardeniz et al., 2006)**. **Sleper et al. (2008)** in their studies found that biological stimulation as influenced by abiotic factors played an important role in optimizing crop in terms of germination. Positive impact on germination and growth of plants mentioned in his study **Aladjadjiyan (2002)** and reported stimulative effect of the energy of magnetic field on the development and morphological characteristics of the plants. According to the same author **(Aladjadjiyan, 2010)** electromagnetic fields have a positive effect on the paramagnetic properties of some atoms in plant cells and pigments in them. In studies of **Rajendra et al., (2005)**, the authors proved that magnetic fields stimulated germination and growth of selected varieties of beans and peas, noting improvement in germination parameters and higher yields. **Masafumi, Takuya and Wataru (1998)** in their study reached better formation of roots of seedlings of maize, compared with the control group as a result of magnetic fields influence. **Fischer et al. (2004)** in their experiment exposed sunflower seedlings to magnetic field, and achieved small yet significant increase in the total green plant weight compared to the control plants. The studies of authors **(Kavi, 1977 and Lebedev et al., 1977)** state that in electromagnetically treated tomato seeds there was confirmed biostimulation effect of those fields in the early stages of germination and growth. **De Souza et al. (2006)** report that electromagnetic tomato seed treatment resulted in a significant increase in leaf area, leaf dry weight, the average weight of the fruits as well as an increase in harvest of tomatoes per unit area. **Martínez et al. (2009)** in their study indicated shortening of seed germination period of magnetically treated of tomato seeds.

The aim of the study was to determine the effects of low frequency electromagnetic fields on germination, growth and fruits of tomato variety „Pavlína".

MATERIAL AND METHODOLOGY

The experiment with tomato variety "Pavlína" was carried out in years 2012 and 2013. Part of the experiment was conducted in laboratory conditions - magnetization of tomato seeds and the magnetization of the young plants until the date of planting in open. Seeds and young plants were exposed to an electromagnetic field (EMF) at the level of induction of 20 mT (variant 1), 40 mT (option 2), or 60 mT (variant 3), with the same frequency (50 Hz) and the exposure time for 20 minutes a day. Magnetization of plants was performed every day within 48 days. Seeds and young plants in the control variant (variant 4) were not exposed to EMF.

The electromagnetic induction coil used in the experiment was made based on own project, taking into account the requirements of the experiment. An induction coil with an internal diameter 2R = 55 cm (Fig. 1) created a magnetic field defined according to **Horak and Krupka** (1976) relationship for the solenoid.

Description electromagnetic inductor

Electromagnetic inductor (coil), which we used in the experiment is composed of:

- Supply network driver,
- Voltage transducer,
- Meter of magnetic induction,
- Leading wires to the induction coil,
- Cylindrical induction coil.

The seeds of tomato were sown on March 10 into trays with universal substrate, and were regularly irrigated afterwards. 2 grams of seed produced in previous year was used for sowing in each of experimental years. After germination and froming of first pair of true leaves young plants were transplanted and grown under cover until May 15. Growing space with young plants after germination was ventilated to maintain a temperature of 15 °C, after transplanting day were increased to approximately 25 °C during night hours 15 °C.

Tomato plants were planted in field conditions for experimental fields on May 15, into light, soil rich in humus, at spacing 0.5 x 0.5 m. Surface irrigation was used according to need (soil moisture level).

In the first phase of the experiment the period of germination and growth of young plants (above-ground and underground part) were monitored, after planting in open field production of fruit was assessed (quantity and weight of ripe fruit). Results of the experiment were evaluated statistically by analysis of variance.

RESULTS AND DISCUSSION

The time required for germination of seeds in the experimental years is given in Table 1a and Table 1b.

As to the time needed for seed germination the shortest period was recorded in the second experimental variant (40 mT) - by 3 days (50%) shorter time compared to untreated seed. The longest period of germination was found in the control variant (0 mT) - 6 days (P <0.001).

Data on growth of young plants under cover in the experimental years are shown in Table 2a and Table 2b. Electromagnetic field had a significant impact on the growth characteristics of both aboveground part and roots of the experimental plants. The strongest growth was recorded in the second experimental variant in which aboveground part reached 0.45 m in average (55.2% compared to the control variant), and root length reached 0.36 m (71.4%).

Figure 1 Block diagram of the electromagnetic inductor device

Table 1a Period of seed germination in experimental variants (year 2012)

Variant	**EV1**	**EV2**	**EV3**	**CV**
Induction level (B)	20 mT	40 mT	60 mT	0 mT
Germination period (days), compared to CV	4,0 66.6% *	3,0 50.0% ***	4,0 66.6% **	6 ***; **; *

Table 1b Period of seed germination in experimental variants (year 2013)

Variant	**EV1**	**EV2**	**EV3**	**CV**
Induction level (B)	20 mT	40 mT	60 mT	0 mT
Germination period (days), compared to CV	3,5 41,6% *	3,0 50,0% ***	3,0 50.0% **	6 ***; **; *

EV1 - the first experimental variant; EV2 - the second experimental variant; EV3 - the third experimental variant; CV - control variant, *** P <0,001; **P <0,1; *P <0,5

Table 2a Growth of plants in experimental variants prior to planting to field conditions (year 2012)

Variant	EV1	EV2	EV3	CV
Induction level (B)	20 mT	40 mT	60 mT	0 mT
Aboveground part (m)	0.34 21,4%	0.44 57,1% ***	0.37 32,1% **	0.28 ***; **
Underground part root (m), relative increase compared to CV	0,20 0.0%	0,37 85.0% ***	0,27 13.5% **	0.20 ***; **

Table 2b Growth of plants in experimental variants prior to planting to field conditions (year 2013)

Variant	EV1	EV2	EV3	CV
Induction level (B)	20 mT	40 mT	60 mT	0 mT
Aboveground part (m)	0,30 0.0%	0,46 53.3% ***	0,39 30.0% **	0,30 ***; **
Underground part root (m), relative increase compared to CV	0,24 9.0%	0,35 59.0% ***	0,31 40.9% **	0.22 ***; **

EV1 - the first experimental variant; EV2 - the second experimental variant; EV3 - the third experimental variant; CV - control variant, *** P <0,001; **P <0,1; *P <0,5

The weakest growth was recorded in the control variant e.g. 0.29 m (aboveground part) and 0.21 m (roots). These differences were significant at P <0.001.

Average weight of tomato fruits harvested at the time of maturity reached in the experimental variants in individual years are shown in Table 3.

The highest mean weight of ripe fruit was observed in the second experimental variant (159 g), which was by 11.2% higher compared with the lowest mean fruit weight achieved in the control variant (143 g), and the difference was significant (P <0.01). In the all experimental variants, the mean fruit weight was higher than that recorded in control variant.

Treatment of seeds of tomato variety „Pavlína“, as well as the young plants with electromagnetic fields at different induction levels (20, 40 and 60 mT), single exposure 20 minutes a day by planting in the eperimental field in open significantly effected seed germination and subsequent growth of young plants, as well as the size of the fruits. The magnetically treated tomato seeds showed shortened germination period, and also faster growth of seedlings was recorded, greater length above ground (stem) and underground (root) systems, and earlier fruit setting up to 14 days compared to tomatoes in the control variant. Plants exposed to EMF produced larger fruit - mean weight of tomatoes in the second experimental variant wa by 17 grams higher than that in the control variant (0 mT). Fruits of the tomatoes ripened evenly, which may have major economic importance.

In all experimental variants (EV1, EV2 and EV3) we observed a positive effect of magnetic fields on all monitored parameters of the experiment, compared to plants and fruits in the control variant.

References on the biological effects of magnetic fields

Table 3a Mean fruit weight in experimental variants (year 2012)

Variant	EV1	EV2	EV3	CV
Induction level (B)	20 mT	40 mT	60 mT	0 mT
Mean fruit weight (g), relative Increase compared to CV	147 4,2%	157 11,3% **	150 6,3%	141 **

Table 3b Mean fruit weight in experimental variants (year 2013)

Variant	EV1	EV2	EV3	CV
Induction level (B)	20 mT	40 mT	60 mT	0 mT
Mean fruit weight (g), relative Increase compared to CV	147 1,4%	161 11,0% **	152 4,8%	145 **

EV1 - the first experimental variant; EV2 - the second experimental variant; EV3 - the third experimental variant; CV - control variant, **P <0,1

have shown that the magnetic field can cause or change number of phenomena, such as the increase in the expression of calcium channels **(Belyavskaya, 2004)**.

Studies carried out by the authors **Aksyonov et al. (2001)** confirmed that the 15 minute treatment of seeds of wheat by the magnetic field of induction of 30 mT caused amplification of root growth by nearly 25%, while the length of the stems of the plants was higher by 40% compared to the control plants. **Alexander et Doijode (1995)** report that the use of the magnetic field within the seed treatment before germination improved root growth and viability of rice and onions. Similar results reported **Murphy (1994) and Phirke et al. (1996)**.

Authors **Bachárová and Valšíková (2013)**, in the experiments found a beneficial effect of magnetic radiation on tomato seed germination at frequencies of 20 mT and 40 mT.

Garcia et al. (2001) in experiments with magnetically treated lettuce seeds have found their earlier germination compared with untreated seeds, which could be due to increasing the amount of water absorbed. Even **Hoff (1981)** in his experimental results indicated the impact of magnetic fields to increase of the intensity of photosynthesis and water penetration during the growth of the treated plants.

Earlier germination in magnetically treated seeds of tomatoes in their study was also mentioned by **Martinez et al. (2009)**. **Vashisth and Nagarajan (2010)** attributed to a shorter period of germination of magnetically treated sunflower seed increased activity of hydrolytic enzymes.

De Souza et al. (2006) and **Socorro et al., (1999)**, **De Koning (1993)**, his experiments with magnetically treated seeds of tomatoes confirmed higher mean fruit weight of tomatoes, as well as their overall higher yields, which is consistent with our findings.

CONCLUSION

In the experiment, we investigated the effect of low-frequency electromagnetic field on seed germination, growth of young plants and fruit size of tomato variety „Pavlína“. We found that:

1. Low-frequency electromagnetic fields had a stimulatory effect on germination, growth characteristics and size of tomato fruit.
2. The effects of magnetic fields on growth apices of tomatoes varied depending on the level of induction.
3. Treatment of tomato seeds and young plants with electromagnetic field at the time before planting in field conditions influences also the production of fruit.
4. Exposure of seeds and young plants to magnetic field in all the variations lead to achieving better quantitative results compared to that obtained with the control treatment, we observed better fruit quality parameters also.
5. Faster germination and growth of young plants during their growing in protected areas can have a major economic impact, whereas shortening of growing period saves energy costs and subsequent earlier ripening can bring direct benefit due to higher prices of produce.

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RELATIONS BETWEEN MUST CLARIFICATION AND ORGANOLEPTIC ATTRIBUTES OF WINE VARIETES

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ABSTRACT

Blowdown musts is important operation performed in winemaking, which can have a major impact on the future quality of the wine. Blowdown of the wine removes components that may carry elements that negatively affect the hygienic and sensory quality of the wine. Fining of musts and wines is carried either by a static method or using different fining preparations. The aim of this work was to evaluate the effect of different methods of decanting on the wine quality varieties of Sauvignon. The overall sensory quality was evaluated (100 - points system, and semantic differential) and the aromatic profile (profile method). All sensory evaluations were practiced by skilled sensory panel in controled conditions of Faculty sensory lab. Wine samples were clarified by static manner or with the assistance of the preparation applied to the clarification of wine in two different doses. By the results and their visualization of flavour and smell profile by spider plots we could conclude that pure cultures have positive effect on processed wine. Based on the results we found a beneficial effect of clearing by the clarification of the preparation based on cellulose, polyvinylpolypyrrolidone, gelatin and mineral adsorbents at 100 g.100 L^{-1} of the sensory quality of the wine.

Keywords: must; wine; organoleptic attributes; Sauvignon

INTRODUCTION

The number of new trends in the processing of grapes plays a big role in the quality of the wine and then the attractiveness of wine to consumers worldwide **(Fotopoulos 2003)**. Wine quality is affected by the composition of grape juice, which changes during the ripening of grapes and this is determined by the complex bundle of environmental factors (soil, slope, climate, technology aside), the genetic material (grape variety) and also the oenological practices and microorganisms represented during fermentation **(Le Moigne, 2008, Callejon, 2010; Bindon et al., 2013)**. In traditional winemaking fermentation is spontaneous, carried through different types of yeast. Fermentation is carried out by wild asporogenous yeast forms *Kloeckera apiculata* a *Candida pulcherrima.* These yeasts with increasing concentration of ethanol die and are replaced by noble cultural yeast alcoholic fermentation *Saccharomyces cerevisiae* **(Zohre and Erten, 2002)**. Yeasts are used in wine making since the ancient times. In modern viticultural practice is now almost exclusively used controlled fermentation process using pure cultures of yeast. For the preparation of these cultures are used noble yeasts *Saccharomyces cerevisiae var. elipsoidesus* and *Saccharomyces oviformis.* Noble yeasts have a large number of strains that differ from each other mainly in physiological and biochemical properties. Worldwide is grown hundreds of yeast strains that are suitable for fermentation of musts and wines. They produce desirable aromas and flavors in wine, and therefore their choice is very important **(Patel and Shibamoto, 2003)**. Fragrance belongs among important organoleptic wine characteristics. Most of the flavors in wine originate just during must fermentation **(Regodón Mateos et al., 2006)**.

A very important role in winemaking is the removal of constituents and impurities that cause turbidity of the must and can be carriers of factors with a negative impact on wine quality. By blowdown the particles are being removed, which got there during the process processing of grapes, sludge particles get into the must even with rotten grapes. On sediment particles are trapped also chemical residues from vine plant protection spraying, which adversely affect the fermentation process. The fermentation process can be adversely affected by the microorganisms that are found on the impurities in the must. Blowdown partially eliminates undesirable microflora and oxidative enzymes. Musts are blowdown immediately after pressing before the start of the fermentation process **(Malík, 1996; Moio et al., 2004, Cosme et al., 2008)**. Based on the gravity takes place the static blowdown, which is carried out by cooling the must for several hours below 10 ˚C. The settled sludge must be cleaned curls and prepared for fermentation. **(Pintér, 2012)**.

Evaluation of wine quality is based on sensory evaluation. Chemical analysis, however, are carried out in addition to explain some sensory observable changes **(Teissedre et al., 2011)**. Relationship between sensory evaluation and chemical compounds of wine is a crucial research subject of oenology **(Colagrande et al., 1988 a Girard et al., 2001)**. The aim is to determine which substances affect the sensory characteristics of the wine and how they relate to them **(Thorngate, 1997)**. Furthermore, the quantitative determination of certain

chemical compounds represents the criterion of origin of the wine **(Chira et al., 2011)**.

The goal of this paper was using different methods of sensory analysis to determine the impact of different methods of decanting must on sensory quality and aroma profile of wine varieties of Sauvignon.

MATERIAL AND METHODOLOGY

The grapes for the production of test samples came from Nitra wine-growing region of Radošinské vineyard turf from year 2012.

Variant **Sauvignon X** was harvested on 4th of September 2012 and reached the sugar content in must 22 °NM, and the average yield per hectare of 1.9 t ha^{-1}, according to the Law no. 313/2009 Coll. meets the classification in the category of „late harvest".

Variant **Sauvignon Y** was harvested seven days later than the first harvest, the sugar content reached 24 °NM, average yields per hectare of 1.8 t ha^{-1} and ripening is classified in category „selection of grapes".

After harvesting the grapes were pressed and got rid of stems. Obtained must was divided into four equal homogeneous parts, of which we have prepared our own experimental samples.

Sample A - must without decanting, without the addition of yeast with spontaneous fermentation.

Sample B - must with static decanting for 12 hours, without adding clarifying preparations, with the addition of active dry wine yeasts *Saccharomyces cerevisiae.*

Sample C - must clarified by the clarification preparation at a dose of 100 g. 100 L^{-1} of must, representing the maximum dose of the clarification preparation. The preparation was applied directly to the must. Yeasts *Saccharomyces cerevisiae* were applied to the clarified must after the must turbidity.

Sample D - must clarified by the clarification preparation at a dose of 30 g. 100 L^{-1} must, with the addition of yeast *Saccharomyces cerevisiae.*

Clarification consisted of preparation of highly pure cellulose, polyvinylpolypyrrolidone, gelatin and mineral adsorbents.

The process of fermentation took place at a standard temperature of 15 °C for 14 days. After the fermentation completion the wine was added and subsequently clarified with bentonite. After clarification was coiled up, filtered, and after thorough preparation to be bottled.

Produced wines were evaluated after finishing of the wine by selected sensory methods - 100-point rating system, profile and semantic differential method.

The 100 point rating system assesses the appearance of wine (max. 15 points), smell (max. 30 points, taste of wine (max. 44 points) and overall impression of wine (max. 11 points).

Profile method is a special quantitative method of descriptive evaluation. It is characterized by the fact that each sample must be from a large number of descriptors defined ones that best match a given sample. Profile method results are the product of intensity scales, which are compiled either for a variety of descriptors or for individual characters.

Semantic differential is widely used technique for treatment of certain stimuli. In this method, in most cases are selected 3 factors: rating scale good - bad, activity on a scale active - passive and robustness on scale strong - weak **(Suzuki et al., 2005)**.

RESULTS AND DISCUSSION

In appearance evaluators followed the clarity and color of the samples, the intensity of its aroma, softness and quality and with smell its intensity, grade, quality of taste and persistence.

The fourth endpoint was overall impression of wine treated in evaluating on the evaluator.

Based on the results (Table 1) of the 100-point evaluation, we can conclude that the sensory evaluators for the best specimens identified production experimental technology sample C, in which the maximum dose used was the clarification preparation of fining agents in must and must was subsequently yeasted with pure culture yeast of Saccharomyces cerevisiae. The second best sample was sample D of Savignon Y with the minimum dose of fining agents. Based on the results we can focus on the fact that variant Sauvignon Y was of better quality for wine production compared to variant Sauvignon X. This argument is reflected in all tested samples.

For the evaluation of the profile method we used descriptors of smell typical for the variety Sauvignon. Wines made from Sauvignon varieties are characterized by distinctive sensory properties. For these wines are characterized fruit and vegetable tones **(Parr et al., 2007; Cozzolino et al., 2011)**. They can contain herbal tones, gooseberry, grapefruit, green pepper, red pepper and also tomato leaf **(Pulko et al., 2012)**. **Swiegers et al. (2009)** argues that the typical Sauvignon aromas are of green pepper, tomato leaves, asparagus, grapefruit, gooseberry and fruit extracts. These tones can be described as green and tropical. **Parr et al. (2007)** found that the variety Sauvignon, the most common descriptors determining were green pepper, herbal and grassy notes. They argue that these so called green tones are an important feature in the evaluation of wines, but should not dominate. These so called green tones dominate especially at lower sugar content, while at higher sugar content are dominated by fruity notes and tropical fruit. Sauvignon varieties are sometimes considered to be simple and non flower white varieties **(Parr et al., 2010)**.

Table 1 Results of sensory evaluation of samples obtained by one hundred point wine rating system

	Sample A	Sample B	Sample C	Sample D
Sauvignon X	79,0	79,2	83,2	78,2
Sauvignon Y	82,0	82,2	85,4	83,2

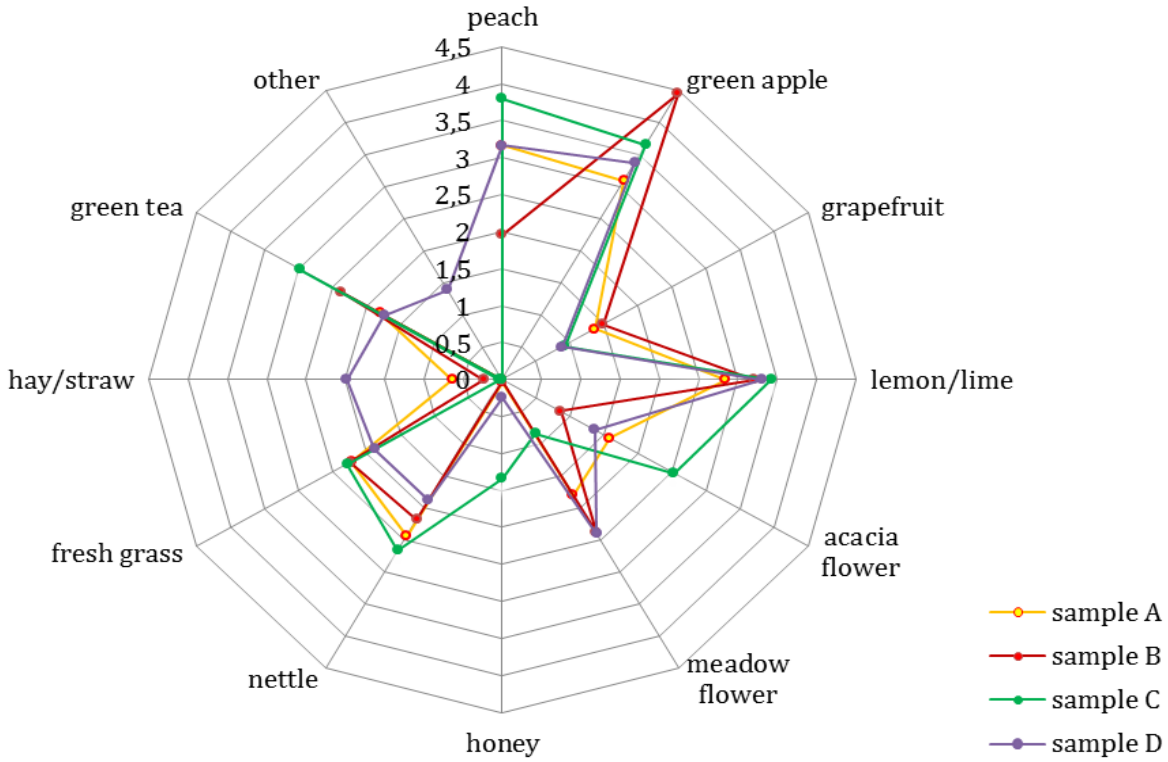

Fig. 1 The sensory profile of wine Sauvignon X variation

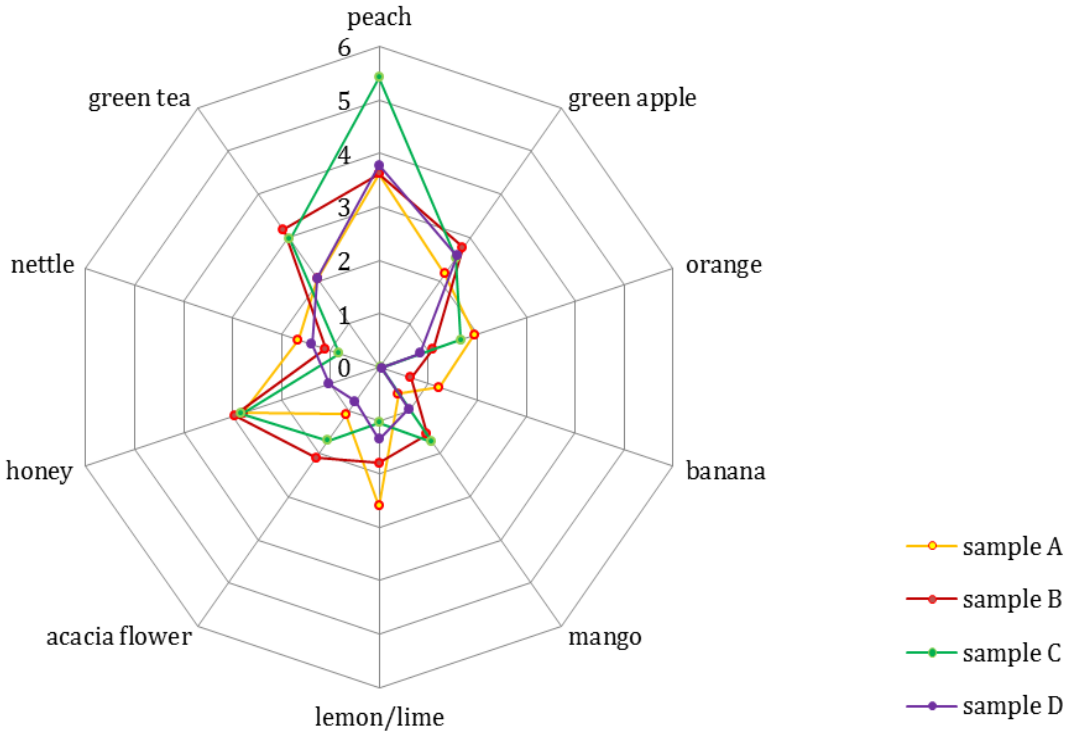

Fig. 2 The sensory profile of wine varieties of Sauvignon harvest Y

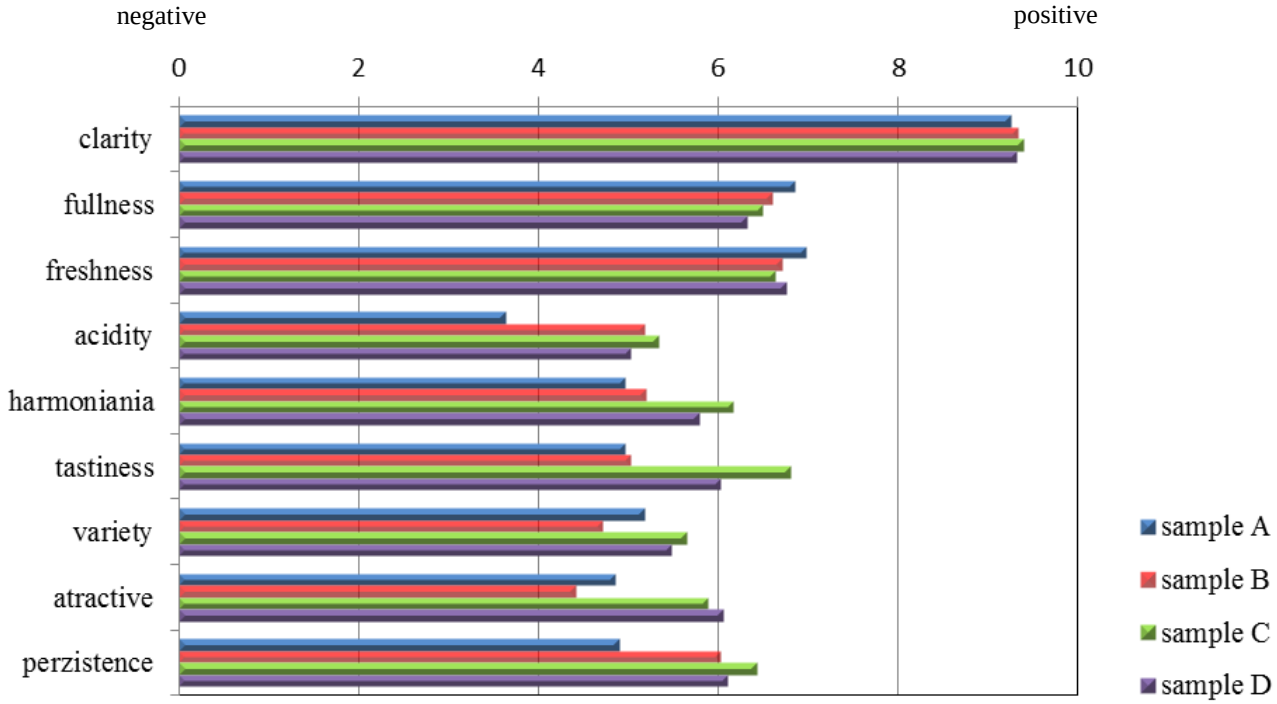

Fig. 3 Semantic differential of Sauvignon X

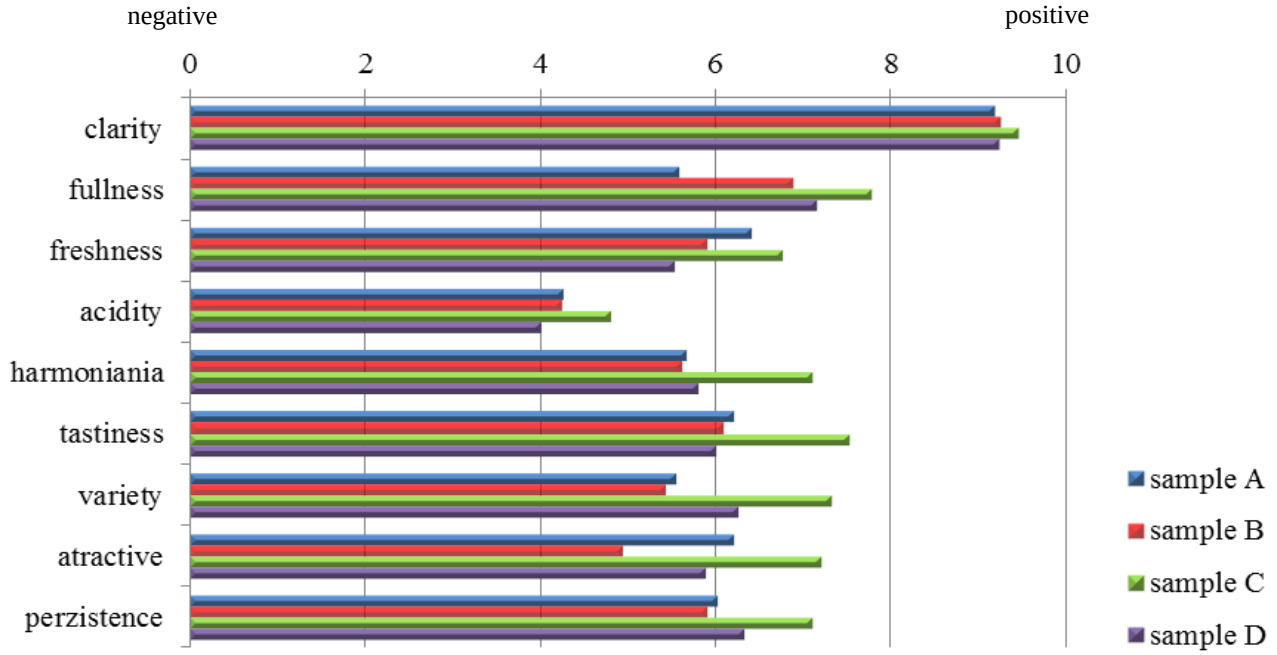

Fig. 4 Semantic differential of Sauvignon Y

Decisive influence on varietal wines Sauvignon have aromas of yeast strain selection and fermentation temperature **(Masneuf - Pomarede et al., 2006)**.

The most striking peach aroma had wine samples C, which were produced under the maximum clearing technology of must using yeast, contrary to the lowest level occurred in the sample B produced by static blowdown using yeast. In the sample B prevailed scent of green apple. Grapefruit has been identified in all samples at low intensity, strongest is in the sample B. Other citrus fruits such as lemon and lime dominated in the sample C produced using the maximum fining agents in must, for which dominated also the smell of acacia flowers. Meadow flowers predominated in the sample D, in which the minimal dose clarified fermentation was used using pure yeast culture. In the sample C evaluators also identified honey aroma, which almost did not occur in other samples. In the same sample were set at a higher level and nettle tones, fresh grass and green tea. Tones of hay and straw, as well as other flavorings prevailed in the sample D, these did not almost occur in other samples almost.

In samples of wines dominated peach flavor, which was most pronounced in the sample C, in the other samples, this was balanced flavor, moderate. Green apple fragrance was present in all samples, this aroma, which was noticeable at a lower level. Citrus notes such as orange, lemon and lime were evident in the sample A and in that sample was present banana aroma, but just like the smell of other citrus fruits was low. Scent of acacia flowers and the scent of green tea was also prevalent in the sample B. Honey scent is equally strong in all samples, except for sample D, which occurs only at very low levels. Nettle tones were present in all samples at very low levels.

Semantic differential is a simpler method of sensory evaluation of wines. The method is derived from a 100 point system, while we determine the intensity of each evaluation descriptors.

Character clarity of wine in all samples was evaluated very positively. All samples were evaluated as sparkly, differences between the samples were minimal. Similarly, in characters fullness and freshness of the samples were balanced.

Significant differences in the quality the evaluators found in the characteristics of acidity, flavor, variety, attractiveness and persistence. Least acidic was sample A, the most pronounced acidity was found in the sample C. All samples except sample A had a pleasant acidity.

The harmony of taste and flavor were the most valuable specimens, which was used for Ciriaco, settling material. Variety for all variants was set at a moderate level n terms of attractiveness for consumers were the best samples C and D blowdown with the help of the clarification plant. Shortest persistence was determined for sample A. The longest persistence had sample C.

When evaluating samples of Sauvignon Y reached the best quality in all the characteristics the sample C, i.e. sample, which was clarified before fermentation using a maximum dose of the clarification plant. Rated variants surpassed in all respects, most notably it was in harmony characteristics, palatability, variety, attractiveness and persistence.

CONCLUSION

The goal was to assess the effect of clearing on the sensory profile of wine varieties Sauvignon. We used two variants with different sugar content of must and 4 different ways of must clarification. Based on the evaluation results, we can conclude that the wines from both alternatives had a very good sensory quality. On the basis of a 100 point system was as a better sample identified the one from a late harvest, which reached a higher sugar content, but also better overall sensory profile of the characteristics flavor.

By evaluation sample profile method, we focused on monitoring the aromatic profile of wines. We found that for evaluators were most attractive wine samples, which musts were before fermentation clarified by the clarification formulation at a dose of 100 g.100 L^{-1} and then leavened by pure culture yeast. These samples were characterized by strong peach flavor and aroma of green apples, which were gently completed by the scent of citrus fruits, acacia flowers and honey. Significant differences in scent-profile were found between samples variation Sauvignon X. Samples Sauvignon variant Y were in the fragrance of wine more balanced.

By semantic differential we evaluated the wines based on the complex sensory site, in more detailed way than the 100 - points system. We found that samples which musts were before fermentation clarified with the help of the clarification plant were better evaluated in palatability traits, harmony, variety and persistence. Samples Sauvignon variant Y also in fullness and attractiveness.

Based on the results of the sensory evaluation methods of wine, we can conclude that the clarification and then fermenting of musts for using pure cultures of yeast has a beneficial effect on the sensory character and overall attractiveness of wine.

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CHANGES OF THE DOUGH RHEOLOGICAL PROPERTIES INFLUENCED BY ADDITION OF POTATO FIBRE

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ABSTRACT

Fibre is an important component of diet and nutrition and is one of the groups of biologically important substances consumed in insufficient quantity. One possibility to increase its intake is to enrich with fibre the food consumed in significant quantities, such as bread and pastries. The aim of this experimental work was to verify the influence of the addition of commercially produced potato fibre Potex to wheat flour and to evaluate the changes in physical properties of subsequently prepared doughs. The addition of 1%, 3%, 5% and 7% of Potex has been chosen and rheological properties were evaluated by the means of E-Farinograph (consistency, water absorption, dough development time, stability and degree of softening), Extensograph-E (resistance to extension, maximal resistance to extension, extensibility, extensibility maximum and energy dough), and Amylograph-E (beginning of gelatinization, viscosity, maximum of gelatinization). The addition of Potex depending on the amount changed the physical dough properties: above 3% of addition significantly increased the beginning of gelatinization and with increasing addition the maximum of gelatinization has increased. Based on the evaluation of farinograph curves it can be concluded that the addition of Potex increased water absorption of composite flours, prolonged dough development time and dough stability. Extensograph measurements showed, with increasing addition of Potex the decrease in dough extension. All doughs were easy to process and with the suitable amount of addition their satisfactory parameters could be maintained. From a technological point of view, the addition of fibre (Potex) up to 3% was fully acceptable and did not significantly alter the rheological properties of processed doughs.

Keywords: bread; potato fibre; kneading; dough rheology

INTRODUCTION

Bread and pastries are basic foods consumption of which in Slovakia, despite the downturn in recent years still reaches high numbers, around 67 kg per person per year (Figure 1). However, considering the technology of milling of cereals, where the milling process changes the appearance and nutrition value of cereal grains by separating the bran and germ from the endposperm and reducing the particle size **(Barbosa and Yan, 2003)**, it is appropriate to consider the enrichment of bread with nutritionally important ingredients that would increase its nutritional benefits for consumers **(Paturi et al., 2012)**. One of the groups of biologically important substances consumed in insufficient quantities is fibre, which is often deficient in the diet. Generally speaking, dietary fibre is the edible parts of plants, or similar carbohydrates, that are resistant to digestion and absorption in the small intestine **(Lattimer and Haub, 2010)**. There are many beneficial effects of increased dietary fibre consumption on human health and body function.

Codex alimentarius Commission **(FAO/WHO 2009)** defines dietary fibre as carbohydrate polymers with ten (or three) or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

Edible carbohydrate polymers naturally occurring in the food as consumed; carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities; and synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

Many studies have confirmed the beneficial effect of fibre on human health, especially on the physiology of digestion **(Fardet, 2010; Yikyung et al., 2011; Gong and Yang, 2012)**. In addition to the favourable preventive effect on development and progression of gastrointestinal diseases, such as colon and rectum cancer, chronic inflammation of the colon, gallbladder diseases, fibre is important in the prevention of so-called lifestyle diseases - type 2 diabetes mellitus, obesity, cardio-vascular diseases, etc. **(Champ et al., 2003; Marcil et al., 2003; Mohamed, 2014)**.

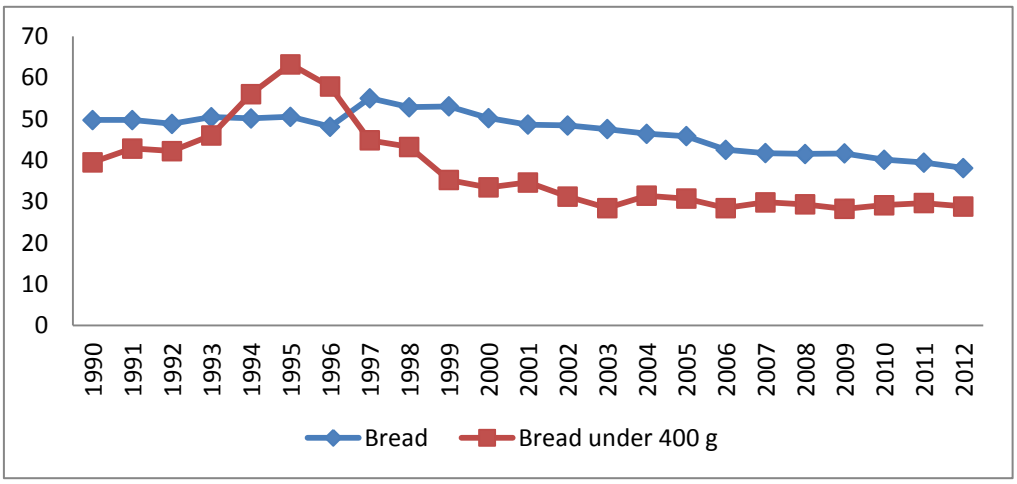

Figure 1 Consumption of bread and bread under 400g per person per year in Slovakia **(URL 1)**

Based on the recommendations (recommended dietary intakes) the daily fibre intake should range from 1 g (children up to 6 months) to 30 g per day depending on age, gender and physical demands of the work. One possibility for securing that intake is consumption of fibre-enriched bread; however, the possibility of its production is affected by the processability of dough. In the frame of our research work the potato fibre Potex has been added to wheat flour what caused the changes in the monitored and evaluated rheological properties of kneaded dough.

MATERIAL AND METHODOLOGY

For the production of dough the wheat flour T650 was used, to which 1%, 3%, 5% and 7% of potato fibre (POTEX, source: LYCKEBY CULINAR, Horaždovice, Czech Republic) was added with the following composition (g per 100 g of product): fibre 65 g, carbohydrates 17 g, 5 g protein, 0.3 g fat, dry matter min 90%)

Composite flour was analysed in order to determine the rheological properties by the means of following devices: Farinograph-E, Brabender OhG, Duisburg, Germany (ICC Standard 115/1, 1992, AACC Method 54-21, 1995). The mixing curve is characterised by an ascending part that indicated the changes during the dough development process, while the subsequent decline in the resistance is taken as a sign of a steady breakdown of the dough structure upon mixing beyond the point of optimum development. Optimum development from the standpoint of bread quality may occur slightly past "mixing peak". The effects of the Potex addition on consistency, water absorption, dough development time, stability and degree of softening were established (FU = farinograph units - unit commonly used for evaluation of rheologic consistency in bakery practice. It was defined by Brabender company. The physical equation is Nm - 100 FU = 0.1 Nm).

Extensograph-E, Brabender OhG, Duisburg, Germany (ICC-Standard 114/1, AACC Method 54-10) measures dough extensibility and dough relaxation behaviours. The effects of the Potex addition on resistance to extension, maximal resistance to extension, extensibility, extensibility maximum and energy dough were established.

Amylograph-E, Brabender OhG, Duisburg, Germany (ICC-Standard 126/1, AACC Method 22-10). The effects of the Potex addition on beginning of gelatinization, viscosity (enzyme activity), maximum of gelatinization were established (AU = amylograph units - unit commonly used for evaluation of rheologic consistency in bakery practice. It was defined by Brabender company. The physical equation is Nm - 100 AU = 0.1 Nm).

Control dough without Potex was also analysed.

RESULTS AND DISCUSSION

The potato pulp and potato peel are formed as a result of industrial processing of potatoes. Potato peel can be used after modification as an addition in wheat bread **(Kaack et al., 2006)**. The major components of commercially produced potato fibre Potex are non-starch polysaccharides. Potex constitutes a potato fibre, preparation widely used as an ingredient to meat and bakery products which with thermal treatment results in creation of new compounds **(Perez-Jimenez et al., 2014)**. Melanoidins are high molecular weight brown end products of Maillard reaction (formed in the process of bread baking), and few data presenting tumour cell growth inhibiting activity of melanoidins have been reported. The results suggest potential application of Potex preparation as a functional food ingredient and chemopreventive agent **(Langner et al., 2011)**.

The mixing process is the crucial operation in bakery industry by which the wheat flour, water, and additional ingredients are changed through the mechanical energy flow to coherent dough. It is well known that dough properties can be affected by many features with different significance, therefore the dough development and processing optimization towards best quality bakery products is quite a difficult problem.

Regarding the rheological properties of prepared doughs with the addition of Potex, based on the amylografic evaluation the significant (double) increase in the beginning of gelatinization was recorded (AU = amylograph units) with the addition of 3% Potex and more (Figure 2). With the increasing amount of addition maximum of gelatinization was increased, for all samples the values were higher than the optimum. (**Bojňanská et al., 2013**). It can be assumed that this

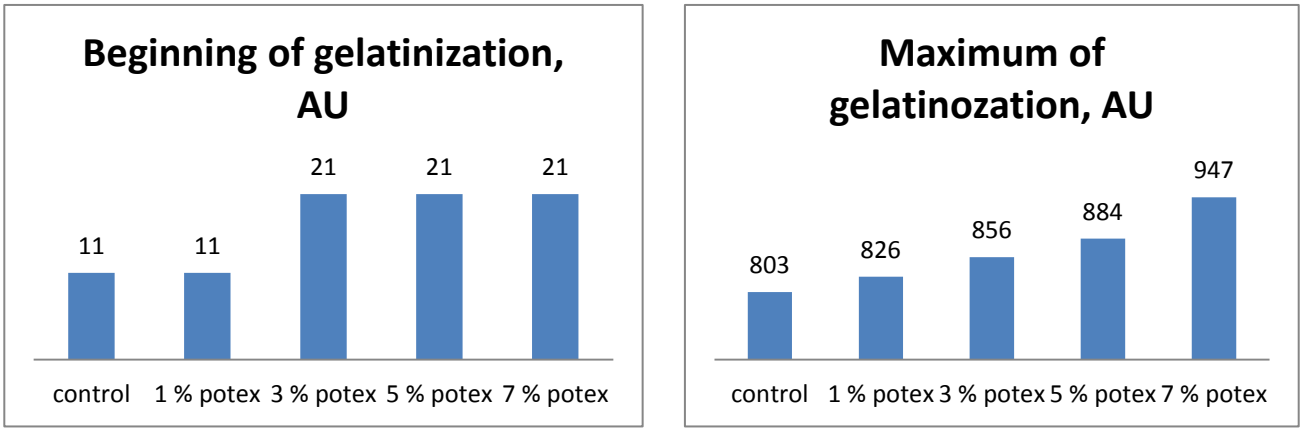

Figure 2 Amylographic properties of prepared doughs with the addition of Potex (AU = amylograph units)

phenomenon could be caused by the increased content of non-starch polysaccharides that form viscous solutions and are part of Potex **(Mohammed et al., 2012; Almeida et al., 2013)**. Obtained values suggest deterioration of properties of breadcrumb, surface and other bread properties.

Changes of **farinograph characteristics** of dough with the addition of Potex are shown in Figure 3a to 3e. With an increasing proportion of Potex slower hydration of the dough components and prolongation of the period of dough development were observed. With the increasing addition of Potex the dough stability increased and dough flexibility slightly decreased compared to control samples. **Almeida et al. (2010)** verified that the fibres studied altered the main farinographic parameter drastically, suggesting that the incorporation of these fibres in bread making processes leads to various consequences to the dough forming stage (mixing), which must be considered for the adjustment of process parameters.

The addition of Potex significantly increased the water absorption of composite flours, which was caused by the presence of fibre components. As mentioned in **Camire et al. (1997)** high water absorption of potato fibre is mainly due to the high proportion of cellulose, hemicellulose and lignin. With increasing addition, the dough development time increased, with 5% and 7% of Potex to unacceptable 15 to 20 minutes. With regard to the development time, the incorporation of fibre increased the time to reach the maximum consistency of dough, as has already been observed by **Sanz Penella et al. (2008)**.

It can be assumed that due to the higher content of fibre components there was a change in the structure and hence delayed hydration and later the creation of compact homogeneous dough mass. This effect could be attributed to a fibre-gluten interaction, which prevents protein hydration **(Gomez et al., 2003; Rosell et al., 2006)**.

Stability of dough was significantly prolonged. Under certain processing conditions this can be interesting, but such dough is harder to process with higher demands for energy.

When analysing the dough **extensografic characteristics** extensografic curves were obtained (Figure 4).

Based on the obtained results it can be pointed out that the changed of dough character with the addition of Potex were in favour of elastic properties. To create an optimal dough structure desirable viscoelastic properties are needed, not just its elasticity or viscosity. Very elongated doughs (low and wide curves of extensograf) are characterized by spreadability (fluidity), which is undesirable because of the low volume of bread, which is caused by leakage of CO2 through the weakened dough structure. On the other hand, very firm doughs (high and narrow curves of extensograf), do not allow the increase of the products volume due to the very solid dough structure. The formed CO2 does not have sufficient overpressure to increase product volume.

With increasing proportion of Potex significant decrease in dough elongation was observed (as reflected in narrowing the curve). **Gomez et al. (2003)** investigated the effect of the addition of different types of fibre on the rheological properties of wheat dough. Their findings show a decrease in elongation rheology of doughs with the addition of fibre compared to the wheat dough, which is consistent with the results obtained by us. The bigger the dough elongation the looser the dough, but too low values are not desirable to produce bread with demanded parameters **(Dodok and Szemes, 1998)**. Fibre incorporation into bread dough systems greatly interferes with protein association and behaviour during heating and cooling, for example, the incorporation of sugar beet fibre into the dough matrix induces the disruption of the viscoelastic system yielding weaker doughs, and it greatly competes for water with starch affecting pasting and gelling. Conversely, inulin in the range tested seems to integrate into the dough increasing its stability **(Rosell et al., 2010)**.

It can be stated that the addition of Potex did not change significantly the extensografic energy, but importantly affected the dough properties in a way that the values of elongation decreased and the extensografic maximum increased. It shows that although the extensografic energy was about the same for all douh samples, its properties were different.

Almeida et al. (2013) verified that, depending on the type and quantity of the dietary fibre source used, different responses can be obtained for process parameters and final quality characteristics of bread. Fibres can be used by the food technologist in bread formulations.

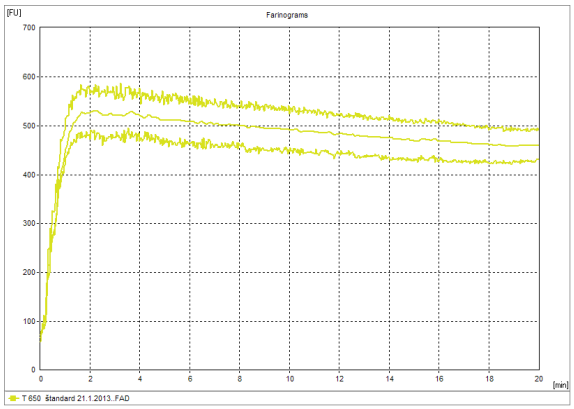

Figure 3a (control)

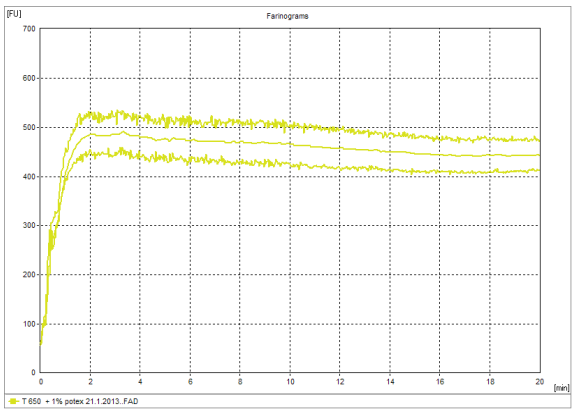

Figure 3b (1 % Potex)

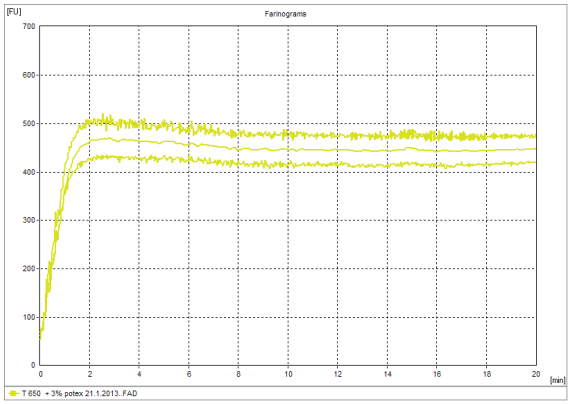

Figure 3c (3 % Potex)

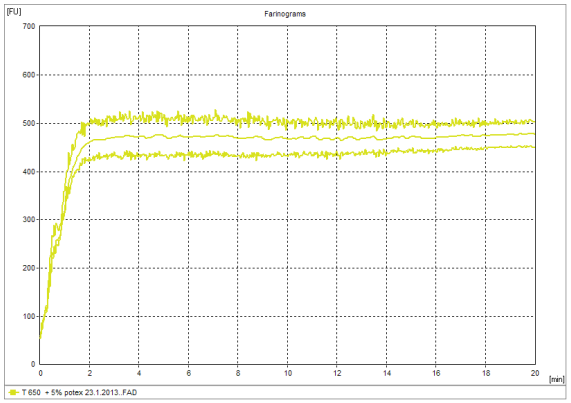

Figure 3d (5 % Potex)

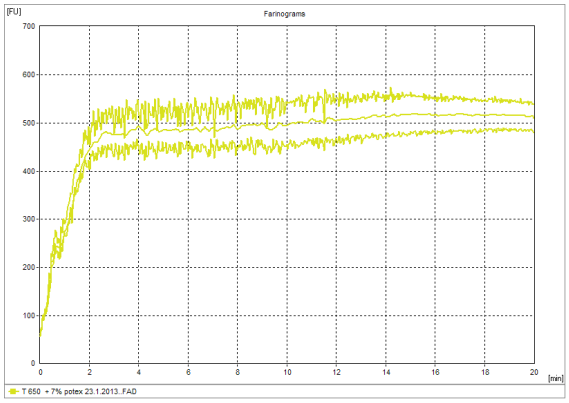

Figure 3e (7 % Potex)

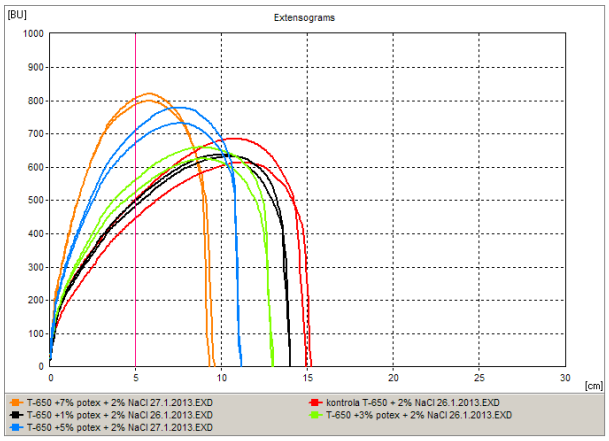

Figure 4 Extensographic parameters

Potato fibre combines the positive properties of insoluble fibre with its innate starch in a perfect way. One key technological property of fibre containing cellulose is the construction of a three-dimensional fibre network in the end product greatly improving the texture and stability of the food. In addition the moisture binding and retention of the cellulose from the potato which are independent of external factors such as temperature, pH value or storage time, are synergically complemented by the temperature-induced water-binding ability of the starch. That is why potato fibre is distinguished by an extremely high water-binding capacity **(Meyer et al., 2009)**.

It has been found that the addition of fibre to the bread dough improves its nutritional value **(Fardet, 2010; Mohamed, 2014)**, and can also positively influence the rheological properties of the dough. Furthermore, the addition of fibre extends product shelf life and ultimately improves qualitative and sensory properties of bread **(Gómez et al., 2003)**. The addition of fibre in the products is particularly important in terms of the required increase of its daily intake and reducing the calorific value of the bakery products. With a suitably chosen amount of addition satisfactory technological parameters of dough can be maintained.

CONCLUSION

Based on the results of evaluation of the physical characteristics of doughs prepared from composite flours with addition of 1%, 3%, 5% and 7% of potato fibre Potex it can be stated that the addition of fibre affects the rheological behaviour of dough during processing.

From a technological point of view, the addition of 3% of potato fibre (Potex) was fully acceptable and did not alter the rheological properties of the processed dough. Higher amounts significantly increased the beginning of gelatinization and with increasing addition the maximum of gelatinization was increased. Based on the evaluation of farinograph curves it can be concluded that the addition of Potex increased water absorption of composite flours, prolonged dough development time and dough stability. Extensograph-E measurements highlighted the decrease in elongation of dough with increasing addition of Potex. All prepared doughs were easy to process and their satisfactory parameters can be maintained with the addition of a suitably chosen amount. From a technological point of view, the addition of 3% of potato fibre (Potex) was fully acceptable and did not alter the rheological properties of the processed dough. Whether the product from such dough is accepted by consumers is at the end decided based on its sensory quality.

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EFFECT OF BEE POLLEN EXTRACT AS A SUPPLEMENTAL COMPONENT OF DIET ON BROILER´S ROSS 308 BREAST AND THIGH MEAT MUSCLES FATTY ACIDS

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ABSTRACT

The present study was aimed to study the effect of the bee pollen extract on the broiler Ross 308 breast and thigh meat fatty acids. The experiment enrolled 90 chicks in one day old, which were divided into 3 groups (control, E1 and E2). The broiler has been bred in a cage condition for 42 days. To the experimental groups were added bee pollen extract in the amount (400, 800 mg.kg^{-1}). The chickens have been bred in a cage conditions, each cage was equipped with feed dispenser and water intake was ensured *ad libitum* through a self feed-pump. The temperature was controlled during the fattening period and it was 33 °C at the first day and every week was reduced about 2 °C the end temperature was 23 °C. At the end of the experiment the fatty acids have beenevaluatedby using Agilent 7890A Gas Chromatograph apparatus (USA). The findings have been shown that the myristoleic acid, linoleic acid, linoelaidic acid, arachidonic acid, and archaic acid were decreased after using the bee pollen into broiler feed mixture otherwise, the bee pollen has been increased the polemic acids and oleic acid and there were found no significant differences (P ≥0.05) among all the experimental groups.From the recent experiment, we conclude that bee pollen extract has decreasedthe fattyacids except palmitoleic acid acid and oleic acid, whichwere higher compared to control groupand there were no significant differences (P ≥0.05) between experimental groups.

Keywords: broiler Ross 308; bee pollen; feed mixture; fatty acid

INTRODUCTION

Fatty acid are important sources of body fuel because, when metabolized they yield large quantities of ATP and many cell types can use either glucose or fatty acid for this purpose in particular heart and skeletal muscle prefer fatty acid, despite longstanding assertions to the contrary, the brain can use fatty acid as a source of fuel in addition to glucose and ketone bodies **(Goodhart and Shils, 1980; Marin-Valencia et al., 2012)**. Fatty acids have been linked with the pathological processes of the various human diseases, particularly cardiovascular disease with the strongest evidence suggesting that saturated fatty acids (SFA) have negative consequences on human health whilst polyunsaturated fatty acids (PUFA) have beneficial effects **(Gibbs et al., 2013)**. One reason for the success of the broiler meat industry has been the consumer perception of a healthy product that contains less fat, most predominantly unsaturated fatty acids as comparable to beef or pork products **(Leeson, 1999; Bonoli, 2007)**. That dietary monounsaturated fatty acid enrichment has a positive effect on cardiovascular health, decreasing low-density lipoprotein cholesterol, but not high-density lipoprotein cholesterol in blood plasma, and decreasing the susceptibility of low-density lipoprotein to oxidation **(Grundy, 1986; Roche, 2001)**. The fatty acid profile of poultry meat is related to the composition of the bird's diet and, as such, dietary alterations can be used to modify the proportion of PUFA in chicken meat **(Rymer and Givens, 2005)**. The enrichment of poultry tissue with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been proposed as a potential vehicle for increasing dietary intakes of long chain n-3 PUFA in the human diet **(Rymer and Givens, 2005; Gibbs et al., 2010)**. Bee pollen is rich of the a major fatty acid, presented as mean values were C18:3 (25.1%), C16:0 (19.6%), C18:1 (17.3%), C18:2 (8.78%), C22:0 (4.07%), and C18:0 (2.96%) acids. The proportions of C18:3 were generally higher than those of C18:2 and the ratio of total unsaturated fatty acid (TUS) to total saturated fatty acid (TS) was >1.0, except for *Nelumbo nucifera* Gaertn, pollen for the characteristic absence of C18:3 acids **(Yang et al., 2013)**.

The present experiment was objected to study the effect of the bee pollen extract on the broiler Ross 308 breast and thigh muscles essential fatty acids.

MATERIAL AND METHODOLOGY

The experiment has been done in the test poultry station of Slovak University of Agriculture in Nitra. The tested chickens were broiler Ross 308. The experiment included 90 chicks in one day-old, which were divided into 3 groups (n=30): control group, E1 and E2, for 42 days. The chickens were bred in a cage conditions. Each cage was equipped with feed dispenser and water intake was ensured *ad libitum* through a self-feed-pump. The temperature was controlled during the fattening period and

it was 33 °C at the first day and every week was reduced about 2 °C and final temperature was 23 °C. The lighting during the experiment period was continuous. Each group was fed by the same starter complete feed mixture (CFM) HYD-01 (loose structure) from 1st day to 21st days of their age, and from the 22nd to 42nd days of their age, chickens were fed by a complete feed mixture (CFM) HYD-02 (loose structure), in all investigated groups of the experiment (Table 1). However, to experimental groups, they were added bee pollen extract in amount (400, 800 mg.kg^{-1}) into feed mixture. The complete feed mixture HYD-01 and HYD-02 has been produced without antibiotic preparations and coccidiostatics. The bee pollen extract was prepared from minced bee pollen (150 g) in the conditions of the 80% ethanol in the 500 cm^3 flasks **(Krell, 1996**). At the end of the fattening period (42 days) from each experimental group have 30 pieces of chickens for slaughter analysis (15 ♂ pieces and 15 pieces ♀), to evaluate the count of fatty acid in broiler muscles were selected. The experimental analyses were done in Animal Production Research Centre Nitra (APRC Nitra, Slovak republic) by using Agilent 7890A Gas Chromatograph (USA) apparatus.

Statistical analysis:

The results of the experiments were statistically analysed by the statistic program Statgraphics Plus version 5.1 (AV Trading Umex, Dresden, Germany). For the determination of significant differences (P ≤0.05) among the tested groups analysis of variance (arithmetic mean, standard deviation) was used.

RESULTS

The results of the effect of bee pollen extract on broiler Ross 308 breast muscles fatty acids (%) have been shown in the Table (2) where were found that the myristoleic acid in the control group (0.715 ± 0.110) was higher compared to E1 (0.554 ± 0.098) and E2 (0.632 ± 0.062) groups. Otherwise, the palmitoleic acid in E1 (6.485 ± 1.325) and (6.295 ± 0.606) were higher compared to the control group (5.577 ± 1.113). Moreover, were found that the oleic acid was higher in E1 (43.583 ± 1.507) and E2 (45.342 ± 0.877) groups than the control group (43.222 ± 4.284). Further, were found that the linoleic acid in the control group (12.348 ± 2.306) was higher compared to E1 (11.289 ± 0.882) and E2 (10.684 ± 0.676) groups. Moreover, the linoelaidic acid in the control group (0.728 ± 0.163) was higher compared to E1 (0.674 ± 0.179) and E2 (0.588 ± 0.123) groups. Also, arachidonic acid in the control group (1.346 ± 0.240) was higher compared to E1 (1.129 ± 0.480) and E2 (1.090 ± 0.376) similar result was found in archaicacid, which was higher in the control group (0.998 ± 0.362) compared to E1 (0.784 ± 0.258) and E2 (0.624 ± 0.121) groups. And there were no significant differences (P ≥0.05) among the groups.

Table 3 shows the data of the effect of bee pollen extract on broiler Ross 308 thigh muscles fatty acids (%) where were found the myristoleic acid in the control group (0.685 ± 0.078) was higher compared to E1 (0.638 ± 0.035) and E2 (0.623 ± 0.0505) groups. Similarly, the palmitoleic acid was higher in the control group

Table 1 Composition of the broiler feed mixture

Ingredients (%)	Starter (1 to 21 days of age)	Grower (22 to 42 days of age)
Wheat	35.00	35.00
Maize	35.00	40.00
Soybean meal (48% N)	21.30	18.70
Fish meal (71% N)	3.80	2.00
Dried blood	1.25	1.25
Ground limestone	1.00	1.05
Monocalcium phosphate	1.00	0.70
Fodder salt	0.10	0.15
Sodium bicarbonate	0.15	0.20
Sodium bicarbonate	0.15	0.20
Lysine	0.05	0.07
Methionine	0.15	0.22
Palm kernel oil Bergafat	0.70	0.16
[1]Premix Euromix BR 0.5%	0.50	0.50
Analysed composition (g.kg^{-1})		
Crude protein	210.76	190.42
Fibre	30.19	29.93
Ash	24.24	19.94
Ca	8.16	7.28
P	6.76	5.71
Mg	1.41	1.36
ME (MJ.kg^{-1})	12.02	12.03

[1] active substances per kilogram of premix: vitamin A 2 500 000 IU; vitamin E 50 000 mg; vitamin D3 800 000 IU; niacin 12 000 mg; d-pantothenic acid 3 000 mg; riboflavin 1 800 mg; pyridoxine 1200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 50000 mg; folic acid 400 mg; biotin 40 mg; vitamin B12 10.0 mg; choline 100000 mg; betaine 50000 mg; Mn 20 000 mg; Zn 16 000 mg; Fe 14 000 mg; Cu 2 400 mg; Co 80 mg; I 200 mg; Se 50 mg.

(8.025 ± 0.636) compared to E1 (7.907 ± 0.925) and E2 (7.689 ± 0.708) groups. Comparably, were found that the oleic acid was higher in E1 (45.186 ± 1.471) and E2 (46.451 ± 1.156) groups than the control group (44.571 ± 2.205). Further, there were found that the linoleic acid was higher in the control group (11.549 ± 1.927) compared to E1 (11.544 ± 0.593) and E2 (11.315 ± 0.775) groups. Moreover, the linoelaidic acid was higher in the control group (0.737 ± 0.103) compared to E1 (0.699±0.0412) and E2 (0.665 ± 0.058) groups. Also, were found that the arachidonic acid was higher in the control group (0.638 ± 0.137) compared to E1 (0.627 ± 0.0649) and E2 (0.567 ± 0.021) groups. Analogous, were found that the arachidonic acid was higher control group (0.737 ± 0.103) compared to E1 (0.699 ± 0.0412) and E2 (0.665 ± 0.058) groups. However, no significant differences among the groups were found.

DISCUSSION

In the recent years there has been an increased interest to study the manipulate fatty acid composition in meat. This is because meat is seen to be a major source of fat in the diet and especially of saturated fatty acids, which have been implicated in diseases associated with modern life, especially in developed countries **(Wood et al., 2003)**. The fatty acid composition and total fatty acid content of broiler breast and thigh muscles retail was shown in Tables (2, 3) where we found that in our finding that following fatty acids such asmyristoleic acid, linoleic acid, linoelaidic acid, arachidonic acid, and archaic acid fatty acids were decreased after using the bee pollen extract into broiler Ross 308 feed mixture, although the bee pollen has content many types of fatty acids **(Yang et al., 2013)**, we suggest the reason which led to decrease the fatty acid, that return to use ethanol for made bee pollen extract and **(Horrobin, 1980; McCarty, 1999)** reported that the ethanol has damage fatty acid.

On the other hand, our results have increased the oleic acid and palmitoleic acid in breast muscles and thigh just oleic acid, which were higher in experimental groups than the control and this is a good result because oleic acid has a positive effect on human health **(Høstmark and Haug, 2013)** which led to an improved insulin sensitivity, and endothelium-dependent flow-mediated vasodilatation **(Ryan et al., 2000; Tholstrupet al., 2004)**, lowering of LDL cholesterol **(Gillingham et al., 2010; Damasceno et al., 2011)** and an increase in HDL cholesterol **(Estévez-González et al., 2010)** and oleic acid enriched LDL in lipids, which that led to particles will be less liable to be oxidized **(Cicero et al., 2008)**. **Bolsoni-Lopes et al. (2013)** found that palamtic acid (16:1n7) has increased fatty acid incorporation into TAG and glycerol 3-phosphate synthesis from glucose in both wild-type and PPARα- deficient, however, palmitoleic acid increases adipocyte lipolysis and lipases by a mechanism that requires a functional peroxisome proliferator-activated receptor alpha (PPARα.). **Paillarda et al. (2008)** said that the palmitoleic acid content a product of SCD activity and this enzyme (SCD) could represent a target for prevention and treatment of these metabolic disorders in particular in subjects at risk of developing a metabolic syndrome.

CONCLUSION

In the present study bee pollen extract treatment decreased the myristoleic acid, linoleic acid, linoelaidic acid, arachidonic acid, and archaic acid fatty acids, on the contrary palmitoleic acid palmitoleic acid and oleic acid were increased in broiler breast muscles also the oleic acid was increased in the thigh muscles.

Table 2 The effect of bee pollen extract broiler Ross 308 breast unsaturated fatty acids (%)

Indicators	Control	E1 Pollen 400mg.kg^{-1}	E2 Pollen 800mg.kg^{-1}
Myristoleic acid	0.715 ± 0.110	0.554 ± 0.098	0.632 ± 0.062
Palmitoleic acid	5.577 ± 1.113	6.485 ± 1.325	6.295 ± 0.606
Oleic acid	43.222 ± 4.284	43.583 ± 1.507	45.342 ± 0.877
Linoleic acid	12.348 ± 2.306	11.289 ± 0.882	10.684 ± 0.676
Linoelaidic acid	0.728 ± 0.163	0.674 ± 0.179	0.588 ± 0.123
Arachidonic acid	1.346 ± 0.240	1.129 ± 0.480	1.090 ± 0.376
Archaic acid	0.998 ± 0.362	0.784 ± 0.258	0.624 ± 0.121

E1, E2: experimental groups; ^{a,b}– means with different superscripts differ significantly; (P ≤0.05) significant.

Table 3 The effect of bee pollen extract broiler Ross 308 thigh unsaturated fatty acids (%)

Indicators	Control	E1 Pollen 400mg.kg^{-1}	E2 Pollen 800mg.kg^{-1}
Myristoleic acid	0.685 ± 0.078	0.638 ± 0.035	0.623 ± 0.0505
Palmitoleic acid	8.025 ± 0.636	7.907 ± 0.925	7.689 ± 0.708
Oleic acid	44.571 ± 2.205	45.186 ± 1.471	46.451 ± 1.156
Linoleic acid	11.549 ± 1.927	11.544 ± 0.593	11.315 ± 0.775
Linoelaidic acid	0.737 ± 0.103	0.699 ± 0.0412	0.665 ± 0.058
Arachidonic acid	0.638 ± 0.137	0.627 ± 0.0649	0.567 ± 0.021
Archaic acid	0.256 ± 0.324	0.122 ± 0.0323	0.129 ± 0.041

E1, E2: experimental groups; a,b– means with different superscripts differ significantly; (P ≤0.05) significant

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MYCOBIOTA OF SPICES AND AROMATIC HERBS

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ABSTRACT

A total of 67 samples of spices and herbs were tested for mould contamination. From 50.7% of samples, moulds were not isolated. The most dominant genera were *Aspergillus* and *Penicillium*. Potential producers of mycotoxins *Aspergillus* spp. and *Penicillium* spp. were tested for the ability to produce some mycotoxins. Isolates of potentially toxinogenic species were found to produce various mycotoxins, namely alfatoxin B_1 (*Aspergillus flavus*), cyclopiazonic acid (*Aspergillus flavus*), sterigmatocystin (*Emericella nidulans*), roquefortine C (*Penicillium allii, P. chrysogenum, P. crustosum, P. expansum*), penitrem A (*P. crustosum*) and patulin (*P. expansum*). Some of the tested isolates produce two mycotoxins: *A. flavus* (aflatoxin B_1 and cyclopiazonic acid), *P. crustosum* (roquefortine C and patulin) and *P. expansum* (roquefortine C and patulin). None of the tested isolates of *Aspergillus* section *Nigri* screened, appeared to produce ochratoxin A. Totally 11 samples were analysed for the presence of aflatoxins and ochratoxin A. Aflatoxin B_1 was found in 5 (45.5%) out of 11 samples analysed with levels ranging from 0.14 to 2.9 $\mu g.kg^{-1}$. In one sample we detected aflatoxin G_1. Ochratoxin A was found in 3 samples (27.3%), with levels ranging from 2.2 to 5.19 $\mu g.kg^{-1}$. No sample was contaminated by aflatoxins or ochratoxin A above the maximum admitted threshold established by the European legislation.

Keywords: mycobiota; spices; aflatoxin; ochratoxin A

INTRODUCTION

Spices have been used for flavour, colours, aroma and preservation of food or beverages for thousands years **(Ozbey and Kabak, 2012)**. Because of their processing and environmental conditions, spices can be heavily contaminated with toxigenic fungi and mycotoxins. Mycotoxins are secondary metabolites produced naturally by filamentous fungi, which are considered toxic substances when present in food for human and food for animals **(da Rocha et al., 2014)**. For spices there are two groups of mycotoxins of concern, aflatoxins and ochratoxin A **(Ozbey and Kabak, 2012)**. Aflatoxins are produced by fungi that belong to *Aspergillus* genus and especially by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* **(Cary and Ehrlich, 2006, Marín et al., 2009)**. Ochratoxin A is a secondary metabolite produced by filamentous fungi of the genera *Aspergillus* and *Penicillium* present in a wide range of foodstuffs. The most relevant ochratoxin A producing species are *Penicillium verrucosum*, *Aspergillus ochraceus*, *Aspergillus niger* and *Aspergillus carbonarius* due to their prevalence in foodstuffs (cereals, grapes, coffee, etc.) and the number of strains are able to produce ochratoxin A **(Amézqueta et al., 2012, Luque et al., 2013, Rodríguez et al., 2011)**. Prevention of microbial contamination in dried herbs and spices lies in the application of good hygiene practices during growing, harvesting and processing from farm to fork **(Sagoo et al., 2009)**.

The aim of the study was the determination of potentially toxigenic filamentous fungi from genera *Aspergillus* and *Penicillium* from spices and herbs. A special emphasis was laid on the ability of isolated *Aspergillus* and *Penicillium* species to produce some significant toxic extrolites - mycotoxins.

MATERIAL AND METHODOLOGY

Samples

Totally 67 samples of spices and herbs from different countries (Table 1) were analysed. The samples (approximately 100 g) were collected from the storage rooms of food factory.

Mycological analysis

Dilute plate technique was used for isolation of fungi from the samples according to **Samson et al. (2002)**. Sample in weigh of 20 g was mixed with 180 ml of saline solution (0.85% sodium chloride) with 0.05% Tween 80 in homogenizer. Then 0.1 ml of appropriate dilution made up to 10^{-2} was applied on DRBC (Dichloran Rose Bengal Chloramphenicol agar). After 5 to 7 days of incubation at 25 ± 1°C, in dark resulting colonies were transferred onto appropriate identification media.

The identification of *Aspergillus* species. Conidial suspensions were inoculated at three equidistant points both on Czapek-yeast Extract agar (CYA), Czapek-yeast with 20% Sucrose (CY20S) and malt extract agar (MEA) and incubated in the dark at 25 ± 1 °C, 7 days. Species identification was done according to **Klich (2002), Pitt and Hocking (2009), Samson et al. (2002, 2010)** and **Samson and Varga (2007)**.

The identification of *Penicillium* species. The penicillia were inoculated at three equidistant points both on Czapek-yeast Extract agar (CYA), Malt Extract agar (MEA) and Creatine Sucrose agar (CREA) and incubated in dark at 25 °C. Sub-cultivation on CYA at 37 °C was

Table 1 Sample of spices and herbs

Sample	Country of origin	Number of samples	Sample	Country of origin	Number of sample
basil	Egypt	2	**crushed bay leaves**	Turkey	1
crushed white pepper	Vietnam	3	**crushed rosemary**	Morocco	2
marjoram	Egypt	2	**crushed thyme**	Poland	2
garlic powder	China	2	**crushed ginger**	Nigeria	5
onion powder	France	2	**nutmeg**	Indonesia	3
crushed black pepper	Vietnam	2	**paprika (spicy)**	Spain	8
granulated onion	France	2	**paprika**	Hungary	7
granulated garlic	China	1	**chive**	China	1
curry	Spain	1	**leaves of parsley**	Slovakia	1
crushed marjoram	Egypt	3	**dill**	Poland	2
cumin powder	Finland	1	**crushed green pepper**	India	2
salvia	Albania	2	**leaves of celery**	Slovakia	2
crushed chillies	China	6	**savory**	Hungary	2

used as well. Species identification was done after 7 days according to **Pitt aHocking (2009), Samson et al., (2002, 2010)** and **Frisvad a Samson (2004)**.

The identification of *Fusarium* species. Potato Dextrose agar (PDA) was used for observation of colony characteristics. "Synthetischer Nährstoffarmer agar" (SNA) was used for micromorphological features. Cultures were incubated at the room temperature and natural light. Species identification was done after 10 days according to **Leslie a Summerell (2006), Nelson et al. (1983), Nirenberg (1981) Pitt a Hocking (2009) and Samson et al. (2002, 2010)**.

Mycotoxins screening by a modified agar plug method

The ability of selected potentially toxigenic isolates to produce relevant mycotoxins in *in vitro* conditions were screened by the means of thin layer chromatography (TLC) according to **Samson et al. (2002)** modified by **Labuda a Tančinová (2006)**.

The cultivation for screening of extracellular metabolites (aflatoxin B_1, aflatoxin G_1, citrinin, patulin, ochratoxin A) were carried out on YES (Yeast Sucrose agar) and for intracellular (cyclopiazonic acid, penitrem A, roquefortin C, sterigmatocystin) on CYA (Czapek-yeast Extract agar); conditions of cultivation in dark at 25 °C, 14 days. In each tested isolate, 3 pieces of mycelium together with cultivation medium of approximately 5 x 5 mm area were cut from colonies and extracted in 1000 ml of chloroform:methanol (2:1, v/v) on vortex for 2 minutes. Then 20 µl of liquid phase of extracts along with standards (Sigma, Germany) were applied on TLC plate (Marchey-Nagel, Germany) and consequently developed in solvent system toluene:ethylacetate:formic acid (5:4:1, v/v/v/). The visualisation of extrolites was carried out as follows: cyclopiazonic acid directly in daylight after spraying with the Ehrlich reagent (violet-tailed spot); patulin by spraying with 0.5% methylbenzothiazolone hydrochloride in methanol, heated at 130 °C for 8 min and then detectable as a yellow-orange spot; penitrem A after spraying with 20% $AlCl_3$ in 60% ethanol, heated at 130 °C for 8 min and then detectable as a dark green to black spot on daylight; roquefortin C after spraying with $Ce(SO_4)_2$ x 4 H_2O visible as an orange spot. Directly under UV light (365 nm) were visualised following mycotoxins: aflatoxin B_1 (blue spot), aflatoxin G_1 (green), citrinin (yellow-green), ochratoxin A (bluish-green), sterigmatocystin (reddish).

The determination of mycotoxins in sample

In the 11 samples were determined following mycotoxins: aflatoxin B_1, B_2, G_1, G_2 and ochratoxin A. Analyses were performed by HPLC method (high-pressure liquid chromatography) in an external accredited laboratory.

RESULTS AND DISCUSSION

In the current study from 50.7% of the samples, moulds were not isolated (basil, crushed black pepper, granulated garlic, curry, cumin powder, salvia, crushed chillies, crushed bay leaves, paprika (spicy), dill, crushed green pepper, savory). These findings are similar to data reported by **Witkowska et al. (2011)**, where in 50% of samples of commercial herbs and spices were detected moulds. The fungal species recovered from the samples are listed in Table 2. Species of 11 genera were isolated and identified. The *Aspergillus* and *Penicillium* were the most common genera. **Hashem and Alamri (2010)** from 15 spices isolated as the most common genera *Aspergillus, Penicillium* and *Rhizopus*. *Rhizopus* (*Rhizopus stolonifer*) was isolated from granulated onion, only. **Hammami et al. (2014), Kong et al. (2014), Yogendrarajah et al. (2014)** and other authors reported *Aspergillus* and *Penicillium* as predominant fungi in spices. With regard to *Aspergillus* genus, the following species were isolated: *A. flavus, A. fumigatus* and *Aspergillus* section *Nigri*. *A. flavus* is a very important producer of aflatoxins and is frequently occurring in food commodities **(Luo et al., 2012)**. Species of *Aspergillus* section *Nigri* are important producers of ochratoxin A.

In the *Aspergillus* section *Nigri*, *A. niger* and *A. carbonarius* produce ochratoxin A **(Almela et al., 2007)**. The isolates of this section were the most frequent in our study.

Table 2 Mycobiota isolated from the samples of spices and aromatic herbs

Sample	Isolated species (group)
crushed white pepper	*Aspergillus* section *Nigri*, *Emericella nidulans*, *Eurotium* sp. *Penicillium crustosum*, *Penicillium expansum*, *Penicillium chrysogenum*
marjoram	*Aspergillus* section *Nigri*, Penicillium expansum
garlic powder	*Aspergillus* section *Nigri*, *Penicillium allii*, *Penicillium chrysogenum*
onion powder	*Aspergillus flavus*, *Aspergillus* section *Nigri*, *Penicillium glabrum*, *Penicillium chrysogenum*
granulated onion	*Aspergillus* section *Nigri*, *Penicillium* sp., *Rhizopus stolonifer*
crushed marjoram	*Aspergillus* section *Nigri*, *Aspergillus fumigatus*, *Cladosporium* sp., *Penicillium atramentosum*, *Penicillium solitum*
crushed ginger	*Aspergillus flavus*, *Aspergillus* section *Nigri*, *Paecilomyces* sp.
nutmeg	*Paecilomyces* sp.
paprika	*Penicillium chrysogenum*
chive	*Fusarium proliferatum*
leaves of parsley	*Alternaria* sp., *Geotrichum candidum*
leaves of celery	*Cladosporium herbarum*, *Geotrichum candidum*

Table 3 Potential ability of moulds isolated from spices and aromatic herbs to produce relevant mycotoxins in *in vitro* conditions, tested by TLC method

Tested isolates	Source of isolates	OTA	AFB1	AFG1	CPA	STER	RC	PA	PAT
Aspergillus section *Nigri*	crushed white pepper	0*/1**	-	-	-	-	-	-	-
	marjoram	0/1	-	-	-	-	-	-	-
	garlic powder	0/2	-	-	-	-	-	-	-
	granulated onion	0/1	-	-	-	-	-	-	-
	crushed ginger	0/1	-	-	-	-	-	-	-
Aspergillus flavus	granulated onion		1/1	0/1	1/1	-	-	-	-
	crushed ginger		0/1	0/1	1/1	-	-	-	-
Emericella nidulans	crushed white pepper	-	-	-	-	1/1	-	-	-
Penicillium allii	garlic powder	-	-	-	-	-	2/2	-	-
Penicillium chrysogenum	crushed white pepper	-	-	-	-	-	3/3	-	-
	garlic powder	-	-	-	-	-	2/2	-	-
	paprika	-	-	-	-	-	1/1	-	-
Penicillium crustosum	crushed white pepper	-	-	-	-	-	1/1	1/1	-
Penicillium expansum	crushed white pepper	-	-	-	-	-	1/1	-	1/1
	marjoram	-	-	-	-	-	1/1	-	1/1

** number of tested isolates, * number of isolates with ability to produce mycotoxin, OTA - ochratoxin A, AFB1 - aflatoxin B_1, AFG1 - aflatoxin G1, CPA - cyclopiazonic acid, STER - sterigmatocystin, RC - roquefortine C, PA - penitrem A, PAT - patulin, TLC - thin layer chromatography

Kong et al. (2014) isolated *Aspergillus* section *Nigri* as the most frequent in spices in China markets. Isolated pencillia were *Pencillium allii*, *P. atramentosum*, *P. crustosum*, *P. chrysogenum*, *P. expansum*, *P. glabrum*, *P. solitum* (in alphabetical order). **Hammami et al. (2014)** detected *P. aurantiogriseum*, *P. charlesii*, *P. verruculosum*, *P. citrinum*, *P. commune*, *P. griseofulvum*, *P. melanoconidium*. **Yogendrarajah et al. (2014)** in their study identified *Penicillium* only as genus.

Apart from the two regulated mycotoxins in European Union (aflatoxins and ochratoxin A), we determined the ability of the isolates obtained from the analysed samples, to produce other mycotoxins (cyclopiazonic acid, patulin, penitrem A, roquefortine C and sterigmatocystin).

Table 4 Contamination of spices with aflatoxins and ochratoxin A

Sample	Mycotoxins (µg.kg^{-1})				
	AFB1	**AFB2**	**AFG1**	**AFG2**	**OTA**
crushed white pepper	<0.10	<0.10	<0.10	<0.10	<0.20
crushed chillies	**0.14**	<0.10	<0.10	<0.10	**5.19**
nutmeg	**0.14**	<0.10	<0.10	<0.10	<0.20
paprika (spicy)	<0.10	<0.10	<0.10	<0.10	**2.2**
paprika	**0.11**	<0.10	<0.10	<0.10	**2.35**
crushed green pepper	<0.10	<0.10	<0.10	<0.10	<0.20
crushed black pepper	<0.10	<0.10	<0.10	<0.10	<0.20
crushed ginger	**2.9**	<0.10	**3.2**	<0.10	<0.20
crushed ginger	<0.10	<0.10	<0.10	<0.10	<0.20
nutmeg	**0.55**	<0.10	<0.10	<0.10	<0.20
paprika (spicy)	<0.10	<0.10	<0.10	<0.10	<0.20

AFB1 - aflatoxin B_1, AFB2 - aflatoxin B_2, AFG1 - aflatoxin G_1, AFG2 - aflatoxin G_2, OTA - ochratoxin A

The ability to produce relevant mycotoxins are shown in Table 3. The isolates of potentially toxinogenic species were found to produce various mycotoxins, namely aflatoxin B_1 (*Aspergillus flavus*), cyclopiazonic acid (*Aspergillus flavus*), sterigmatocystin (*Emericella nidulans*), roquefortine C (*Penicillium allii, P. chrysogenum, P. crustosum, P. expansum*), penitrem A (*P. crustosum*) and patulin (*P. expansum*). Some of the tested isolates produce two mycotoxins: *A. flavus* (aflatoxin B_1 and cyclopiazonic acid), *P. crustosum* (roquefortine C and patulin) and *P. expansum* (roquefortine C and patulin). None of the tested isolates *Aspergillus* section *Nigri* screened appeared to produce ochratoxin A.

Totally 11 samples were analysed for the presence of aflatoxins and ochratoxin A (Table 4). Aflatoxin B_1 was found in 5 (45.5%) out of 11 samples analysed with levels ranging from 0.14 to 2.9 µg.kg^{-1}. No sample was contaminated by aflatoxin B_1 above the maximum admitted threshold established by the European legislation **(Commission regulation, 2010b)**. In one sample we detected aflatoxin G_1. Ochratoxin A was found in 3 of samples (27.3%), with levels ranging from 2.2 to 5.1 µg.kg^{-1}. No sample was contaminated by ochratoxin A above the maximum admitted threshold established by the European legislation **(Commission regulation, 2010a)**. **Prelle et al. (2014)** showed that 15.4% and 23.8% of samples were contaminated with aflatoxins and ochratoxin A, respectively. In our study, 2.3% of spice samples contaminated by ochratoxin A get over the threshold admitted by European Regulation. **Zhao et al. (2013)** presented that about 11% of the 480 Chinese spices samples tested contained detectible levels of aflatoxin B_1, with the highest concentrations found in chili, prickly ash and pepper. **Zinedine et al. (2006) reported** the higher level of aflatoxin B_1 contamination in red paprika (9.68 µg.kg^{-1}). The analysis of the spice samples contamination (in Morocco) with aflatoxin B_1 revealed that paprika is frequently contaminated, since 95% were contaminated with that mycotoxin and 40% of samples exceeded European regulation for that contaminant **(Mahgubi et al., 2013)**. Co-occurrence of aflatoxin B_1 and ochratoxin A in samples of crushed chillies and paprika was detected in our study. **Ozbey a Kabak (2012)** reported co-occurrence of these mycotoxins in 62.5% of red chilli flake, 40.9% of red chilli powder and 4.3% pepper of powder samples.

CONCLUSION

From 50.7% of samples, moulds were not isolated. The most dominant genera were *Aspergillus* and *Penicillium.* The isolates of potentially toxinogenic species were found to produce various mycotoxins (alfatoxin B_1, cyclopiazonic acid, sterigmatocystin, roquefortine C, penitrem A and patulin). None of the tested isolates *Aspergillus* section *Nigri* screened appeared to produce ochratoxin A. Totally 11 samples were analysed for the presence of aflatoxins and ochratoxin A. Aflatoxin B_1 was found in 45.5% out of 11 samples analysed. Ochratoxin A was found in 27.3% of samples.

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COMPARISON OF THE CARBOHYDRATE CONTENT IN APPLES AND CARROTS GROWN IN ORGANIC AND INTEGRATED FARMING SYSTEMS

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ABSTRACT

The aim of this study was to compare some quality parameters of apples and carrots from organic and integrated farming systems. In addition, the cultivars of carrots were grown in two plant densities (600 or 900 thousand plants per hectare). The fructose, glucose, saccharose and dry matter content of seven apple cultivars (Florina, Zvonkové, Topaz, Šampion, Ontario, Melrose and Idared) and two carrot cultivars (Afalon F1 and Cortina F1) were analysed by HPLC and gravimetric methods, respectively. Significant differences were found between organic and integrated apple samples. The interactions between cultivars and farming methods were also significant (p <0.0001). The dry matter and sugar level tendencies were not the same for all apple cultivars. Conversely, more consistent data were obtained for the two carrot cultivars. The bio carrots of both cultivars showed significantly lower dry matter content (p = 0.0004) and higher carbohydrate content ($p_{fructose}$ = 0.0303, $p_{glucose}$ = 0.0003, $p_{sucrose}$ = 0.0083) than the samples from integrated production. Other factors like cultivar and plant density also played an important role in sugar content in carrots. Different densities of plants significantly affected the glucose content (p = 0.0373). Cultivar Aftalon F1 showed higher concentration of monosaccharides compared to Cortina F1 ($p_{fructose}$ = 0.0001 and $p_{glucose}$ <0.0001).

Keywords: fructose; glucose; sucrose; dry matter; organic farming

INTRODUCTION

There has been an increasing interest in organic farming in recent years. One of the main positives of organic production is its friendly approach to the environment. There are several studies dealing with the assessment of the quality of products from organic and integrated agricultural farming systems in order to objectively evaluate possible differences in the quality parameters **(Hogstat et al., 1997; Bavec et al., 2010; Bertazza et al., 2010)**. Organic farming aims to eliminate the application of agricultural chemicals and use natural materials both in plant protection products, as well as in animal nutrition. Organic products may contain lower levels of pesticide residues in comparison with conventional or integrated production **(Róth et al., 2007)**. It should be noted however, that studies published so far have not clearly proved higher nutritional quality of organic or conventional production.

Apples and carrots are among the most commonly grown fruits and vegetables in our climate. They represent very tasty food from the sensory point of view due to the presence of carbohydrates and organic acids. Carbohydrates are the main source of energy in human nutrition necessary for normal muscle and brain functions. They also protect cells against some external harmful effects **(Clarková, 2000)**. Fructose, glucose and sucrose (saccharose) belong to the main available carbohydrates in apples and carrots.

According to **Kopec (1998)** the total carbohydrate content in apples is 144 $g.kg^{-1}$ and the value for carrots is 97 $g.kg^{-1}$. The composition of carbohydrates in fresh apples was also analyzed by **Suni et al. (2000)**. Total carbohydrate content ranged from 615 to 716 $g.kg^{-1}$ of dry matter; fructose was the dominant component (57%). **Soria et al. (2009)** analysed the carbohydrate content in carrots by gas chromatography. The concentrations of fructose were 20 - 244 $mg.g^{-1}$, glucose 17 - 245 $mg.g^{-1}$ and sucrose 137 - 689 $mg.g^{-1}$ of dry matter.

Cultivar, climate conditions, soil type, method of cultivation and storage conditions are very important parameters for the internal quality of apples and carrots. **Weibel et al. (2000)** tested "Golden Delicious" apples. They grew simultaneously organic and integrated apples in a similar microclimate. They compared the following quality parameters: fruit hardness, content of carbohydrates, malic acid, minerals, phenols and vitamins C and E. Sensory analysis was also carried out. All samples from organic production had significantly harder fruit flesh and also had a better taste than conventional fruits. Bio apples contain more phosphorus and phenolics. **Róth et al. (2007)** analyzed "Jonagold" apples from organic and integrated production from three different regions in Belgium with similar climatic and soil characteristics. The analysis did not prove that there is convincing evidence of a difference in nutrient content between organic and conventional samples. **Bordeleau (2002)** compared organic and conventional varieties of

"Golden Delicious" apples. Significant differences were found in the acidity, size and colour of the fruit. In contrast, no difference was detected in carbohydrates, pH, dry matter content or the hardness of the apples. The author, however, stated that there are differences between organic and conventional food for only some parameters, but the final quality is affected by many other factors. In addition, the organoleptic survey of **Nagy et al. (2012)** did not identify any differences between organic and integrated products.

Rembialkowska (2003) analyzed the effects of organic methods on the quality of vegetables during production, storage, and on the way to the consumer. The nutritional and sensory qualities of carrots and potatoes from organic and conventional farming were compared. It was found that the organic vegetables had lower yields, but some of its nutritional and sensory quality attributes were better than those conventional products. But according to **Seljasen et al. (2013)** sensory and chemical quality parameters of carrots are determined mainly by genetic and climate-related factors and only to a minor extent by cultivation method.

Although experiments with animals fed with organic feed, showed that animal health and reproductive performance had been slightly improved, similar findings have not been identified in humans yet **(Magkos et al., 2003)**.

The aim of this paper was to study selected quality parameters (concentration of carbohydrates and dry matter content) of the organic and integrated production of seven apple and two carrot varieties and to establish the similarities and differences between these farming systems. The main hypothesis was whether products from organic farming have a higher nutritional value than products from integrated systems.

MATERIAL AND METHODOLOGY

Material

Seven apple cultivars, "Florina", "Zvonkové", "Topaz", "Šampion", "Ontario", "Melrose" and "Idared", were selected for the experiment. All these cultivars were grown in organic and integrated farming systems. The apples from organic farming were from certified orchards near the town of Chrudim. Apples of the same cultivars from an integrated system were harvested at the same year (2012) in a demonstration orchard at the Czech University of Life Sciences in Prague. Two cultivars of carrots ("Afalon F1" and "Cortina F1") were grown at the same place. The density of carrots was 600 or 900 thousand plants per hectare. Integrated samples were fertilized using 80 kg of nitrogen (80% in the form of urea before sowing and 20% in the form of ammonium nitrate with calcite during vegetation) per hectare; organic carrots were treated by 1.5 $t.ha^{-1}$ of Organica fertilizer (Agro CS, Czech Republic).

Dry matter content determination

The dry matter content of samples (1 g) was analysed by using infrared balances (Precisa 310M, Precisa HA300, Precisa Instruments AG, Switzerland). Drying under infrared light was performed at 105 ºC for about 20 minutes to a constant weight with a difference of less than 1 mg min^{-1}. Each sample was measured three times.

Instrumental analysis of sugars

A high-performance liquid chromatographic (HPLC) method was used to determine the fructose, glucose, and sucrose content of apples and carrots. The HPLC system (Varian Star 9010) consisted of ion exchange resin-based column (Aminex HPX-87H) heated to 55 °C, a solvent system of 0.005M H2SO4, a flow rate of 0.6 $mL.min^{-1}$, and a refractive index detector (Varian RI-4) heated to 35 °C. The calibration curve was determined using standard solutions of sucrose, glucose and fructose at concentrations of 0.5, 1.0, 2.0 and 3.0 g.100 g^{-1}. Their retention times were 8.09, 9.62 and 10.45 minutes respectively. The correlation coefficients of the calibration curves (R^2) were 0.9932 for sucrose, 0.9973 for glucose and 0.9960 for fructose.

Samples preparation

All fruits were cleaned before analysis. Parts that are not consumed were removed. The edible parts were homogenised by a Fagor B-515M blender (Electrodomésticos, Spain). Thirty grams of the homogenised sample plus 60 g of demineralised water were thoroughly mixed and taken for the analysis. The sample was filtered through the filter paper and then through the Simplepure NY 0.45 μm filter. The filtrate was injected into HPLC loop (20 μL). The analysis of one sample took 15 min.

Statistical analysis

Linear regression equations, regression coefficients (R^2) as well as other results were calculated from the data using Microsoft Office Excel 2007. The impact of factors of "cultivar", "farming system" and "density" was evaluated using multi-way (two-way for apples and three-way for carrots) analysis of variance (ANOVA). Tukey's test was used to calculate statistically significant differences between samples using statistical software Statistica 12 (StatSoft Inc.). For all statistical tests, a 5% level of significance was used.

RESULTS

Dry matter content of apples and carrots grown under organic and integrated systems are given in Tables 1 and 2. The results are expressed as arithmetic mean ± standard deviation. They were in the range of 9.63% - 17.41% for apples and 10.74% - 14.39% for carrots. The sucrose, glucose and fructose content of apples and carrots grown under organic and integrated systems are given in Tables 3 and 4. The results are expressed as arithmetic mean ± standard deviation. It is seen from Table 3 that fructose content was the highest in all varieties of apples followed by glucose and sucrose, which is in line with **Soria et al. (2009)** and **Nagy et al. (2012)**.

Cultivar "Šampion" from organic farming had the highest total carbohydrate content (160 $g.kg^{-1}$) (Figure 1). The lowest value was recorded in the cultivar "Topaz" from organic farming (65 $g.kg^{-1}$). Cultivar "Aftalon F1" (density 600) from organic production showed the highest value of carbohydrates among carrots (Figure 2, Table 4).

Contrary to other analysed samples this variety had the highest content of glucose, which was even higher than the fructose content. Cultivar "Cortina F1" (density 600) from integrated production had the lowest carbohydrates value.

Table 1 Dry matter content (%) of apples grown under organic and integrated systems.

Cultivar	Farming system	
	interated	organic
IDARED	9.6 ±0.3	13.5 ±0.1
MELROSE	12.6 ±0.1	16.0 ±0.2
ONTARIO	17.4 ±0.1	13.5 ±0.1
ŠAMPION	13.4 ±0.1	15.7 ±0.2
TOPAZ	15.4 ±0.2	14.4 ±0.2
ZVONKOVÉ	13.5 ±0.1	14.5 ±0.2
FLORINA	13.6 ±0.1	13.7 ±0.1

Table 2 Dry matter content (%) of carrots grown under organic and integrated systems.

Cultivar	Density of plants (1000/ha)	Farming system	
		organic	integrated
AFALON F1	600	10.8 ±0.8	14.4 ±0.8
AFALON F1	900	10.7 ±0.8	13.5 ±0.4
CORTINA F1	600	12.9 ±0.5	11.5 ±0.8
CORTINA F1	900	13.6 ±0.8	13.4 ±0.8

Table 3 Carbohydrates content of apples grown under organic and integrated systems.

Cultivar	Sucrose ($g.kg^{-1}$)		Glucose ($g.kg^{-1}$)		Fructose ($g.kg^{-1}$)	
	integrated	organic	integrated	organic	integrated	organic
IDARED	1.14 ±0.06	1.24 ± .08	12.88 ±0.75	18.01 ±1.66	76.81 ±2.60	87.30 ±9.83
MELROSE	0.45 ±0.04	0.43 ±0.05	29.82 ±0.75	35.89 ±0.81	84.52 ±1.88	112.20 ±11.01
ONTARIO	0.14 ±0.01	0.04 ±0.01	12.03 ±0.48	8.36 ±0.81	88.46 ±14.06	78.89 ±2.12
ŠAMPION	0.47 ±0.02	0.79 ±0.05	9.84 ±1.48	25.69 ±0.59	99.29 ±2.74	133.01 ±4.87
TOPAZ	2.37 ±0.05	1.85 ±0.22	6.18 ±0.44	2.15 ±0.37	65.59 ±0.20	60.97 ±1.24
ZVONKOVÉ	0.55 ±0.01	0.26 ±0.02	6.01 ±0.60	7.48 ±0.83	59.21 ±2.60	85.17 ±2.14
FLORINA	1.62 ±0.10	0.42 ±0.01	16.24 ±0.14	5.34 ±0.65	74.08 ±2.05	66.44 ±2.96

Table 4 Carbohydrates content of carrots grown under organic and integrated systems.

Cultivar (Density)*	Sucrose ($g.kg^{-1}$)		Glucose ($g.kg^{-1}$)		Fructose ($g.kg^{-1}$)	
	integrated	organic	integrated	organic	integrated	organic
AFALON F1 (600)	0.44 ±0.09	0.94 ±0.41	6.65 ±2.76	14.45 ±4.24	8.98 ±1.72	11.40 ±4.65
AFALON F1 (900)	0.31 ±0.05	0.26 ±0.02	6.65 ±1.08	7.07 ±0.58	9.93 ±1.88	11.73 ±1.37
CORTINA F1 (600)	0.18 ±0.12	0.41 ±0.05	2.51 ±0.47	6.34 ±0.46	5.49 ±0.99	8.57 ±0.80
CORTINA F1 (900)	0.44 ±0.11	0.54 ±0.09	4.31 ±0.96	5.08 ±0.66	5.33 ±1.59	6.19 ±1.88

* Density of plants (thousands per hectare)

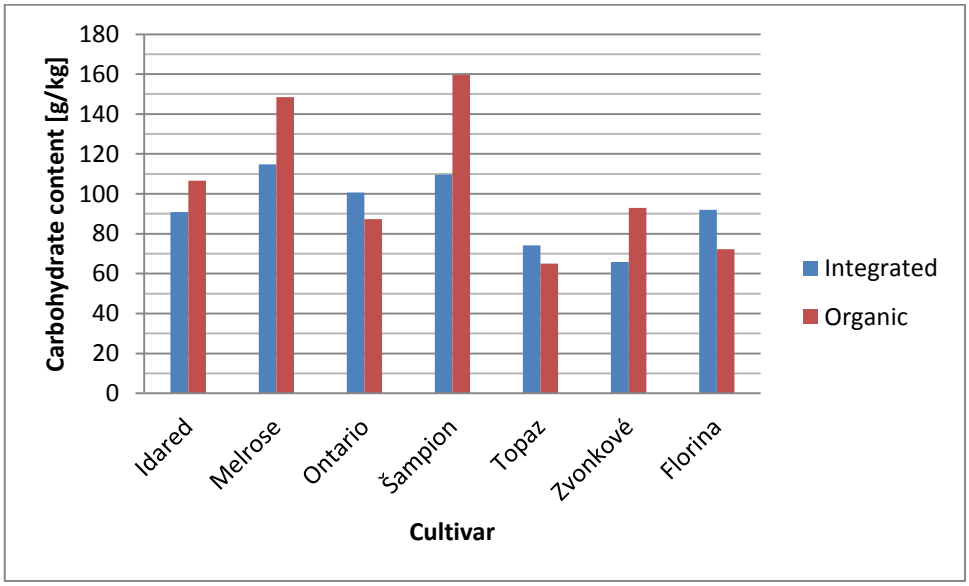

Figure 1 Total carbohydrate content of apples grown under organic and integrated systems

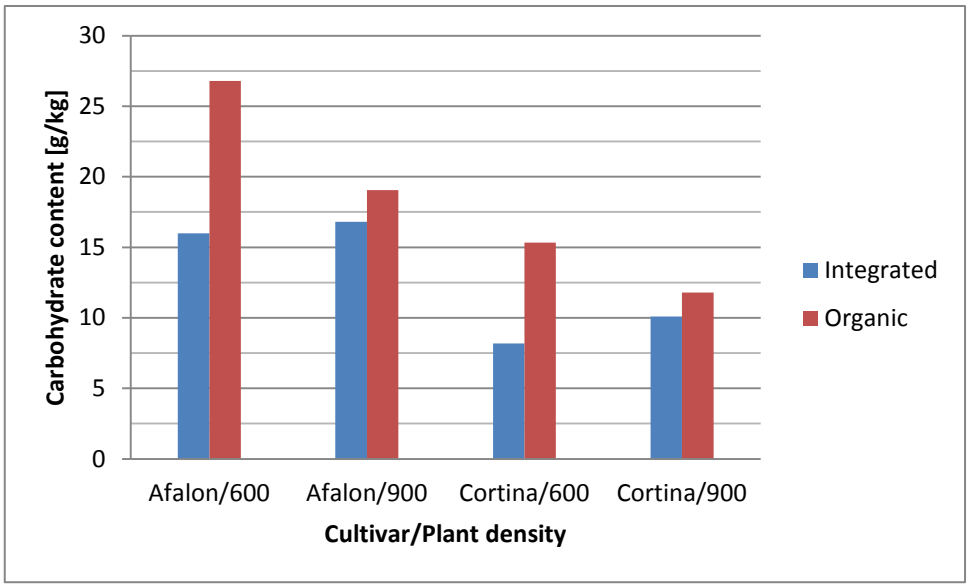

Figure 2 Total carbohydrate content of carrots grown under organic and integrated systems

Comparing all types of carrots, fructose was the most represented carbohydrate followed by glucose and sucrose, which corresponds with the same representation as in the case of analysed apples. Unlike apples, where the fructose content is markedly higher than the glucose content, the content of these monosaccharides in carrots is more or less comparable.

DISCUSSION

The results of dry matter content in the varieties of apples (Table 1) were slightly lower than in **Kopec (1998)**, but generally in line with **Nour et al. (2010)** and **Kourimska et al. (2013)**. There were statistically significant differences between the dry matter content in organic and integrated apples (p <0.0001) as well as between the dry matter content and cultivar (p <0.0001). Apples from organic farming had, on average, a higher amount of dry matter than apples from integrated system, which is in line with findings of **Bertazza et al. (2010)** who found a significantly higher content of dry matter in organic "Golden Delicious" apples. But this tendency was not observed in all cultivars. "Ontario" and "Topaz" apples showed the opposite trend. The joint effect of cultivar and farming method was confirmed by the significant interaction of these two factors (p <0.0001).

The values of the dry matter content of carrots (Table 2) are consistent with the literature **(Vogel, 1996; Kopec, 1998)**. No significant differences between the different density of plants per hectare (600 or 900 thousand) (p = 0.1704) or between cultivars (p = 0.1233) were found, but the interaction between these two factors was significant (p = 0.0062). Carrots from integrated farming showed a significantly (p = 0.0004) higher amount of dry matter content (13.20%) compared to the organic carrots (12.01%). The interaction between cultivars and farming systems was also significant (p <0.0001).

The measured concentrations of carbohydrate content in apples (Table 3) correspond with the values given by **Kopec (1998)** and **Ma et al. (2014)**. Significant differences between the farming systems as well as between cultivars were calculated comparing the content of sucrose, glucose and fructose in all seven organic and non-organic samples. The calculated *p*-values were in all cases below the significance level $\alpha = 0.05$ (they were even less than 0.0001), except the effect of farming systems and glucose content where it was 0.0001. The *p*-values were also less than 0.0001 for all interactions between cultivar and farming system. The content of prevailing carbohydrates (fructose and glucose) was reasonably higher in organic apples "Idared", "Melrose", "Šampion" and "Zvonkové", which is consistent with **Bertazza et al. (2010)** who also detected higher contents of monosaccharides in organic fruit. On the other hand, **Prugar (2000)** found no significant differences in carbohydrate content between organic and non-organic systems. This could be supported by our results from cultivars "Ontario", "Topaz" and "Florina" where the contents of glucose and fructose showed an even higher content in the case of integrated systems. These controversial tendencies support the importance of many factors affecting the carbohydrate content in apples.

The carbohydrate content in carrots (Table 4) was slightly lower than in literature sources **(Vogel, 1996; Kopec, 1998; Soria et al.; 2009)**, but it could vary depending on the cultivar **(Cefola et al. 2012)**. According to **Bufler (2013)** starch content is a key reserve of carbohydrates in carrot. The most represented sugars are fructose and glucose. All bio cultivars of carrots had higher concentrations of carbohydrates than integrated samples. Statistically significant differences were calculated for sucrose ($p = 0.0083$), glucose ($p = 0.0003$) and fructose ($p = 0.0303$). **Hogstad et al. (1997)** indicated that the amount of fertilizer used is one of the most important factors for sugar content in carrots. Carrots grown with no fertilizer and carrots fertilized with 40 - 80 kg nitrogen ha^{-1} as mineral fertilizer or 20 - 72 $t.ha^{-1}$ of organic fertilizer contained more total sugars compared with carrots fertilized with 100 - 192 kg nitrogen ha^{-1} as mineral fertilizer. Carrots in our experiment were fertilized by a slightly higher dose of nitrogen in the case of integrated samples (80 kg. ha^{-1}) compared to samples from organic farming production (54 $kg.ha^{-1}$). The differences, however, are in the form and thus the availability of nitrogen. The organic form of nitrogen prevails in Organica fertilizer and it must first be mineralized to the form acceptable for plants. In this way, a steady supply of lower levels of nitrogen for a longer period is ensured, which may have a significant influence on carbohydrates' formation and levels.

Different densities of plants also affected the carbohydrate content in carrots, but significant difference was calculated only in the case of glucose ($p = 0.0373$). The cultivar was a very important factor for the final sugars content. "Aftalon F1" showed higher concentration of monosaccharides compared to "Cortina F1" ($p = 0.0001$) for fructose and $p < 0.0001$ for glucose). No significant difference was found for sucrose ($p = 0.1657$) but the level of this disaccharide is reasonably lower and less important than levels of monosaccharides. **Seljasen et al. (2013)** also reported that the genetic factor shows the highest impact on quality variables in carrots. But they also found that climate-related factors may cause a difference of up to 82% for total sugars. The combined action of multiple factors is also supported by significant interactions calculated between variety and density ($p < 0.0001$ for sucrose and $p = 0.0176$ for glucose), as well as the density of plants and growing conditions ($p = 0.0186$ for sucrose and $p = 0.0026$ for glucose).

Our findings from apples and carrots analyses are not generally in line with **Prugar (2000)** who found no significant differences between products from organic and conventional growing systems in the dry matter content or total carbohydrates, but it must be stated that the problem is more complex and the effect of other factors, such as cultivar or density of plants, were also very important. This corresponds with **Nagy et al. (2012)** who stated that, in the case of apples, their sugar content was more affected by cultivars than by production systems. **Róth et al. (2007)** and **Bordeleau (2002)** reported the same conclusions. They agree that the quality is affected by many other factors such as cultivar, climate, soil type and subsequent storage conditions. These parameters often have a greater impact than the production method **(Bordeleau, 2002)**.

CONCLUSION

Many statistically significant differences were found comparing carbohydrates and dry matter content of apples from organic and integrated farming systems, but the tendencies were not the same for all cultivars. Some differences were also calculated between organic and non-organic carrots. Samples from integrated farming system showed significantly higher dry matter content than organic carrots. Bio cultivars of carrots had higher concentrations of sucrose, glucose and fructose than integrated samples. Our findings showed that farming systems could affect the composition of apples and carrots, but also some other factors like cultivar or density of plants significantly influence their quality parameters.

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SENSORY EVALUATION OF COBB 500 CHICKEN MEAT AFTER APPLICATION OF DIFFERENT ADDITIVES IN THEIR NUTRITION

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ABSTRACT

The objective of the experiment was to verify the effect of different feed additives in nutrition of Cobb 500 broiler chickens on the sensory quality of breast and thigh muscle modified by baking at temperature 200 ºC for 60 minutes. The experiment included 250 one-day-old Cobb 500 hybrid chickens, which were divided into 5 groups (n=50): control (I) and experimental groups (E1 with Agolin Poultry at doses of 100 $mg.kg^{-1}$, E2 with Agolin Tannin Plus at doses of 500 $mg.kg^{-1}$, E3 with Biostrong 510+FortiBac at doses of 1000 $mg.kg^{-1}$ and E4 with Agolin Acid at doses of 1000 $mg.kg^{-1}$). The chickens were fed during 42 days of age by *ad libitum* system with feed mixtures: BR1 starter feed mixture (until the of 10^{th} day of age), BR2 growth feed mixture (from 11^{th} to 20^{th} day of age), BR3 growth feed mixture (from 21^{st} to 35^{th} days of age) and BR4 final feed mixture (from 36^{th} to 42^{nd} days of age). Feed mixtures were produced with coccidiostats in powder form. Panellists evaluate aroma, juiciness, taste and tenderness on 5 point hedonic scale where 1 (the worst) and 5 (the best) were the extremes of each characteristic. Significant differences were found between control and experimental group E3 in juiciness and tenderness of breast muscles and between control and experimental group E2 in smell of thigh muscles. Sensory evaluation of breast and thigh muscles in Cobb 500 chickens after application of different feed additives indicated that these additives have not worsened the quality of meat. The highest sensory score was obtained in experimental group E4 (with addition of Agolin Acid at the dose of 1000 $mg.kg^{-1}$).

Keywords: sensory analysis; chicken meat; broiler chicken; feed additives

INTRODUCTION

Processed chicken products' consumption has also dramatically increased over the last decades **(Bianchi et al., 2009)**. Poultry meat is a very popular food commodity around the world due to its low cost of production as compared to meat products as beef, lamb or pork, low fat content, high nutritional value and distinct flavour **(Barbut, 2002; Chouliara et al., 2007; Patsias et al., 2008)**.

Food safety is an important aspect of food quality and efforts should be led to safety of new functional products from poultry meat **(Burdock et al., 2006)**. Meat quality may be affected already by manipulation of animal feeding **(Kennedy et al., 2005; Assi and King, 2007)** or *post mortem* manipulation of carcass body. Poultry meat and meat products are important source of proteins, but other components as fats have an important role in their composition, too. Nutrient content in meat products is between 40% and 50% **(Ordõnez et al., 1999)**, and fat performs the primary role in sensory aspects as taste and juiciness of all meat products **(Lucca and Tepper, 1994; Hughes et al., 1997; Cofrades et al., 2000)**.

New legislation, EU regulation and bans regarding the use of animal meal, classical antibiotic stimulators for growth and antimicrobial substances in feeds of animal including poultry lead to application of new supplements and biotechnological products in science as well as in practice **(Haščík et al., 2006, 2007; Bobko et al., 2009)**.

Maintaining of appropriate technological, nutritional and sensorial properties in meat is one of the conditions for new component integration in animal nutrition, because different supplements can cause the deterioration of meat quality, mainly in term of sensory properties **(Aleson-Carbonell et al., 2004; Pérez-Alvarez, 2006)**.

In recent years, products containing essential oils derived from several spices and herbs could be used in animal nutrition as feed additives to promote the growth. These phytogenic additives may have more than one mode of action, including improving feed intake and flavour, stimulating the secretion of digestive enzymes, increasing gastric and intestinal motility, endocrine stimulation, antimicrobial, anti-viral, anthelminthic and coccidiostat activities, immune stimulation, and anti-inflammatory and anti-oxidative activity and pigments **(Kırkpınar et al., 2011)**.

Many studies have also been conducted on the effects of dietary essential oils or combinations thereof on the performance of poultry but with varying and conflicting results. While some reports **(Hertrampf, 2001; Alçiçek et al., 2003)** demonstrated that essential oils improved animal performance, some researchers **(Schiavone et al., 2001; Lee et al., 2003a,b; Papageorgiou et al., 2003; Botsoglou et al., 2003, 2004)** reported that these additives were not effective in this regard.

The evaluations of properties as taste, smell, juiciness and tenderness, which are subject of sensory analysis, are

important factors that consumers will consider before making a decision to buy poultry **(Liu et al., 2004)**.

Quality assessment parameters of chicken meat, including sensory flavour and texture profiles, have been widely used in scientific studies to validate pre-processing treatments and postharvest processing technologies for chicken meat **(Swatland, 1999; Lyon et al., 2001)**.

According to **Augustin and Fischer (1999), Brestenský (2002), Mojto and Zaujec (2003), Haščík et al. (2004)**, evaluated sensory properties are dependent on type of used feed mixture, content of intramuscular fat in meat, way of meat preparation, genetics and many others intra-vital and extra-vital factors.

The objective of present study was to evaluate the effect of different additives as a dietary supplement added to feed mixtures on sensory quality of broiler chicken meat.

MATERIAL AND METHODOLOGY

Animals and diets

The experiment was undertaken in poultry test station Zamostie Company. The experiment included 250 pcs of one-day-old hybrid chickens Cobb 500, which were divided into 5 groups (n=50): control (I) and experimental groups (E1, E2, E3 and E4).

Experimental broiler chickens were fed during 42 days of age by *ad libitum* system with feed mixtures: BR1 starter feed mixture (until the of 10th day of age), BR2 growth feed mixture (from 11th to 20th day of age), BR3 growth feed mixture (from 21st to 35th days of age) and BR4 final feed mixture (from 36th to 42nd days of age). Feed mixtures were produced with coccidiostats in powder form.

Nutritional value (Table 1) of feed mixtures were the same in each group during the whole experiment. However, the diet of broiler chickens in experimental groups were supplemented by feed additives on base of acids and plant essential oils: Agolin Poultry at doses of 100 mg.kg^{-1} (E1); Agolin Tannin Plus at doses of 500 mg.kg^{-1} (E2); Biostrong 510+FortiBac at doses of 1000 mg. kg^{-1} (E3) and Agolin Acid at doses of 1000 mg. kg^{-1} (E4).

Sample analysis

At the end of the fattening (42nd day) and after slaughtering, 15 pieces of chickens halves were chosen from each group and were heat-treated at 200 °C for 60 minutes. From each halves, part from a thigh and breast muscle were separately evaluated in sensory analysis. Sensory evaluation of anonymous samples was performed by six-member committee and five-point scale was used

Table 1 Composition of the basal feed mixtures

Ingredients (%)	Starter (1 to 10 days of age)	Grower I. (11 to 20 days of age)	Grower II. (21 to 35 days of age)	Finisher (36 to 42 days of age)
Maize	46.33	48.50	50.05	50.91
Wheat	14.00	15.00	15.00	15.00
Soybean meal (45% CP)	30.00	26.60	28.00	26.70
Fiesh meal (72% CP)	2.50	2.00		
Dried blood	2.00	2.00		
Soybean oil	1.00	1.80	2.80	3.00
Monocalcium phosphate	1.60	1.25	1.30	1.48
Calcium carbonate	1.37	1.55	1.50	1.56
Fodder salt	0.20	0.30	0.35	0.35
Lysine	0.27	0.15	0.15	0.16
Methionine	0.27	0.18	0.17	0.20
Threonine	0.09	0.10	0.08	0.07
Vitamin premix	0.05	0.04	0.04	0.03
Micromineral premix	0.04	0.04	0.04	0.04
Enzyme phytase	0.015	0.015	0.015	0.015
Wheat meal	0.215	0.12	0.10	0.135
Maxiban (Narasin+Nicarbasin)	0.05			
Sacox (salinomycin sodium)		0.055	0.055	
Analyzed composition (g.kg^{-1})				
Crude protein	220.00	207.00	197.00	188.00
Fibre	20.00	24.00	28.00	29.00
Lysine	14.00	12.50	12.50	11.50
Methionine	6.00	5.20	5.20	5.00
Ca	9.00	8.50	8.50	8.50
P (non-phytate)	4.20	4.00	4.00	4.00
Na	1.60	1.60	1.60	1.60
[1]ME$_N$ (MJ.kg^{-1})	12.30	12.75	13.15	13.15

[1]ME$_N$ - Metabolizable energy, CP - Crude protein

for the self-assessment. Panelists evaluate aroma, juiciness, taste and tenderness on 5 point hedonic scale where 1 (the worst) and 5 (the best) were the extremes of each characteristic.

Statistical analysis

The results of experiment were processed in statistical programme Statgraphics Plus version 5.1 (AV Trading, Umex, Dresden, Germany). The variables statistical values (arithmetic mean, standard deviation) were calculated and to determine the significant differences among groups was used variance analyses with subsequent Scheffé's test.

RESULTS AND DISCUSSION

Results from sensory evaluation of valuable parts of carcass (breast and thigh muscles of Cobb 500 broiler chickens carcasses) after application of aditives in the feed mixtures at the doses of Agolin Poultry 100 mg.kg^{-1} (E1); Agolin Tannin Plus 500 mg.kg^{-1} (E2); Biostrong 510+FortiBac 1000 mg. kg^{-1} (E3) and Agolin Acid 1000 mg. kg^{-1} (E4) are recorded in Table 2 and 3.

Firstly, the properties of sensory quality in breast muscle were evaluated. We found the highest score in the control group (4.20 points) and lowest in the group E3 (4.00 points) in sensory evaluation of smell in breast muscle. Taste of breast muscle was the best in the E4 group (4.13 points) and the worst in the group E2 (3.83 points). Juiciness, which depends on the water content and fat content in muscle, was highest in the group E3 (4.26 points) and the lowest in the group E2 (3.53 points). Tenderness was highest in the group E3 (4.36 points).

In the terms of overall sensory assessment in breast muscles of Cobb 500 chickens after baking, we found the highest value in the group E3 (16.90 points) and the lowest in the group E2 (15.06 points). From a statistical point of view, balanced values in individual variables were achieved between groups, but the significant differences ($p \leq 0.05$) were found in juiciness and tenderness of breast muscle between control group and experimental group E3.

Smell of thigh muscle was 4.40 points in control group and it ranged from 3.96 points (E2) to 4.43 (E1) points in experimental groups. The results for taste were comparable between the control and experimental groups (3.93 - 4.20 points), what was confirmed also in the juiciness of thigh muscles (from 3.93 points in control group to 4.33 points in E1). The highest score of tenderness was recorded in tested group E1 (4.26 points) and the lowest score in tested group E4 (4.06 points). In term of the overall sensory assessment of thigh muscles we found the highest score in the experimental group E1 (17.20 points) and the lowest in the tested group E2 (16.23 points). The significant differences ($p \leq 0.05$) we found in taste of thigh muscles between control group and group E1 and between control group and group E2.

Obtained results from sensory evaluation of most valuable carcase parts of Cobb 500 chickens with application different feed additives on base acids, plant extracts and oils are in accordance with tendencies which were found by **Połtowicz (2000), Osek et al. (2001), Barteczko et al. (2003), Haščík et al. (2004, 2007, 2013, 2014), Bobko et al. (2006, 2009), Baracho et al. (2006), Chekani-Azar et al. (2008), Kim et al. (2009), Marcinčák et al. (2009), Mihok et al. (2010)** in

Table 2 Sensory evaluation of chicken breast muscles

	Control	E1	E2	E3	E4
Smell	4.20 ±0.41^{a}	4.13 ±0.39^{a}	4.00 ±0.65^{a}	4.10 ±0.60^{a}	4.16 ±0.52^{a}
Taste	4.06 ±0.46^{a}	4.10 ±0.54^{a}	3.83 ±0.81^{a}	4.10 ±0.60^{a}	4.13 ±0.63^{a}
Juiciness	3.76 ±0.65^{a}	3.73 ±0.62^{a}	3.53 ±0.72^{a}	4.26 ±0.53^{b}	4.00 ±0.71^{ab}
Tenderness	3.83 ±0.52^{a}	3.86 ±0.69^{a}	3.70 ±0.75^{a}	4.36 ±0.55^{b}	4.03 ±0.55^{ab}
Suma	15.86 ±1.70^{ab}	15.83 ±1.97^{ab}	15.06 ±2.65^{a}	16.90 ±1.57^{b}	16.33 ±1.94^{ab}

n = 15 pcs per group, E1 - Agolin Poultry at doses of 100 mg.kg^{-1}, E2 - Agolin Tannin Plus at doses of 500 mg.kg^{-1}, E3 -Biostrong 510+FortiBac at doses of 1000 mg. kg^{-1}, E4 - Agolin Acid at doses of 1000 mg. kg^{-1}, ^{a,b}- means with different superscripts differ significantly ($P \leq 0.05$).

Table 3 Sensory evaluation of chicken thigh muscles

	Control	E1	E2	E3	E4
Smell	4.40 ±0.54^{a}	4.43 ±0.37^{a}	3.96 ±0.51^{b}	4.13 ±0.54^{ab}	4.10 ±0.54^{ab}
Taste	4.10 ±0.54^{a}	4.16 ±0.41^{a}	3.93 ±0.62^{a}	4.20 ±0.56^{a}	4.00 ±0.56^{a}
Juiciness	3.93 ±0.67^{a}	4.33 ±0.55^{a}	4.10 ±0.43^{a}	4.23 ±0.59^{a}	4.10 ±0.66^{a}
Tenderness	4.13 ±0.58^{a}	4.26 ±0.53^{a}	4.23 ±0.41^{a}	4.13 ±0.44^{a}	4.06 ±0.59^{a}
Suma	16.56 ±2.04^{a}	17.20 ±1.47^{a}	16.23 ±1.69^{a}	16.96 ±1.94^{a}	16.26 ± 1.85^{a}

n = 15 pcs per group, E1 - Agolin Poultry at doses of 100 mg.kg^{-1}, E2 - Agolin Tannin Plus at doses of 500 mg.kg^{-1}, E3 -Biostrong 510+FortiBac at doses of 1000 mg. kg^{-1}, E4 - Agolin Acid at doses of 1000 mg. kg^{-1}, ^{a,b}- means with different superscripts differ significantly ($P \leq 0.05$).

application of different feed additives in chicken nutrition. In general, we found higher score of tenderness in thigh muscle than in breast muscle in the experiment. It is in accordance with results published by **Scholtyssek and Sailer (1986), Kofrányi and Wirths (1994)** and **Guéye et al. (1997)**, **Haščík et al. (2013, 2014)** because thigh muscles contain more internal fat and blood capillaries. Authors stated that availability and correctness of technological, nutritional as well as sensory quality in chicken meat is possible to achieve only by verified feed supplements, because any additive substances have not a positive impact on sensory properties of meat and may show an opposite trend, which somewhat reflected ($p \geq 0.05$) in the group E2 with the addition of Agolin Tannin Plus for both breast and thigh muscle both breast and thigh meat.

CONCLUSION

In this experiment, we examined the influence of feed additives applied in chicken nutrition on sensory properties of breast and thigh muscles after meat baking. Based on obtained results, we can conclude we did not find negative influence on sensory properties of breast and thigh muscles after application of chosen feed additives in Cobb 500 chicken nutrition. The best of tested feed additive was group with application of Biostrong 510+FortiBac at doses of 1000 mg. kg^{-1}.

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COMPARATIVE ANALYSIS OF ACETIC AND CITRIC ACID ON INTERNAL MILIEU OF BROILER CHICKENS

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ABSTRACT

The aim of the present study was to analyse the effect of two organic acids (acetic and citric acid) inclusion on serum parameters and the level of antioxidant status of broiler chickens. Some organic acidifiers reduce the growth of many intestinal bacteria, reduce intestinal colonisation and reduce infectious processes, decrease inflammatory processes at the intestinal mucosa, increase villus height and function of secretion, digestion and absorption of nutrients. Broiler chickens hybrid Ross 308 (n=180) were divided into 3 groups: one control (C) and two experimental groups (E1, E2). Experimental animals received acetic and citric acid per os in water in single dose 0.25% for 42 days. After 42 days of feeding blood samples were collected (n=10 in each group). Significant decrease of serum triglycerides in citric acid group when compared with the control group was recorded. Acetic acid administration resulted in increased sodium level. Significant increase of albumin content in both experimental groups and increase of bilirubin content in citric group was recorded. Acids administration had no significant effect on other serum and antioxidant parameters. Acetic and citric acid had no harmful influenced on internal milieu of broiler chickens. The research on the field of organic acid will be worthy of further investigation.

Keywords: acetic acid; citric acid; serum parameters; antioxidants; broiler chickens

INTRODUCTION

Organic acids are weak acids and do not dissociate completely in water. They are widely distributed in nature as normal constituents of plants or animal tissues and formed through microbial fermentation of carbohydrates mainly in the large intestine **(Partanen and Mroz, 1999)**. Organic acids such as citric and acetic acids have successfully been supplemented in feed in broiler production **(Kirchgessner and Roth, 1982; Hassanpour et al., 2009)** also as alternatives to antibiotic growth promoters **(Gunal et al., 2006)**. They have mainly been used in order to sanitize the feed having problem with bacterial infection **(Thompson and Hinton, 1997)**. Several organic acids have been reported to improve growth performance, feed efficiency and mineral absorption when supplemented in nonruminant diets **(Boling et al., 2000)**, are able to inhibit microorganism growth in the food, and consequently to preserve the microbial balance in the gastrointestinal tract and improve the solubuility of the feed ingredients, digestion and absorption of nutrients **(Brown and Southern, 1985; Boling et al., 2000; Farran et al., 2005; Yesilbag and Colpan, 2006; Deepa et al., 2011)**. Citric acid is a weak organic acid and has been reported to cause a decrease in the pH of intestinal contents by contributing hydrogen ions to the intestinal environment in chickens **(Brown and Southern, 1985)** thus may have antimicrobial effects **(Barnhart et al., 1999)**. It has been reported that citric acid improves phytate P utilization in broiler chickens **(Snow et al., 2004; Rafacz-Livingston et al., 2005)**, increased the feed intake and lowered feed conversion ratio **(Deepa et al., 2011)**, improved weight gain **(Nezhad et al., 2007)**, feed efficiency and feed consumption, increased the immune status of the broilers **(Abdel-Fattah et al., 2008)**.

It was reported that vetch soaked in 1% acetic acid at room temperature for 24 h improved performance of broilers and laying hens **(Farran et al., 2001)** and at 40°C for 24 h enhanced hen performance **(Farran et al., 2005)**. **Adenkola et al. (2008)** reported that acetic acid may be of value in combating adverse effects of stress in turkeys reared during the hot-dry season.

Aerobic organisms are exposed to reactive oxygen species (ROS). In healthy organisms, their production is balanced by the antioxidant defence system. Antioxidants within cells, cell membranes and extracellular fluids can neutralize excessive ROS formation (**Halliwell, 2007)**. Cells seem to use several systems for protection against oxidative stress **(Sedlak and Snyder, 2004)**. Examples include repair enzymes (to repair damaged biomolecules), preventative antioxidants as albumin (to prevent the formation of free radicals) and scavenging antioxidants as bilirubin (to remove reactive species once formed). Data obtained from the literature indicated that only certain food components are protective against ROS (reactive oxygen species) in humans and animals **(Chakraborty et al., 2009)**. Our previous results reported that various feed additives have antioxidant properties and affect the internal milieu of poultry **(Capcarova et al., 2008; 2010a, b; 2012)**.

Many studies consider mainly the effect of organic acid on chicken performance, production parameter and its

antimicrobial effects. To our knowledge only few data regarding the effect of organic acids as acetic and citric acids on serum parameters and antioxidant status of chicken blood are available. Base on this our study was designed to determine the effect of acetic and citric acids on internal milieu of chickens.

MATERIAL AND METHODOLOGY

Animals and diets

The experiment was conducted on broiler chickens, hybrid Ross 308 (n=180). Each group included 60 chickens. Chickens (no sexed) were divided into three groups (control - C and two experimental groups E1 - E2). Experimental chickens received an organic acids inclusion in water as follows: E1 - acetic acid in concentration 0.25%; E2 - citric acid in concentration 0.25%. The group of chickens received feed mixture without organic acid addition served as control.

The feeding period lasted 42 days. Chickens were fed ad libitum with complete feed mixture (CFM) (Biofeed a.s., Kolarovo, Slovakia) as follows: CFM starter (powdery form) from Day 1 till Day 21 and CFM grower (granula form) from Day 22 till Day 42. Water was provided *ad libitum*. Ingrediens and nutrient composition of diets is shown in Table 1. CFM starter contains of cereal grains, soybean meal, fish products, minerals, vitamin-mineral premix and CFM grower includes cereal grains, products and by-products from oil plants, soybean meal, fish products, minerals, vitamin-mineral premix.

Animals were kept in thermoneutral hall (from Day 1 33 °C until 21 °C at the end). In closed hall thermo aggregate was installed and experimental conditions with defined temperature and humidity were simulated by sensor. Simulated conditions were continually monitored using electronic recorder (Hivus s.r.o., Zilina, Slovak Republic). Animals were stabled cage technology (MBD). The measurements of the cage were 75x50 cm (0.370 m^2). The experiment was realized in approved breeding hall of Department of Poultry and Small Animal Husbandry in Nitra, Zobor unit.

Chickens were healthy and their condition was judged as good at the commencement of the experiment. Conditions of animals care, manipulation and use corresponded with the instruction of ethical commission. Care and use of animals and experimental devices met the requirements of the certificate of Authorization to Experiment on Living Animals no. SK PC 30008 (State Veterinary and Food Institute of Slovak Republic).

Blood sampling and analyses

After 42 days of feeding blood samples were collected (n=10 in each group). The blood serum was separated from whole blood by centrifugation at 3000g for 30 min. The concentrations of serum parameters: glucose, total cholesterol, total proteins, triglycerides, calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), chlorides (Cl), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) in blood serum of broiler chickens were analysed. Ecoline kits on automatic analzyer Microlab 300 (Merck, Germany), spectrophotometer Genesys 10 (Thermo Fisher Scientific Inc., USA) were used according to manufacturer condition.

The activity of antioxidant enzyme superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), bilirubin, and albumins content were assayed by spectrophotometer Genesys 10 (using antioxidant RANDOX kits (Randox Labs., Crumlin, UK) according to the manufacturer's instructions.

Statistical analysis

SAS software and Sigma Plot 11.0 (Jandel, Corte Madera, USA) were used to conduct statistical analyses. One-way ANOVA was used to calculate basic statistic characteristics and to determine significant differences among the groups. Data presented are given as mean and standard deviation (SD). Differences were compared for statistical significance at the level $P < 0.05$.

RESULTS AND DISCUSSION

Organic acids given in animal diet improved digestibility of proteins and amino acids and the absorption of minerals **(Omogbenigum et al., 2003)**. Citric and acetic acids have been examined many times for its efficacy in improving growth performance, however little work has detected their effects on internal milieu of animals. The poultry sector is searching for new feed additives in order to improve the feed efficiency and the animal health **(Shane, 1999)**. Among various compounds, organic acids are promising alternatives **(Hyden, 2000)**.

Table 1 Diet composition of feed mixture KKZ HYD-01 and HYD-02

Ingredient	KKZ HYD-01	KKZ HYD-02
Dry matter (g.kg-1)	917.3	913.3
Crude protein (g.kg-1)	211.3	199.7
Fat (g.kg-1)	25.5	23.0
Starch (g.kg-1)	413.0	434.8
Total sugar (g.kg-1)	49.5	31.7
ME (MJ)	11.69	11.56
Ca (g.kg-1)	12.121	8.207
P (g.kg-1)	7.833	6.834

Ca, calcium; P, phosphorus

Table 2 Effect of organic acids on serum parameters of chickens

Parameter	C	E1	E2
Glucose (mmol.L^{-1})	11.31 ±0.60	11.09 ±0.72	11.29 ±0.56
Triglycerides (mmol.L^{-1})	0.78 ±0.16^{a}	0.68 ±0.15	0.60 ±0.09^{b}
Cholesterol (mmol.L^{-1})	5.60 ±0.61	5.41 ±0.54	5.72 ±0.23
Total proteins (g.L^{-1})	37.85 ±2.86	36.45 ±3.27	39.20 ±3.40

C - control group (without organic acid supplement); E1 - E2 experimental groups with organic acid addition, values shown as means ± SD, superscripts with different letters $^{(a-b)}$ within the same row differ significantly (P <0.05).

Table 3 Effect of organic acids on serum mineral parameters of chickens

Parameter (mmol.L^{-1})	C	E1	E2
Ca	2.73 ±0.22	2.55 ±0.29	2.77 ±0.20
P	2.34 ±0.19	2.29 ±0.25	2.36 ±0.17
Mg	1.02 ±0.301	0.92 ±0.14	1.01 ±0.16
Na	145.6 ±2.6^{b}	146.3 ±1.8	148.9 ±3.3^{a}
K	5.26 ±0.61	5.19 ±0.28	5.26 ±0.45
Cl	115.5 ±3.0	114.8 ±1.6	116.5 ±2.3

Ca - calcium, P - phosphorus, Ma - magnesium, Na - sodium, K - potassium, Cl - chlorides, C - control group (without organic acid supplement); E1 - E2 experimental groups with organic acid addition, values shown as means ± SD, superscripts with different letters $^{(a-b)}$ within the same row differ significantly (P <0.05).

Table 4 Effect of organic acids on enzymatic activity of chickens

Parameter (μkat.L^{-1})	C	E1	E2
ALT	0.10 ±0.03	0.09 ±0.02	0.11 ±0.02
ALP	34.96 ±13.71	34.47 ±6.58	42.24 ±15.84
GGT	0.37 ±0.07	0.34 ±0.10	0.29 ±0.10

ALT - alanine aminotransferase, GGT - gamma glutamyl transferase, ALP - alkaline phosphatase, C - control group (without organic acid supplement); E1 - E2 experimental groups with organic acid addition, values shown as means ± SD, differences were not significant (P >0.05)

Table 5 Effect of organic acids on antioxidant parameters of chickens

Parameter	C	E1	E2
SOD (U.ml^{-1})	15.36±2.11	13.17±2.52	16.32±2.91
GSH-Px (U.L^{-1})	524.35±69.49	526.35±59.65	495.37±77.26
Albumins (g.L^{-1})	13.41±0.93^{b}	14.26±1.06^{b}	15.60±1.02^{a}
Bilirubin (mmol.L^{-1})	3.37±0.72^{b}	5.05±2.21	6.66±3.24^{a}

SOD - superoxid dismutase, GSH-Px - glutathion peroxidase, Fe - iron, Cu - copper, E1 - E4 experimental groups with propolis addition in various doses, values shown as means ± SD, superscripts with different letters $^{(a-b)}$ within the same row differ significantly (P <0.05).

Effect of organic acids on serum parameters of broiler chickens

Results of this study are shown in Table 2. Dietary supplementation with both acids resulted in decrease in blood triglycerides content of broiler chickens.

Statistical analyse showed significant difference (P <0.05) between the control and E2 (citric acid) group. Increase of glucose in acetic acid group in comparison with the control group was insignificant (P >0.05). Blood glucose, cholesterol and total proteins were not influenced by

organic acids inclusion. Serum cholesterol content was not influenced by acids inclusion. In the study with mice a mixture of thiamine, arginine, caffeine, and citric acid resulted in decrease of triglyceride content **(Muroyama et al., 2003)**. The results of **Štukelj et al. (2010)** indicated that the diet with citric acid had no deleterious effects on various blood parameters of pigs and no changes have been determined. Adding of acidifier to the chicken diet caused decrease of serum cholesterol, total lipids and triglycerides **(Abdel-Fattah et al., 2008)**. **El-Afifi et al. (2001)** reported no significant effect on serum lipids after citric acid treatment. The discrepancies in literature may be due to different time of feeding, kind of animal involved in the experiments, concentration of acid and environmental conditions. The mechanism of action through organic acids can decrease serum triglyceride content and possibly other parameters of lipid profile may be interpreted through influence in decreasing the microbial intracellular pH. Thus, inhibits the action of important enzymes and forces the bacterial cell to use energy to release the acid protons, leading to an intracellular accumulation of acid anions **(Abdel-Fattah et al., 2008)**.

Effect of organic acids on serum mineral parameters of broiler chickens

The results are presented in Table 3. The addition of citric acid caused significant (P <0.05) increase of Na content in comparison to the control group. Slight increase of this parameter was found also in E1 group (acetic acid), however without significant difference (P >0.05). Other serum mineral parameters were not affecting by organic acids and the values of both experimental groups were similar to those found in the control group.

Citric acid is absorbed across the intestinal brush border membrane via a Na+-dependent transport mechanism that seems to be specific for tri- and dicarboxylic acids **(Wolffram et al., 1992)**.

Effect of organic acids on serum enzymatic activity of broiler chickens

Table 4 presents activity of selected enzymes ALT, ALP, GGT in blood serum of broiler chickens after organic acid supplementation. Among the enzymatic profile, there was no variation in enzymes activity in experimental groups when compared to the control group. Control group had similar level of enzymatic activity to those of the experimental groups and differences among the groups remained insignificant (p >0.05).

Some authors reported significant changes of both acids on cholesterol, total proteins content, Ca and P content, enzymatic activity **(El-Afifi et al. 2001; Muroyama et al., 2003; Abdel-Fattah et al., 2008; Štukelj et al., 2010)**. The discrepancies in literature may be due to different time of feeding, kind of animal involved in the experiments, concentration of acid and environmental conditions.

Effect of organic acids on parameters of antioxidant status of broiler chickens

Addition of citric and acetic acids resulted in increase in albumins, bilirubin and D-3 hydroxybutyrate in chicken blood when compared to the control group (Table 5). Albumins content was increased in both experimental groups against the control, significantly (P <0.05) in the groups with citric acid addition. Bilirubin concentration tended to increase in E1 group with comparison to the control group, however the differences remained insignificant (P <0.05). In E2 group the increase in blood bilirubin was confirmed also statistically (P <0.05). Activities of antioxidant enzymes (SOD, GSH-Px) in experimental groups were similar to those from control group and differences among the groups were insignificant (P >0.05). SOD and GSH-Px are important antioxidant defences, as these enzymes are involved in the clearance of superoxide and hydrogen peroxide **(Mates and Sanchez-Jimenez, 1999)**. Our study demonstrated no diet related changes in SOD and GSH-Px activity. Similar results were reported by **Štukelj et al. (2010)** on pigs. Serum albumin an important protein that presents direct protective effects **(Bourdon et al., 1999)** represents a very abundant and important circulating antioxidant **(Roche et al., 2008).** In our study significant increase (P <0.05) in the content of serum albumins in citric acid group E2 versus control group was measured. Acetic acid also increased albumins content when compared to the control group, however without significant difference (P >0.05). **Yesilbag and Colpan (2006)** found that organic acid mixture in laying hen diet for 18 weeks significantly enhanced serum total protein and albumin concentrations what could be related to improvement of intestinal amino-acids absorption in acidic conditions that consequently enhances protein synthesis.

The combined evidence from animals and human studies indicates that bilirubin, member of the antioxidant family, is a major physiologic cytoprotectant and might alleviate oxidative stress in the blood **(Sedlak and Snyder, 2004)**. In our study both acids increased the bilirubin content in chickens against the control group, significantly (P <0.05) in case of citric acid (E2 group). Organic acids can supress the growth of pathogenic bacteria, encourage the growth of beneficial microflora and ensure that the enzymes function is at maximal capacity **(Ghazalah et al., 2011)** The way of action of organic acids seems to be related to a reduction of pH in the upper intestinal tract, interfering with the growth of undesirable bacteria and modifying to intestinal flora **(Kirchgessner and Roth, 1982)**. An optimal balance in the intestinal flora is beneficial for health and development of the chickens **(Garrido et al., 2004)**. Some data indicated that addition of acetic and citric acids to the diet for broilers resulted in the increase of villus height of the small intestine. Organic acidifiers reduce the growth of many pathogenic or non-pathogenic intestinal bacteria, therefore reduce intestinal colonisation and reduce infectious processes, ultimately decrease inflammatory processes at the intestinal mucosa, which increase villus height and function of secretion, digestion and absorption of nutrients than can be appropriately performed by the mucosa **(Ghazalah et al., 2011)**. However, there is need to conduct more research in order to establish the suitability of adding organic acids to broilers diet **(Nourmohammadi et al., 2010)**.

CONCLUSION

In conclusion, these results show a beneficial effect of organic acids consumption, mainly citric acid, on serum triglycerides concentration, Na content and antioxidant

status in broiler chickens. To widen this idea, more experiments should be performed with various combinations and doses of organic acids.

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VEGETABLE OIL BASED EMULSIONS IN MILK

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ABSTRACT

Milk and dairy products represent an important part of functional food in the market. Based on their positive health and nutritional benefits, they have gained popularity and their consumption as well as production is on the rise in the last few decades. As a result of this trend, milk-based products are being used for the delivery of bioactive food ingredients. This study is devoted to the formulation of stable emulsions containing grape seed oil dispersed with several emulsifiers (Tween 80, monocaprylin, and lecithin) in milk. Photon correlation spectroscopy was used to evaluate the characteristics of the emulsions in terms of mean droplet size, droplet size distribution and polydispersity index. Emulsions were prepared using 2% and 5% w/w grape seed oil, and 3%, 5%, or 8% w/w emulsifier, and these were homogenized at two different rates of 1050 and 13400 rpm. Parameters influencing emulsion particle size and particle size distribution were identified, which included emulsifier type, its HLB value, oil type (virgin, refined), homogenization rate and the fat content in the milk. Homogenization at 13400 rpm for 10 min. produced fine emulsions with small mean particle sizes and monomodal distribution of droplets. Regarding emulsifier type, the smallest droplet sizes were obtained with formulations containing Tween 80 (250-315 nm), whereas lecithin primarily accounted for the monomodal particle size distributions.

Keywords: grape seed oil; photon correlation spectroscopy; milk; encapsulation; emulsions

INTRODUCTION

Oil in water (o/w) emulsions are systems suitable for encapsulating lipophilic substances in various food products, such as milk, yoghurt, ice-cream, etc. **(Shahidi and Bailey 2005, Chee et al., 2005, 2007)**. A suitable method of emulsification thus leads to fortifying selected foods with bioactive substances of a hydrophobic nature. The substances mostly used are, due to their biological effects, polyphenols, fatty acids, phytosterols and carotenoids **(McClements et al., 2007)**. Accessible sources of these bioactive substances include minor and non-typical vegetable oils. These are usually obtained under mild conditions with a view to preserving the specific properties of the original oils. They are usually only available in a limited amount, and include oils from grape seeds, almonds, borage, avocados, macadamia nuts, mangos, Camelina, marigolds and pumpkins **(Shahidi and Bailey 2005)**.

Grape seed oil represents a raw material of significant dietetic value. It is characterized by a high content of nutritionally important compounds, such as essential fatty acids, tocotrienols and polyphenols **(Bail et al., 2008; Madawala, 2012)**. It is a side-product of the wine industry and is made from pomace, i.e. pressed grapes used for wine. The seeds are removed from the pomace, and used to obtain oil by pressing or extracting the same with the use of solvents. Pressed oils are of higher quality, with the superior-quality oils being obtained by cold pressing in hydraulic presses. However, this method produces lower yields. The actual amount of oil obtained depends on many factors. Crucial to this are soil and climate conditions, as well as the characteristics of the variety utilized (the seeds of white grapes contain more oil than those of blue grapes). Its favourable properties make it a popular material in gastronomy, the cosmetics industry and in the production of paints and varnishes **(Burg and Zemánek, 2012)**.

In recent years, the functional foods market has witnessed a rise in the popularity of milk-based products, especially milk drinks. In addition to the preferences of consumers, their advantage lies in the fact that milk is a rich source of nutrients **(Sharma, 2005)**. Therefore, milk products are a suitable food matrix for encapsulating bioactive substances. By dispersing vegetable oils in milk, a system can be created that combines the benefits of both its components.

The aim of the work presented here was to develop stable grape seed oil emulsions in milk, and to evaluate their behaviour as regards the composition of said formula.

MATERIAL AND METHODOLOGY

Emulsion preparation

The model emulsions were prepared via the method of mechanical homogenisation, with the use of two devices possessing different speeds of revolution - a Heidolph mechanical overhead stirrer (1050 rpm; type RZR 2020, Heidolph Instruments GmbH) and an Ultra-Turrax disperser (13400 rpm; type T 25 digital, IKA Labortechnik). All the emulsions so prepared contained as a the basis the original, natural emulsion phase of milk with the fat contents of 0.5%, 1.5% and 3.5%, hereinafter referred to as the water phase (Laclel, Kunín Dairy). The dispersed phase of the emulsion was formed by the one of grape seed oil samples that had been processed in various ways; either extra virgin, cold-pressed, unrefined (Saint

George's), or refined (M+H, Míča a Harašta). Due to the inherent emulsifying properties of milk, emulsions of both types of oil were first prepared without emulsifiers. In order to improve their final stability, food emulsifiers were later added to the formula; these comprised lecithin (Mogador), monocaprylin (MAG C8:0) **(Janiš et al., 2000)** and Tween 80 (Polyethylene glycol sorbitan monooleate, Sigma-Aldrich).

In the first set of experiments a total of 60 emulsions were made with 2% oil concentration and varied emulsifier concentration (3%, 5%, 8% w/w). All the emulsions prepared were visually observed for stability immediately after emulsification and again after one day of being stored at the temperature of 25 °C. Emulsions with a composition that ensured stability and the lowest concentration of emulsifiers were selected out of this group.

Afterwards, these selected emulsions were prepared with 5% concentration of grape seed oil, i.e. with the highest possible concentration that enables creation of a homogeneous emulsion while preserving the original emulsifier concentration. Only the most stable emulsions were selected for analysis of particle size and particle size distribution. Visually registered phase separation was chosen as the stability indicating parameter, which pointed to disintegration of the system representing an undesirable situation from the perspective of consumers.

The following procedure was used with the Heidolph overhead stirrer. Two beakers were filled with the appropriate amounts of emulsifier, and the water and oil phase. These were subsequently warmed in a water bath at a temperature of 85 °C. The formula for emulsion with 5% oil concentration is provided in Table 1**.** The water phase of milk alone, or of the emulsifier dissolved in milk, was gradually added into the oil phase, under stirring. After all the components had blended together, the emulsion was homogenised for 10 minutes at the constant speed of 1050 rpm.

Preparing emulsions with the use of the Ultra-Turrax disperser was carried out as follows: the components of water and oil phase were weighed and filled into the dispersion test tube and then warmed in a water bath. The amounts needed for preparing the emulsions with 5% oil concentration are given in Table 2.

Once the temperature of the water bath had reached 85 °C, the mixture was homogenized for 10 minutes at the speed of 13400 rpm.

Emulsion analysis by photon correlation spectroscopy (PCS)

In the selected emulsions, the size (z-average diameter) and particle size distribution were analysed by photon correlation spectroscopy with the use of the Zetasizer Nano ZS device (Malvern Instruments). The emulsion analysed was first homogenised by manually shaking it. A measured sample was then put into a plastic cuvette - 1 ml of distilled water was filtered through a 0.22 μm (Millipore) filter while 3 μl of emulsion were added to it using a pipette. In order to ensure the constant temperature of the sample, the cuvette was covered with a lid and heated to 25 °C. The following measurement parameters were set on the device - viscosity of the dispersion medium: 0.8872 cP, refractive index of the dispersion medium: 1.330 and refraction index of the dispersed phase: 1.450. The measured data were processed by the Zetasizer software.

RESULTS AND DISCUSSION

Out of 60 emulsions prepared in the first set of experiments (including control emulsions without any added emulsifier), 24 samples were proposed for the experiment itself on the bases of primary selection. These were subsequently evaluated visually and analysed by photon correlation spectroscopy.

Visual observation

Emulsions were evaluated immediately after emulsification and again after one day of being stored at 25 °C. The parameters monitored included visual appearance, colour and stability of the emulsions.

As the formula contained milk, all the emulsions were of noticeable milky white colouring. The stable emulsions were homogeneous, without any signs of phase separation. Instability in the emulsions prepared was revealed by gradual separation of phases. This means that so-called creaming was observed, i.e. a layer of oil accumulated in the top part of the emulsion system due to its lower density.

Table 1 Formula for emulsion prepared with a stirrer and 5% (w/w) grape seed oil

Emulsifier concentration [%]	Emulsifier [g]	Water phase [g]	Oil phase [g]
0	-	47.5	2.5
3	1.5	46.0	2.5
5	2.5	45.0	2.5
8	4.0	43.5	2.5

Table 2 Formula for emulsion prepared with an Ultra-Turrax and 5% (w/w) grape seed oil

Emulsifier concentration [%]	Emulsifier [g]	Water phase [g]	Oil phase [g]
0	-	19.0	1.0
3	0.6	18.4	1.0
5	1.0	18.0	1.0
8	1.6	17.4	1.0

Visual observation revealed that each kind of the milk tested demonstrated sufficient capacity for emulsification and stabilization, which enabled creation of homogeneous emulsion with 5% concentration of grape seed oil (refined or raw) within a single day. It was possible to form emulsion without emulsifiers thanks to the presence of the surface-active agents - casein and whey protein - in the milk serum. In order to increase stability, the basic formula consisting of milk and 5% (w/w) grape seed oil was enriched with one of the food emulsifiers (monocaprylin, lecithin, Tween 80). The 3% concentration of emulsifiers enabled creation of a homogeneous emulsion in the system, with the exception of monocaprylin.

Photon correlation spectroscopy

Many physical, chemical and sensory properties of food emulsions depend on the size and nature of the particles they contain. The important characteristics of emulsion particles also include the parameters determined in this work. Namely, these were the mean particle size and size distribution, which both influenced the properties, stability and application of emulsions.

All the emulsions prepared can be classified as typical food emulsion systems, whose particle size varies in dependence to the composition - between 250 and 1,000 nm **(Shahidi and Bailey, 2005)**. As analysis revealed, the milk samples tested showed a mean particle size of 200 to 350 nm and can be hence ranked as macroemulsions; i.e. the size characteristics in the emulsions prepared concur with expectations.

The first set of experiments showed that in order to create emulsions with the required stability it is optimal to carry out homogenization with the use of the Ultra-Turrax disperser for 10 minutes at 13400 rpm. In this way, stable emulsions can be obtained with a low mean size of particles and monomodal size distribution, which complies with the theoretical expectation of a higher efficiency of homogenization caused by more intensive stirring **(Akoh and Min, 2008)**.

When the z-average diameter of emulsion particles was evaluated more thoroughly, the initial theoretical premise was confirmed, i.e. the size of emulsion particles depends on the type of emulsifier used. As is obvious from Figure 1, 3% concentration of both lecithin and Tween 80 led to a visible decrease in particle size z-average in comparison with the control emulsions prepared without any emulsifier, irrespective of what kind of milk was used. When 3% concentration of Tween 80 was used, the size of emulsion particles was reduced most noticeably. In samples with 8% concentration of monocaprylin, an opposite tendency was observed (with the exception of the emulsion in which whole milk was used as the dispersion environment) - namely, the particle size grew in comparison with the control emulsions. This diversity of results received from the emulsions tested can be explained by the specific values of the hydrophilic-lipophilic balance (HLB) of particular emulsifiers. Tween 80 with HLB 15 is a hydrophilic surfactant with high solubility in the water phase, which is why it efficiently stabilizes o/w systems, as was manifested in the resulting small size of emulsion particles. Nevertheless, monocaprylin (HLB ≈ 6), due to its larger lipophilic part, dissolves in water less readily, hence is better for stabilizing w/o emulsions. The HLB value for soya lecithin equals 8 and is on the border between both the situations mentioned above **(Bartovská and Šišková, 2005)**. In conformity with the theory of hydrophilic-lipophilic balance, the size of the emulsion particles prepared with this emulsifier, represents the transition between the two previous systems.

Figure 1 also shows that the rising amount of milk fat in the system results in larger sized particles. Deflection from this behaviour was again seen in emulsions with 8%

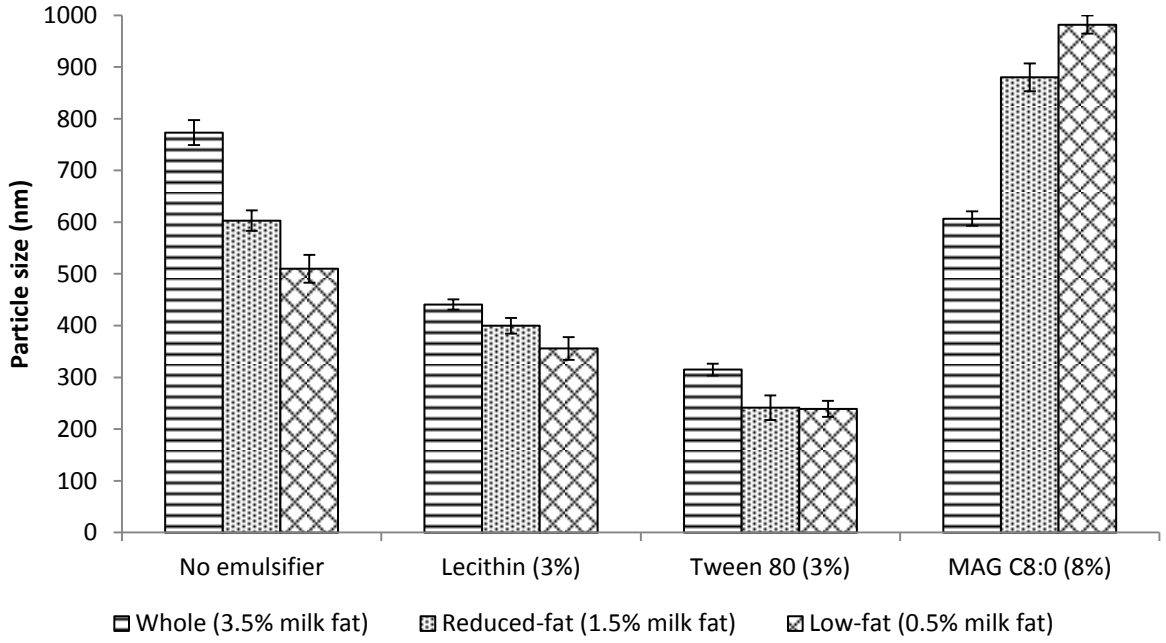

Figure 1 Impact of using various emulsifiers on particle size in 5% concentration of unrefined grape seed oil

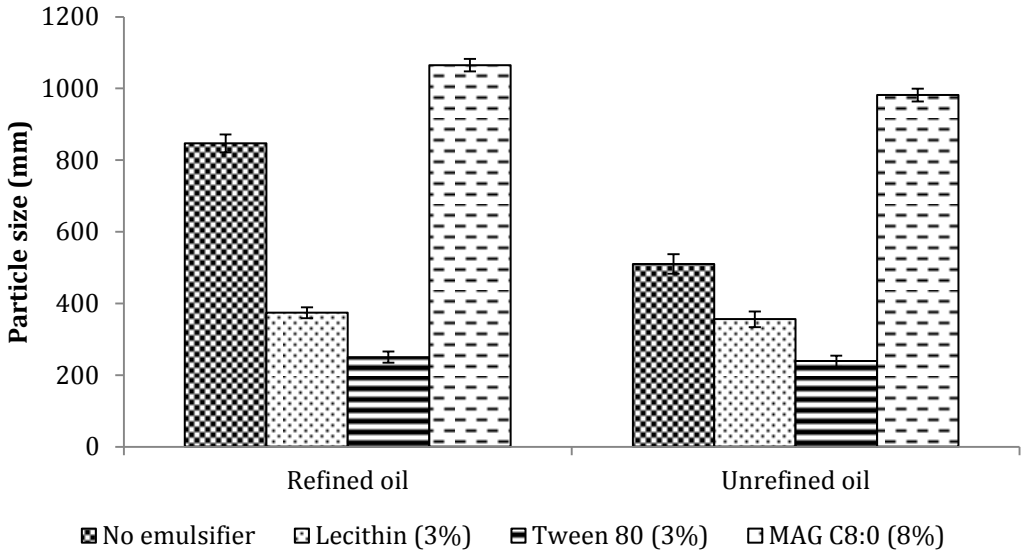

Figure 2 Impact of the kind of grape seed oil (5% w/w) on particle size in low-fat milk serving as the continuous phase of emulsion

concentration of monocaprylin, where an opposite tendency was observed.

Adding grape seed oil (5% w/w) into used kinds of milk resulted, with no exception, in increasing the size of the emulsion particles. An interesting finding arose from comparing the sizes of particles in two different grape seed oils: unrefined extra virgin oil formed emulsion particles of smaller size than refined oil (see Figure 2). This result could be related to the presence of surface-active agents, free fatty acids, proteins and phospholipids, which are present in raw oil, and thus can contribute to better emulsification. In refined oil, the content of these substances is reduced to the minimum concentrations.

The influence of the emulsifiers used on particle size and distribution in the prepared emulsions containing fat-reduced milk is shown in Figure 3. The development of the recorded distribution curves proves that using 3% concentration of Tween 80 resulted in a significant decrease in the mean value of particle sizes in comparison with control emulsions. As in the previous cases, unrefined oil formed emulsions with particles of a smaller z-average. Although the distribution curves were mostly bimodal, one fraction was strongly predominant. Emulsions with 3% lecithin concentration primarily registered one particle population. In comparison with control samples that did not contain emulsifiers, the z-average of particles was smaller. In this group, refined oil also formed emulsions with larger particles. In emulsions with 8% monocaprylin the distribution curves were multimodal, which proves the high polydispersity in the system. Compared to the other emulsifiers, monocaprylin did not cause a decrease in the size of the emulsion particles.

For comparative purposes, photon correlation spectroscopy was used to analyse not only the emulsions prepared but also the kinds of milk used. The received distribution curves in low-fat milk and whole milk could be regarded as monomodal. Of the two identified populations of particles present in reduced-fat milk, the size of a smaller fraction was just within the measuring range of the device being of 5 μm. Consequently, it can be

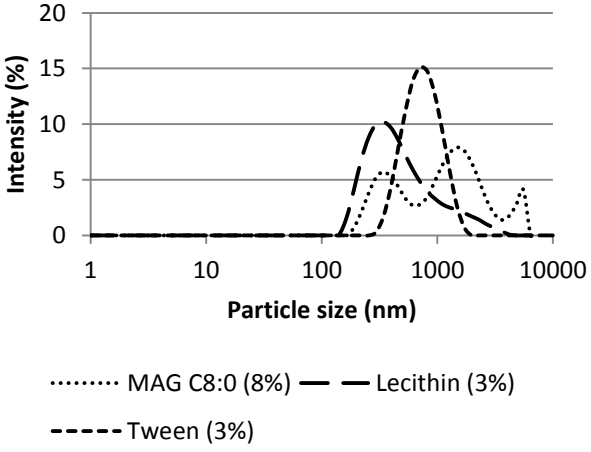

Figure 3 Influence of an emulsifier on the particle size distribution of selected emulsions

assumed that the samples of low-fat and whole might also be polymodal. However, the instrument is not able to detect the particle size >5 µm. The findings from the analyses of milk samples also revealed that the fat content influences the resulting size of particles. Alongside a rising volume of fat, the z-average increased.

CONCLUSION

This experimental work focused on preparing stable emulsions of milk and grape seed oil with the use of lecithin, monocaprylin and Tween 80 as food emulsifiers. The emulsions prepared were subsequently evaluated visually and *via* photon correlation spectroscopy. The experiments conducted revealed that in order to create a model emulsion with small mean particle sizes and monomodal size distribution, the optimal method is homogenization taking 10 minutes at 13400 rpm. The resultant size of particles in the emulsions prepared was most significantly influenced by the type of emulsifier, its respective HBL value, the fat content in the milk and the method of oil processing. In terms of impact on particle size, the optimal emulsifier appeared to be Tween 80. When it was used in 3% concentration, the z-average of the particle size was in the narrowest interval of all the sets of emulsions tested (250-315 nm). With regard to particle size distribution in emulsions, the most suitable emulsifier was lecithin, as it enabled creation of homogeneous emulsions, which was proven by the presence of predominantly monomodal distribution curves. Lecithin as a part of the formula can thus contribute to the nutritional quality of the given system.

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THE INFLUENCE OF GUAR GUM ON TEXTURAL AND SENSORY PROPERTIES OF ROLLS MADE FROM SEMI-FINISHED FROZEN PRODUCTS

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ABSTRACT

Textural and sensory properties of rolls produced from semi-finished frozen products with additions of guar gum were assessed. A commercial wheat flour T 512, ingredients such as yeast *Saccharomyces cerevisiae*, rapeseed oil, sodium chloride were used for preparing dough and final products. Guar gum and commercial additive Trial RC2 were used as additives for production. The textural properties of rolls were measured by a TA-XT Plus Texture Analyzer. All samples were evaluated by selected assessors. A five-point hedonic scale was used for evaluation characteristics such as taste, pliancy, texture, porosity, stickiness, gumminess, crispness and quality. The obtained data showed nonsignificant difference in moisture of rolls after baking and 3 days after baking. Control sample of rolls had higher firmness in comparison to other samples with guar gum after baking. Samples of rolls with higher addition of guar gum (10 $g.kg^{-1}$ and 15 $g.kg^{-1}$) had less firmness in comparison to control sample and sample with 5 $g.kg^{-1}$ of guar gum. Sensory analysis showed negligible differences among all samples in monitored characteristics. Sensory assessors evaluated all samples as identical. Increased addition of guar gum in rolls led to the extension of shelf life (lower firmness) at unchanged sensory properties of the rolls.

Keywords: guar gum; roll; sensory analysis; texture

INTRODUCTION

The baking industry is constantly trying to offer benefits to consumers, including freshly baked breads. The partially baked frozen process provides control over the processes that are partial baking, freezing and storing of bakery products and re-baking at the point of sale or before selling to the end user **(Škara et al., 2013)**.

The redistribution of water and ice recrystallization in dough during frozen storage leads to changes in the arrangement and structure of amylopectin and amylose molecules. Other changes occur during starch gelatinization and retrogradation.

The longer dough remains in frozen conditions, the more pronounced the degree of starch retrogradation. Bread made from frozen dough also exhibits faster starch retrogradation on low temperature (4 °C) storage in comparison to bread made from non-frozen dough, causing an increase in bread firmness **(Ribotta et al., 2001, 2003; Selomulyo and Zhou, 2007)**.

The influence of frozen storage on bread dough and bread affects gradual loss of the dough strength which is related to decrease the retention capacity of CO_2 and longer fermentation time and it also reduce yeast activity. Frozen storage affects the quality of the final product by lowering of loaf volume and deterioration in the texture of the final product **(Selomulyo and Zhou, 2007)**.

Hydrocolloids are used in food products to modify their texture, control water mobility, improve moisture retention and observe overall product quality during storage **(Linden and Lorient, 1999)**. Hydrocolloids have a neutral aroma and taste thereby flavour and all recipe components are able to reflect in the taste of the product **(Kohajdová et al., 2009)**. Hydrocolloids affect the baking performance of dough and also the shelf life of stored bread **(Armero and Collar, 1998; Davidou et al., 1996; Selomulyo and Zhou, 2007)**. Hydrocolloids are able to influence gelatinization, melting, fragmentation and retrogradation processes of starch **(Fanta and Christianson, 1996)**. When used in small quantities (<10 $g.kg^{-1}$) in dough, hydrocolloids are expected to increase water retention and loaf volume, as well as to decrease firmness and starch retrogradation **(Collar et al., 1999; Kohajdová et al., 2009)**. The addition of hydrocolloids into frozen products can provide stability during freeze-thaw cycles and help to minimize the negative effects of freezing and frozen storage on starch-based products **(Ferrero et al., 1993)**. It also decreases water activity due to the competition for water by the hydrocolloids with the bread polymers like protein and starch **(Selomulyo and Zhou, 2007; Schiraldi et al., 1996)**.

Guar gum is commonly used to improve recipe tolerance and mixing. Gums, like guar gum are able to extend the shelf life of products due to moisture retention and thus prevent syneresis in frozen foods and pie fillings **(Maier et al., 1993; Selomulyo and Zhou, 2007)**. Products were achieved with less desirable properties compared with control samples because it lowers the specific porosity and volume of bread and creates a rubbery crust with low crust thickness **(Mandala, 2005)**. **Mettler and Seibel (1993, 1995)** and **Ribotta, et al., (2001)** found that guar gum in frozen dough made a bread with more open crumb structure with higher percentage of gas cells and higher volume unlike products without added guar gum. This result was substantiated by **Ribotta, et al., (2004)**, who

observed that guar gum improved the volume and texture of bread made from frozen dough frozen for 60 days, but the negative effect of frozen dough storage on the dynamic rheological parameters and microstructural damage was not avoided.

Objective of this paper is to present an analysis of the influence of guar gum on textural and sensory properties of rolls made from semi-finished frozen products.

MATERIAL AND METHODOLOGY

Materials

Dough samples were prepared from wheat flour (Mills Kojetín, Kojetín, Czech Republic, ground T 512, moisture 13.5%; ash 0.55%; gluten 34.1% as an amount of wet gluten in DM; falling number 296 s; P 71 mm H_2O; L 105 mm; P/L 0.68, W - 21.8 mJ; I_e - 49.9%;). Alveograph analysis (Chopin - Tripette & Renauld, France) was used for determination of basic characteristics according to the methods **ISO 5530-4 (2002)**. Yeast *Saccharomyces cerevisiae* (Uniferm, Paniferm, Werne, Germany), rapeseed oil (Rosa market, Kroměříž, Czech Republic) and sodium chloride (without iodine, anti-caking agent E 535 sodium ferrocyanide (Solivary Trade, Prešov, Slovakia) were used. Commercial additive for the production of leavened dough intended for freezing Trial RC2 (Irca S.r.l., Gallarate, Italy, wheat flour, malted wheat flour, mono- and diglycerides of fatty acids with tartaric acid acetal, α-amylase, ascorbic acid emulsifier) and additive guar gum (Sigma-Aldrich, Steinheim, Germany; moisture 12%, protein max. 4.5%, fiber max. 2%, fat max. 0.6%, ash max. 1.5%, arsenic max. 3.0 ppm, heavy metals max. 20.0 ppm, lead max. 5.0 ppm) were used.

Methodologies

Preparation semi-finished frozen products

All samples were prepared from 1500 $g.kg^{-1}$ wheat flour (T512), 60 $g.kg^{-1}$ of yeast, 60 $g.kg^{-1}$ of oil, 750 $g.kg^{-1}$ of water, 22.5 $g.kg^{-1}$ of salt, 22.5 $g.kg^{-1}$ of Trial RC2 and individual amount of guar gum (control product-without addition of guar gum (0 $g.kg^{-1}$) and samples with 5, 10 and 15 $g.kg^{-1}$ of guar gum, respectively). The dough samples were mixed in a spiral machine (ALBA, Hořovice, Czech Republic) for 8 min (4 min slow mix, then 4 min fast mix). Oil was added gradually in the prepared dough. The dough samples were left to rise for 10 min in environment of bakery at temperature 30 °C. After that, the dough samples were divided into pieces (60 g) in the divider (Klonek maxRED/36), left to rise for 5 min and shaped on the roll machine (T-682.0, both from ARTHOS, Újezd u Mohelnice, Czech Republic). The pieces of the dough were put into the trug on a baking tray and left to rise for 45 min at a temperature of 40 °C and at a humidity of 80% in the proofer (KA-E1V, Kornfeil, Čejč, Czech Republic). They were baked for 4 min at a temperature 245 °C and 4 min at 250 °C. The semi-finished products were left to cool down in environment of bakery for 20 min, put on a transport tray and gave into the freezer (temperature -22 °C, MTH, Fojtách, Velký Ořechov, Czech Republic). They were sorted and packed in a freezing chamber environment to special PE bags used for the storage of frozen semi-finished frozen products to prevent them from freeze drying of water from the dough and prevent them from damage gluten network. They were labeled and stored in a storage freezer for 48 hours.

Baking of semi-finished frozen products

Semi-finished frozen products were taken out from PE bags, left to stand for 30 min in environment of bakery and baked in a Rotomax rotary gas furnace (Kornfeil, Čejč, Czech Republic) for 1 min at a temperature 280 °C and 3 min at a temperature 260 °C.

Chemical and sensory analysis of rolls after baking and 3 days after baking

Moisture of rolls was determined according to **ISO 712 (1998)**. Each sample was measured five times and all measurements were repeated twice. All samples were evaluated by „15 selected assessors“ (employees of bakery Topek, Topolná, Czech Republic) trained according to **ISO 8586-1 (1993)**. The samples (sample A without guar gum, samples B - D with 5, 10 and 15 $g.kg^{-1}$ guar gum) were coded and served anonymously at room temperature (25 ±1 °C). A five-point hedonic scale was used for the taste, pliancy, texture, porosity, stickiness, gumminess, crispness and quality.

Texture analysis of rolls after baking and 3 days after baking

The texture of the rolls was evaluated by a TA-XT Plus Texture Analyzer (O.K. SERVIS BioPro, Prague, Czech Republic), and conducting a ”measure force in compression” test with an AACC 36 mm cylinder probe with radius (P/36R) using 5 kg load cell. The rolls were divided into five slices of 25 mm thick and subjected to texture analysis after baking and 3 days after baking. The rolls were kept in plastic bags at room temperature for three days. The analyzer was set at a ”return to start’ cycle”, a pre-test speed of 1 $mm.s^{-1}$, a test speed of 1.7 $mm.s^{-1}$, a post-test speed 10.0 $mm.s^{-1}$ and a distance of 10 mm. Firmness F - power which is necessary for achievement of deformation or penetration of the product (initial strength), Firmness A - total force which is necessary for deformation (total strength) were measured in duplicate.

Statistical data analysis

The results of the basic chemical analyses (moisture), the texture and the sensory analyses were statistically evaluated by STATISTICA CZ (Statsoft, Inc., Tulsa, USA), ver. 9.1. The results of sensory analyses were statistically evaluated by means of non-parametric analysis of variance (Kruskal-Wallis test), Friedman test **(Agresti, 1984)**. Differences had to achieve $P < 0.05$ to show significance in all cases.

RESULTS AND DISCUSSION

Chemical and textural analysis of rolls after baking and 3 days after baking

Insignificant differences were found among individual samples in moisture of rolls after baking and 3 days after baking ($P > 0.05$). Additions of guar gum did not influence moisture of rolls, but it decreased within three days as can be seen in Table 1.

Table 1 Moisture of rolls made from semi-finished frozen products after baking and 3 days after baking.

Value (unit)	Day	Samples with guar gum (g.kg^{-1})			
		A	B	C	D
Moisture (%)	0	27.7 ±2.2^{a}	28.1 ±1.2^{a}	28.5 ±2.1^{a}	27.8 ±3.8^{a}
	3	25.4 ±2.1^{a}	26.0 ±1.7^{a}	25.8 ±1.9^{a}	26.2 ±1.8^{a}

* Day after baking

A-samples without guar gum, B-D samples with 5, 10 and 15 g.kg^{-1} of guar gum superscripts expressing diversity (b, c; b, a) or identity (a, a) between samples; samples were compared with each other in rows

Our results are in agreement with authors **Ribotta, et al., (2001, 2003)** and **Selomulyo and Zhou (2007)** who find that bread made from frozen dough exhibits faster starch retrogradation, but they are in disagreement with those of **Maier, et al., (1993)** who stated that guar gum is used to extend the shelf life of products through moisture retention. Figure 1 showed that samples of rolls with additions of guar gum (5-15 g.kg^{-1}) had less firmness (A, F) in comparison to control sample of rolls after baking. 3. days after baking, control sample of rolls and sample with the lowest addition of guar gum had higher firmness (A, F) in comparison to samples of rolls with higher addition of guar gum (10 g.kg^{-1} and 15 g.kg^{-1}) (Figure 2). This agree with the statement that guar gum improved the volume and texture of bread made from frozen dough **(Ribotta et al., 2004)** and with the findings that small quantities (<10 g.kg^{-1} in flour) of hydrocolloids decrease firmness and starch retrogradation **(Collar et al., 1999; Kohajdová et al., 2009)**. It was partially confirmed that hydrocolloids are used in food products to modify texture and improve moisture retention **(Linden and Lorient, 1999)**.

Sensory analysis of rolls made from semi-finished frozen products after baking and 3 days after baking

Insignificant differences were found among samples of rolls (P >0.05) in Table 2.

Sensory assessors evaluated all samples as identical in

Table 2 Results (expressed as median) of the sensory analyses of the tested rolls (samples A-D) after baking and 3 days after baking.

Characteristics	Samples							
	After baking				3 day after baking			
	A	B	C	D	A	B	C	D
taste	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}
pliancy	3^{a}	2^{a}	2^{a}	3^{a}	2^{a}	2^{a}	2^{a}	3^{a}
texture	3^{a}	3^{a}	4^{a}	4^{a}	3^{a}	2^{a}	3^{a}	2^{a}
porosity	2^{a}	2^{a}	2^{a}	2^{a}	3^{a}	3^{a}	2^{a}	3^{a}
stickiness	3^{a}	3^{a}	3^{a}	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}
gumminess	3^{a}	3^{a}	3^{a}	3^{a}	4^{a}	4^{a}	5^{a}	4^{a}
crispness	2^{a}	3^{a}	3^{a}	3^{a}	2^{a}	3^{a}	2^{a}	2^{a}
quality	3^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}

Hedonic scales used: Taste: 1-very good to 5 very bad. **Pliancy:** 1-very high to 5 very low. **Evaluation of texture:** 1-very high to 5 very low. Porosity: 1-very porous to 5 small porosity. Stickiness: 1-very sticky to 5 not sticky. **Gumminess of crumb:** 1-very high to 5 hardly noticeable. **Crispness:** 1- little flexible, rather crisp to 5 very tough. Quality: 1-excellent to 5 very bad, unacceptable.

** Median values having the same superscript letter in each row are not significantly different ($P \geq 0.05$); each group was evaluated separately. Refer to Table 1 for samples A-D

monitored characteristics (taste, pliancy, texture, porosity, stickiness, gumminess, crispness and quality). Our results disagree with those of **Selomulyo and Zhou (2007)** who found that effects of frozen storage on bread include lowering of loaf volume and deterioration in the texture of the final product.

CONCLUSION

Semi-finished frozen products allows easier and more profitable baking, as bread can be made available in few minutes, reducing labour and production costs while facilitating transportation. However, the quality of final bakery products is not good, especially flaking and crackling crust is very often after freezing for several

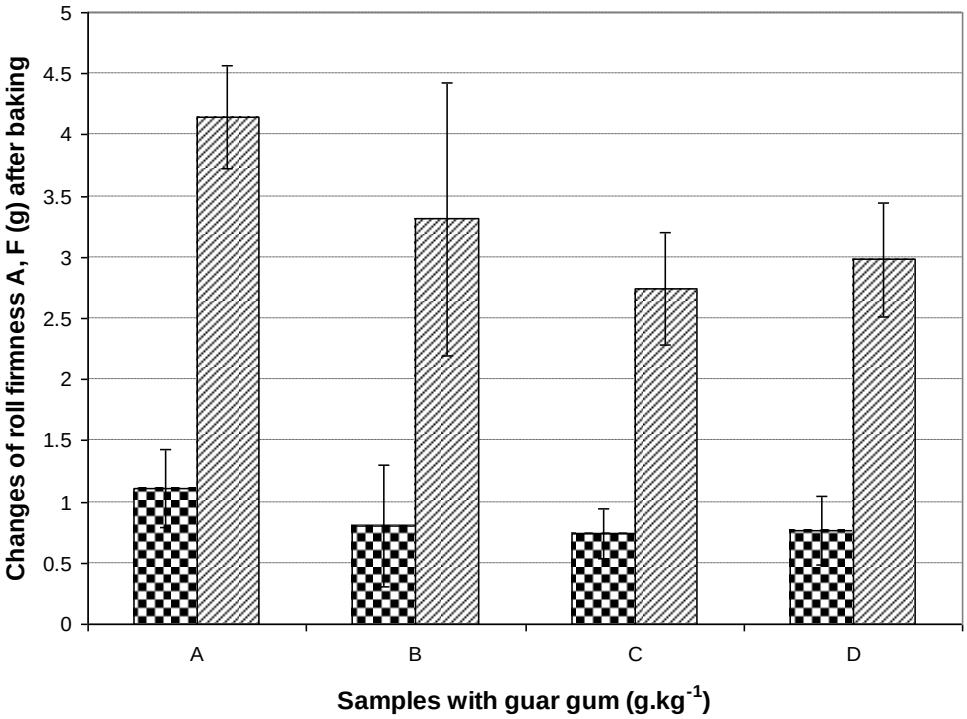

Figure 1 Firmness A, F of rolls after baking

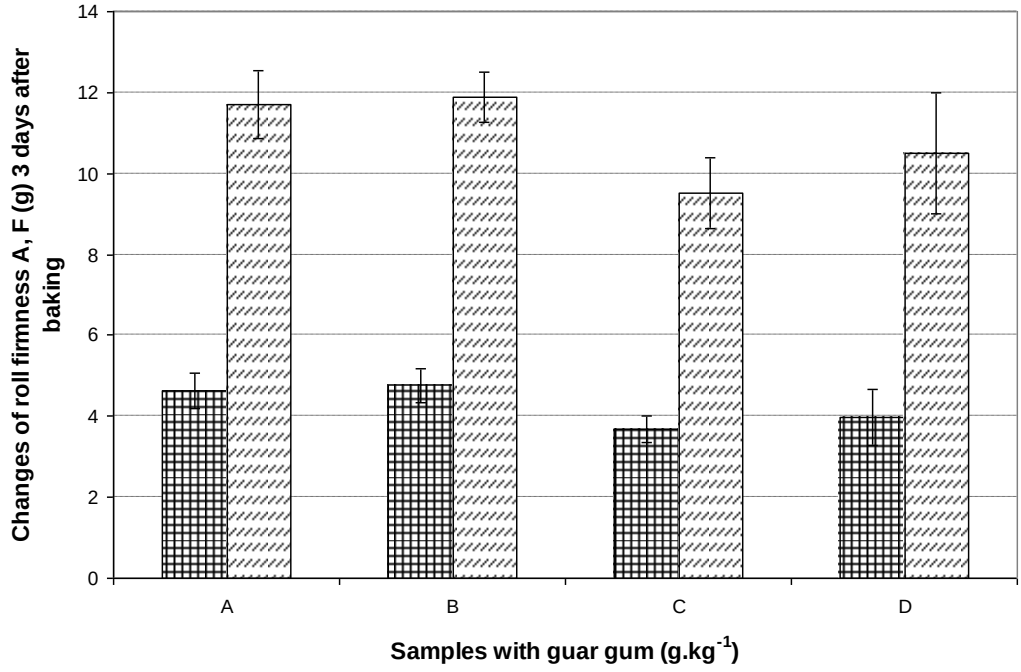

Figure 2 Firmness A, F of rolls 3 days after baking

months. Therefore it is very important to eliminate these problems associated with freezing and frozen storage, thus, the guar gum have been used in individual amount to improve the baking quality and extend the shelf life of bakery products made from semi-finished frozen products. Results of chemical analysis showed no significant differences in moisture of rolls after baking and 3 days after baking. Measurement of texture properties, firmness A and firmness F, showed that samples of rolls with additions of guar gum had lower firmnesses (A, F) in comparison to control samples of rolls after baking. But 3 days after baking, samples of rolls with higher additions (10 and 15 $g.kg^{-1}$) of guar gum had lower firmness (A, F) in comparison to control sample and rolls with the lowest addition of guar gum. Sensory analysis showed that sensory assessors evaluated samples of rolls as the same in all monitored characteristics. But visual assessment of rolls showed that rolls with lower additions of guar gum (5 and 10 $g.kg^{-1}$) had better surface of crust and porosity of crumb and they have a longer shelf life. Solution of problem as a flaking and crackling crust or irregular porosity of crumb may be the guar gum. Moreover, lower additions of guar gum did not increase the final price of the bakery product so much.

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DETECTION OF LUPINE (*LUPINUS* SPP. L.) AS A FOOD ALLERGEN USING THREE METHODS: END-POINT PCR, REAL-TIME PCR AND ELISA

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ABSTRACT

The aim of this work was to compare three methods for the detection and quantification of lupine as an allergen in food. The methods that were used in this work were the direct method: ELISA and the indirect methods: end-point PCR and real-time PCR. We examined the detection limit (the sensitivity with which we can detect the presence of the allergen in a sample) and the reliability for performing an analysis. We used 17 samples of plant species from a processing plant for dehydrated soups production and lupine samples from lupine processing companies. Its practical use is wide and it is used mainly in the bakery industry, in the manufacture of confectionery, pasta, sauces, as a substitute for soy and also in the production of gluten-free food, because it does not contain gluten. Lupine, however, is also included in the list of 14 allergenic substances, which in accordance with the EU legislation must be listed on food labels. The high risk group, which suffers from primary sensitization or cross-reaction with peanuts, are allergic patients. In the EU, people who are allergic to peanuts range from 0.7 to 1.5%. In experiment 1, we detected the presence of lupine using primers for the detection of α- and δ-conglutine in the samples, using the end-point PCR method and the detection limit of this reaction was at the level of 100 ppm. For the vizualization of the DNA fragments, we used a 2% agarose gel and UV visualizer. In experiment 2 we detected lupine using the TaqMan real-time PCR reaction and primers for the detection of α and δ-conglutine at the level of 10 ppm of lupine in sample. The CP values of lupine using primers for the detection of α-conglutine was 24.85 ±0.12 and the reliability equation was R^2 = 0.9767. The CP lupine values using primers for the detection of δ-conglutine was 22.52 ±0.17 and the reliability equation was R^2 = 0.9925. In experiment 3, we used a sandwich ELISA method for the detection of lupine and the detection limit was within the range of 2-30 ppm and the reliability of the method according to the reliability equation was R^2 = 0.9975. The high sensitivity and equation of reliability justify the use of these methods for the detection and quantification of lupine in practice. The most sensitive indirect method for the detection of lupine in our study was the method of real-time TaqMan PCR with a detection limit 10 000-10 ppm of lupine. The most sensitive direct methot was ELISA with detection limit 2-30 ppm of lupine.

Keywords: lupine; food allergy; end-point PCR; real-time PCR; ELISA

INTRODUCTION

Lupines are a relatively modern leguminous plants that can grow in a variety of soil conditions and can be particularly useful with regard to the fixation of nitrogen in the soil, respectively, in the decontamination of heavy metals from the soil **(Herridge and Doyle, 1988; Petterson and Harris, 1995)**. Lupine seeds are rich in protein, which can be used for food production, or the production of animal feed **(Edward and van Barneveld, 1998)**. There are four different types of lupines, which are of agricultural importance: White lupine (*Lupinus albus*), Narrow-leaf lupine (*Lupinus angustifolius*), Yellow lupine (*Lupinus luteus*), Andean lupine (*Lupinus mutabilis*). Lupine is considered as an inexpensive source of protein and can be grown in colder climates, which makes it an attractive crop in comparison to other protein-rich plants, such as soybean **(Holden et al., 2005)**. Lupine seeds are a rich source of non-carbohydrate polysaccharides (30-40%), oil (5-15%) with a high content of polyunsaturated fatty acids and proteins at approximately the same level as soybeans (30-40%) depending on the genotype and location **(Martínez -Villaluenga et al., 2006; Erbas et al., 2005)**. As a substitute for soybean, its popularity has grown in popularity mainly in Europe as lupine is not a genetically modified plant **(Peeters et al., 2009)**.Whereas lupine does not contain gluten and is also used in gluten-free diet and gluten-free products, for example in the productions bread or other bakery products **(Ziobro et al., 2013)**. Despite these positive aspects lupine was added to the list of allergens. Major allergens of Lupinus are storage proteins and conglutines. The two main factions are the α-and β-conglutine, the minor include γ- and δ-conglutine **(Melo et al., 1994)**. In recent years, increasingly lupine has been used mainly in the manufacture of bakery products, but also in the production of health-promoting foods in Europe. In parallel with this the number of cases of allergy to lupine also increases. Although the recorded primary sensitization to lupine occurs more often as cross-reaction in patients with pre-existing allergy to peanuts. Furthermore, a number of studies indicate that the risk of cross-reactions, clinically manifested in patients allergic to peanuts after exposure

lupine is relatively high **(Dooper et al., 2009)**. In recent years, primary and secondary sensitization of lupine was studied mostly through molecular approaches. These studies were conducted in patients allergic to peanut and lupine, and point out that a number of allergic polypeptides belongs to the following lupine protein families (α, β, γ-conglutines), which are responsible for allergic responses *in vitro* and *in vivo* **(Ballabio et al., 2013)**.

MATERIAL AND METHODOLOGY

Isolation DNA from samples

DNA was isolated using commercial GeneMATRIX Food-Extrakt DNA Purification Kit (Molecular Genetic Resources, USA). DNA obtained from isolations were used in experiment 1 and 2. In the analysis we used 17 samples of plant species from a processing plant for dehydrated soups production and lupine samples from lupine processing companies (Table 1).

Organization of experiment 1

In experiment 1 classical end-point PCR was used for detection lupine in samples. Primers for detection α- and δ-conglutine in lupine were used according to **Gomez-Galan et al., (2010)**. Length of base pair for α-conglutine was 153 bp and 150 bp for the δ-conglutine that serve to detect specific DNA species of the genus Lupinus (*Lupinus albus* and *Lupinus angustifolius*) (Table 2). Primers were manufactured by General Biotech (Czech Republic).

In the optimization of experiment 1 the mixture (mastermix) polymerase GoTaq® Hot Start Polymerase (Promega, Medison, USA) was used as follows in the production of the polymerase reaction. Another polymerase that was used in the preparation of the reaction mixture (mastermix) polymerase was Isis™ DNA polymerase (MP Biomedicals, Europe). To dye the agarose gel electrophoresis, we used the following dyes: Ethidium Bromide (EtBr) and GelRed™.

The mixture of classical (end point) PCR reaction using polymerase GoTaq ® Hot Start and primers for detection of α-and δ-conglutine contain the following ingredients: 2 μL of template DNA, 0.45 μL of dNTPs, 1.20 μL of $MgCl_2$, 1.50 μL of reverse and forward primers, 0.1 μL GoTaq® Hot Start polymerase (Promega, Medison, USA), 6 μL of buffer GoTaq Flexi Buffer (Promega, Medison, USA), made up to final volume of 30 μL by means of 17. 25 μL of PCR grade water.

The composition of the reaction mixture using Isis™ DNA polymerase was as follows: 2 μL of template DNA, 0.45 μL of dNTPs, 3 μL Isis Buffer 1x (20 mM Tris-HCl pH 9.0, 25 mM KCl, 10 mM (NH_4) 2 SO_4, 1.5 mM $MgSO_4$, 0.1% Tween 20, and stabilizers), 1.50 μL of reverse and forward primers, 0.1 μL of Isis™ DNA polymerase (MP Biomedicals Europe) made up to the final volume of 30 μL with 21. 35 μL of PCR grade water.

The amplification was conducted with a thermal cycler (PCT-150™ MiniCycler, MJ Research, Watertown, USA). Course of the PCR reaction was as follows: The PCR cycle begins by pre-incubation at 95 °C for 5 min, followed by 40 cycles of its repetition with temperature profile: denaturation at 95 °C for 15 seconds, annealing, and polymerization at 62 °C for 30 seconds. The final step in the PCR reaction was the cooling to a temperature of 25 °C for 1 second.

Table 1 Samples used for detection lupine

Serial number	Sample	Country of origin
1	Lupine (A)	Austria
2	Lupine (NL)	Netherland
3	Lupine (SK)	Slovakia
4	Whole Soy chunks	Austria
5	Yellow pea flour milled	Germany
6	Chickpea flour milled	Germany
7	Green pea flour milled	Poland
8	Bean flour brown milled	Spain
9	Defatted soybean powder	Austria
10	White bean flour milled parboiled	Poland
11	The whole pre-cooked brown lentils	Germany
12	Dried bean pods	Germany
13	Whole dried green peas	Poland
14	Freeze-dried green peas	Germany
15	Yellow peas cooked dehydrated	Spain
16	The whole pre-cooked brown beans	Poland
17	Soy lecithin powder	Austria

Table 2 Primers used for lupine detection-experiment 1 and 2

Primer	Sequence	ID NCBI
CongA-F (exp. 1)	5'- AGAAACGACTTGAGGAGACA- 3'	NCBI U74384
CongA-R (exp. 1)	5'- AGCAGCAAGTCCAAGCCA- 3'	
CongD-F (exp. 1)	5'- TGTGAGCAACTGAATGAGCTTAA-3'	NCBI X53523
CongD-R (exp. 1)	5'-AAACCCACAAGTCCTAGGCAAA3'	
Acon.-F (exp. 2)	5'- AGAAACGGACTTGAGGAGACA -3'	NCBI U743844
Acon.-R (exp. 2)	5'- AGCAGCAAGTCCAAGCCA -3'	
Acon. probe (exp.2)	FAM-ACATCTCCTGATGCATACAACCCTCAAGCTGGTAGG-TAMRA	
Dcon.-F (exp. 2)	5'- TGTGGAGCAACTGAATGAGCTTAA -3'	NCBI X53523
Dcon.-R (exp. 2)	5'- AAACCCACAAGTCCTAGGCAA -3'	
Dcon. Probe (exp. 2)	FAM-AGCCAGAGATGCCAGTGCCGTGCATT-TAMRA	

Agarose gel electrophoresis and - to visualize the DNA fragments of the PCR reaction - we used a 2% agarose gel. When optimizing, the methods we used were ethidium bromide dye and dye GelRed™. Electrophoresis was performed in electrophoresis trough the direct-current voltage of 75 V, for 60 minutes.

DNA samples were gel volume of 15 µL, 2 µL EtBr, or GelRed™ 1 µL weight DNA marker (ruler). In visual processing images electropherograms (Figures of agarose gel after visualization by UV emitter) were processed using the freeware software Irfanview version 4.28.

In identifying the the limit of detection, we used a decimal dilutions of samples. Decimal dilutions are made in the following way: we took 2 µL of DNA sample and have it mixed with µL ml of water bidestilovanej PCR. In this way we prepared by diluting the concentration of 10^{-1} to 10^{-6}. These samples were used in determining the limit of detection in experiment 1 and 2.

Organization of experiment 2

In experiment 2, we have optimized the Taq Man® real-time PCR reaction. Primers for α- and δ-conglutine and Taq Man® probe were designed according to **Gomez- Galan et al., (2010)** (Table 2).

Mastermix for detection lupine - experiment 2: total reaction volume was 20 µL, PCR grade water -CYBR 10 µL H_2O, LightCycler® Taq Man® Master (Roche, Germany) 4 µL, Taq mix for uni (α and δ-conglutine) (Mastermix were used separately for detection of α and δ-conglutine) 4 µL (forward and reverse primer, 15 pmol.μL^{-1} and probe 3 pmol.μL^{-1}), 2 µL of template DNA.

The analysis of samples using the method of Taq Man® real-time PCR, we used the LightCycler capillary Cycler® 1.5 (Roche, Germany) and results were analyzed using the LightCycler software version 4.5 (Roche, Germany). Protocol Taq® Man real-time PCR reaction was as follows: predenaturation 2 minutes at 50 °C, denaturation for 10 min at 95 °C, annealing and polymerization: 95 °C for 15 seconds, 63 °C for 1 minute, which were repeated 50 times, the last step was cooling to 37 °C for 1 second.

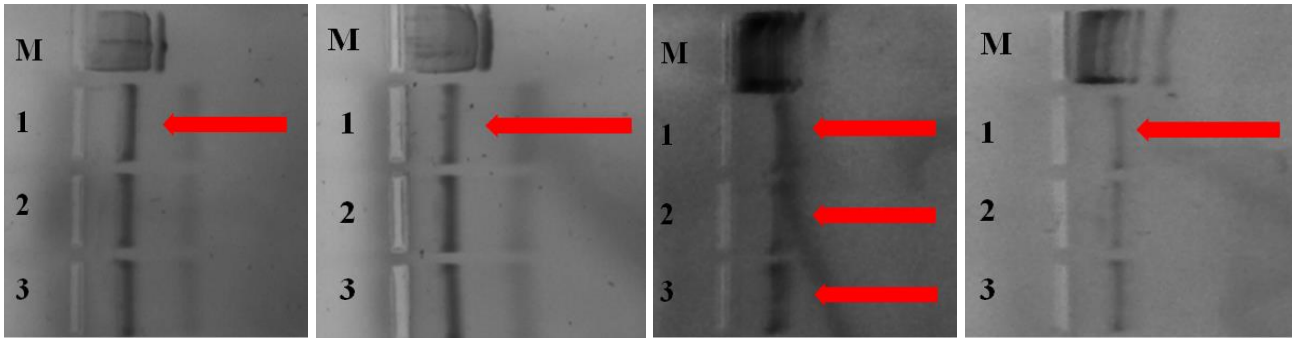
Organization of experiment 3

Experiment 3 focused on quantitative detection of lupine in samples using immuno-enzymatic assay ELISA. For the detection, we used 96-well ELISA (Immunolab GmbH, Germany), catalog number LUP-E01.

The limit of detection (i.e. sensitivity of the method) is 0.2 ppm for the standard curve. Limit of quantification of the assay is 2 ppm, and range generally from 2 ppm to 30 ppm. The optical density was measured at a wavelength of 450 nm at Neogen® Stat Fax® 303 ELISA reader. Calculation of concentration of lupine in the sample was performed by a calibration curve of well-defined standards, which have been a ELISA kit (n=2).

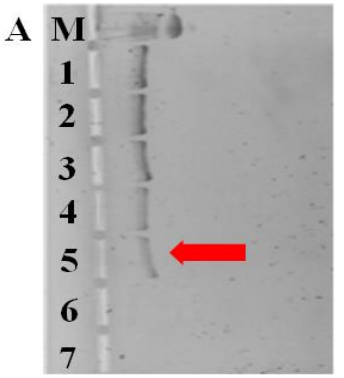
RESULTS AND DUSCUSSION

In experiment 1, we compared the use of polymerase Isis and GoTaq Hot Start GelRed combination of dyes and ethidium bromide. The results are documented in Figure 1. The combination of polymerase and dye Isis GelRed showed better quality of DNA fragments. These same results were obtained with the primers for the α-conglutine (Figure 1A), as well as the use of primers for the δ-conglutine (Figure 1B). Whereas the results using polymerase GoTag Hot Start and ethidium bromide did not show good results (Figure 1 C, D) such the results of the first combination, we did not use it further. The limit of detection end-point PCR using Isis polymerase and GelRed dye to detect lupine using primers to detect α- and δ-conglutine is documented in Figure 2, the detection limit using alpha conglutine stood at 0.01% (Figure 2 A). The same limit of detection was also detected using primers for the detection of δ-conglutine (Figure 2 B). The limit of detection end-point PCR reactions was at level 100 ppm lupine in the sample. The results of tested samples by the end-point PCR reaction (n=3) using the primers for the detection α- and δ-conglutine are shown in Figure 3. Red arrows indicate the DNA fragments of α or δ-conglutine of lupine. Yellow arrows indicate the DNA fragments that have a larger fragment of DNA fragments of DNA than of lupine. The blue arrow indicates the presence of dimer DNA. Green arrows indicate DNA fragments that have a lower DNA fragment as a DNA fragment of lupine.



1 A **1 B** **1 C** **1 D**

Figure 1 A-D Comparison of combination of polymerases Isis and GoTaq , dye's GelRed and Ethidium Bromide (EtBr). Electroforeogram of PCR: samples 1-3 using Isis and GelRed and primers for α-conglutine (A) δ-conglutine (B). Electroforeogram of PCR: samples 1-3 using GoTaq® HotStar and EtBr and primers for α-conglutine (C) δ-conglutine (D).
Legend: M lane - weight marker, lane 1 - sample lupine from Austria, lane 2 - sample lupine from the Netherlands, lane 3 - sample lupine from Slovakia, red arrow shows the DNA fragment



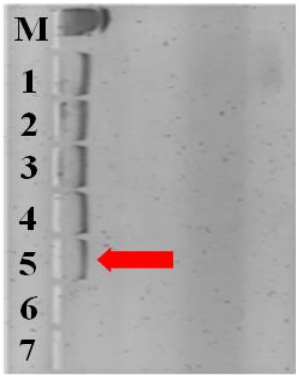


Figure 2 A, B

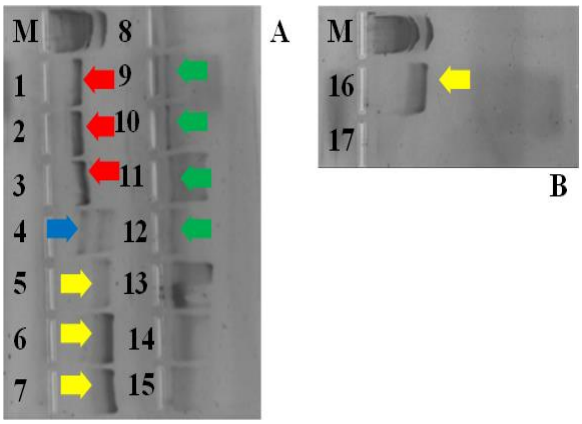


Figure 3 A, B

Figure 2 A, B
Electroforeogram of PCR: sample of lupine using Isis ™ polymerase and dye GelRed ™ and primers for α-conglutine (A) and δ-conglutine (B) decimal dilutions. Legend: lane M-weight marker, lane 1 - 100% of the DNA, lane 2 - 10% of the DNA, lane 3 - 1% of the DNA, lane 4 - 0.1% of the DNA, lane 5 - 0.01% of the DNA, lane 6 - 0.001% DNA, lane 7 to 0.000% 1 DNA lupine, red arrow indicates a DNA fragment of DNA concentration of 0.01% lupine (100 ppm).

Figure 3 A, B
Electroforeogram PCR samples 1-15 (A), 16-17 (B) using the polymerase Isis™ and dye GelRed™ and primers for α-conglutine. Legend: M-lane weight marker, lane 1 - lupine (Austria), lane 2 - lupine (Netherlands), lane 3 - lupine (Slovakia), lane 4 - soya chunks, lane 5 - yellow pea flour, lane 6 - chickpea flour ground, lane 7 - green pea flour, lane 8 - brown bean flour, lane 9 - defatted soybean powder, lane 10 - pre-cooked white bean flour, lane 11 - pre-cooked brown lentils, lane 12 - string beans dried, lane 13 - green peas dried, lane 14 - green peas freeze-dried, lane 15 - yellow peas cooked dehydrated, lane 16 - whole bean brown parboiled, lane 17 - soy lecithin powder, red arrows indicate the presence of DNA fragments of α-conglutine, yellow arrows show the DNA fragments are larger than fragments of α-conglutine green arrows indicate the DNA fragments that are smaller than α-conglutine, black arrow shows the sequence of dimer DNA of soy.

As shown in Figures 3 A, B using the polymerase Isis™ and dye GelRed ™ and primers for α-conglutine are securely detect the presence of the samples of lupines. In lane 4, we recorded dimer. It was a sample of soy. In assessing the need to monitor very closely the position and size of the fragments. In lanes 5, 6, 7 and 16, we have seen more than a fragment of fragments of α-conglutine, the size if which is 153 bp. In lanes 9, 10, 11 and 12 are recorded in reverse fragments, which are less than a length of said fragment of the α-conglutine. Using the same combination using primers for the δ-conglutine are shown in Figure 4 A, B.

Using primers for alpha and delta conglutine brought the same results in detection of lupine. The result of the experiment 1 was to reach the detection limit at 100 ppm level of lupine.

In experiment 2 we use Taq Man real-time PCR method for detection of lupine. We used primers for α- and

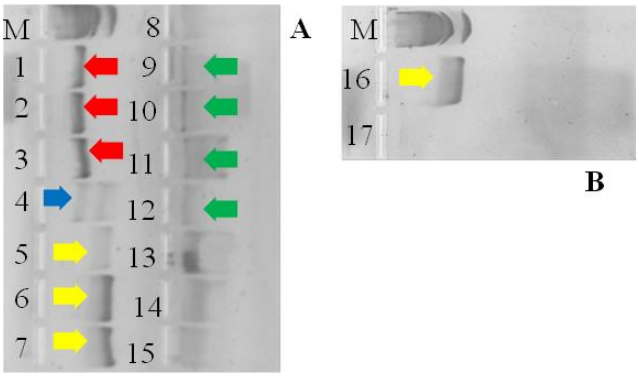


Figure 6 A, B
Electroforeogram PCR samples 1-15 (A), 16-17 (B) using the polymerase Isis™ and dye GelRed™ and primers for δ-conglutine. Legend: M-lane weight marker, lane 1 - lupine (Austria), lane 2 - lupine (Netherlands), lane 3 - lupine (Slovakia), lane 4 - soya chunks, lane 5 - yellow pea flour, lane 6 - chickpea flour ground, lane 7 - green pea flour, lane 8 - brown bean flour, lane 9 - defatted soybean powder, lane 10 - pre-cooked white bean flour, lane 11 - pre-cooked brown lentils, lane 12 - string beans dried, lane 13 - green peas dried, lane 14 - green peas freeze-dried, lane 15 - yellow peas cooked dehydrated, lane 16 - whole bean brown parboiled, lane 17 - soy lecithin powder, red arrows indicate the presence of DNA fragments of δ-conglutine, yellow arrows show the DNA fragments are larger than fragments of α-conglutine green arrows indicate the DNA fragments that are smaller than δ-conglutine, black arrow shows the sequence of dimer DNA of soy.

δ-conglutine and Taq Man probe to detect lupine. As in experiment 1, we used a decimal dilutions to determine the detection limit for the detection of lupine in samples. The result of detection limit using α-conglutine and Taq Man probe show Figure 5. Figure 6 show reliability of used method (primers for α-conglutine and Tag Man probe).

As Figure 5 demonstrates, the reaction showed a very high reliability, the reliability of which is documented by the equation R^2 = 0.9767. Thus, a sample of lupine concentration of 0.001% (10 ppm) can be detected with reliability of 97.67% using primers for the detection of α-conglutine. For the detection of lupine by Taq Man real-time method, we used the primers for the detection of delta conglutine. Figure 6 shows the progress of the reaction, where we have used a decimal dilutions of the sample for the detection of lupine.

As seen in Figure 6 as well as the use of primers for the detection of delta conglutine, we obtained the same result; that is, 0.0001% (10 ppm). Reliability of the method is presented in Figure 6.

When comparing the use of primers for the detection of α and δ-conglutine, the delta conglutine we achieved better results (better than CP values) and also better values of reliability R^2, than when we used primers for detection α-conglutine.

Table 3 is a table of values of CP (i.e. crossing-point values that indicate where within the cycle of the PCR reaction nonspecific background was exceeded) that we obtained a PCR reaction in all tested samples.

The result of experiment 2 was to achieve a detection limit of 10 ppm using primers alpha and delta conglutine. The CP values of lupine using primers for the detection of α-conglutine was 24.85 ±0.12 and the reliability equation was R^2 = 0.9767. The CP lupine values using primers for the detection of δ-conglutine was 22.52 ±0.17 and the reliability equation was R^2 = 0.9925.

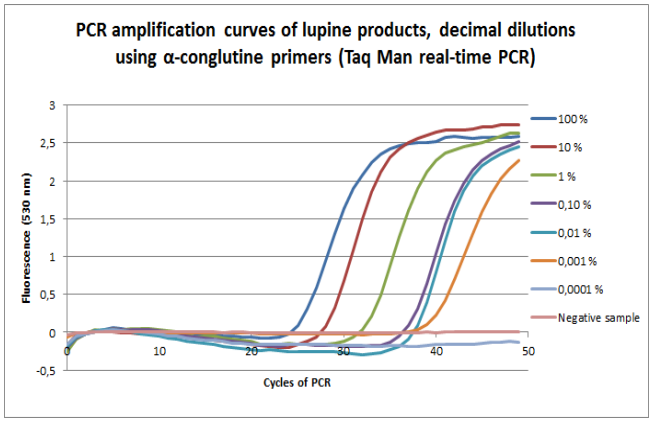


Figure 4 Progress of the increase in fluorescence of PCR products samples of lupine using α-conglutine and Taq Man probe

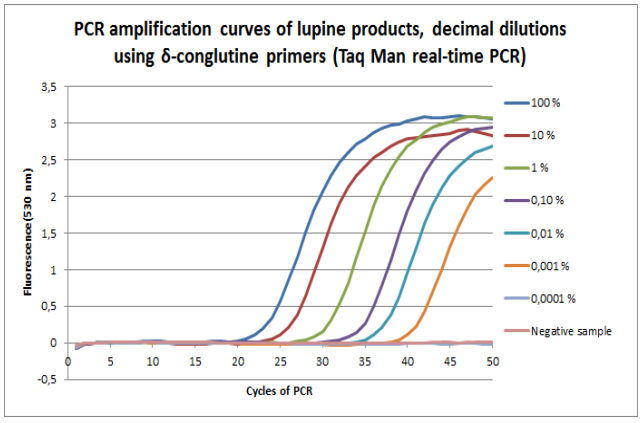


Figure 5 Progress of the increase in fluorescence of PCR products samples of lupine using δ-conglutine and Taq Man probe

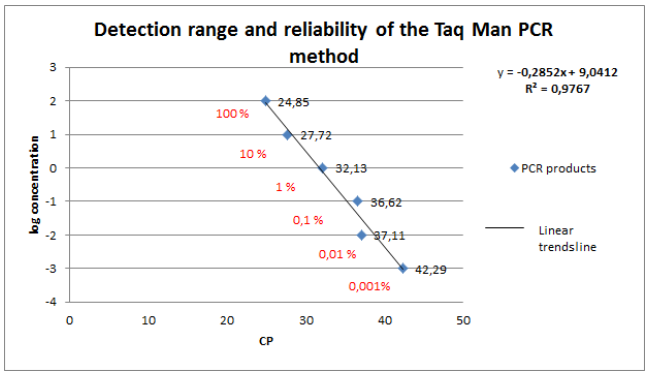


Figure 7

Detection range and reliability Taq Man method using α-conglutine for the detection of of lupine

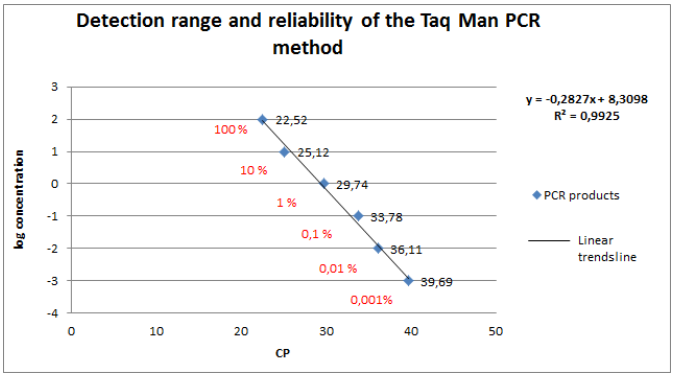


Figure 8

Detection range and reliability Taq Man method using α-conglutine for the detection of of lupine

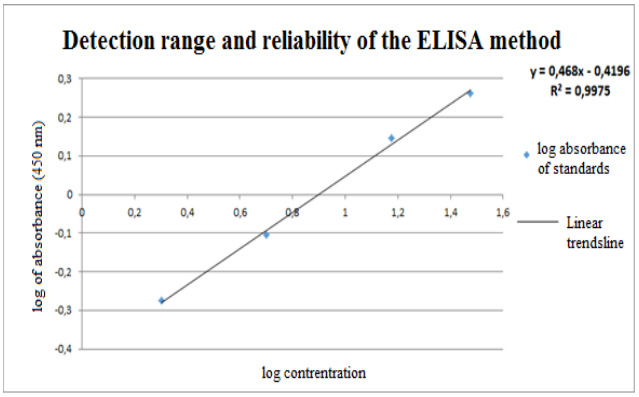


Figure 9 Reliability and detection limit of the ELISA method (n=2)

In experiment 3 we used commercial ELISA kit for the detection of lupine in food. We measured the absorbance of the sample and then interpolated the results after which we got lupine concentration in ppm ($mg.kg^{-1}$). The number of measurements was (n = 2). Measurement results shown in Table 4. Samples 1-3 had more than 30 ppm of lupine in the sample, because these samples were samples of lupine. Sample 4 had more than 30 ppm although they were soybean, and thus presents a potential risk. We found traces of lupine in sample 7 pea - green (2.6 ppm), 12 - dried bean pods (2.3 ppm). Sample 12 - white bean was 22.55 ppm lupine in the sample, which also carries a potential risk.

Red numbers in Table 4 indicate values under detection limit. The result of this ELISA test was to confirm the presence of the traces of lupine, which we already observed in experiments 1 and 2.

The result is 3 experiment detected the presence of lupine traces in 5 samples, which could potentially pose a risk to sensitive consumers. High detection sensitivity and detection limit of 2-30 ppm predisposes ELISA as a highly effective tool in the detection of lupine in samples. High level of reliability (R^2 = 0.9975) makes ELISA highly reproducible and an accurate method for the determining of the presence of lupine as a food allergen.

Tools for the detection and quantification of lupine have been developed in various forms such as food matrix, using wheat flour **(Scarafoni et al, 2009, Demmel, et al., 2012)**, detection of traces of lupine in food **(Gomez-Galan et al., 2010)**, the methods for simultaneous detection of lupine and soybean using the mitochondrial DNA in processed foods **(Gomez-Galan et al., 2011)**. Methods have been developed for the detection of lupine flour as **(Gomez-Galan et al., 2010)**, but also as processed foods containing lupine examples: "Lupine tofu" cookies containing lupine bread containing lupine **(Gomez-Galan et al., 2010)** ice cream containing lupine **(Demmel, 2013)** or pizza **(Demmel et al., 2011)**.

Suitability using primers for the detection of α-and δ- conglutine was confirmed in the work of **Gomez-Galan et al., (2010)**. Besides the above mentioned primers **Demmel, et al. (2011)** used for the construction of the target DNA molecules with overlapping ends of the amplicons primers Limo-162 and ov'-Limo-62 to identify *Lupinus angustifolius* and primer length of the product was 101 bp.

Compared with the results of the authors **Scarafoni et al., (2009)**, who used to detect primer pair for the detection of γ-conglutine in the sample, we have achieved better results. We used a modified end-point PCR method and sensitivity reached, as mentioned above, a 100 ppm detection of lupine in the sample using a primer pair for both δ-conglutine, as well as α-conglutine. When using γ-conglutine, **Scarafoni et al. (2009)** were able to detect more types of lupines in samples - *Lupinus angustifolius*, *Lupinus albus* and *Lupinus luteus* (our chosen method is capable of detecting the presence of *Lupinus albus* and *Lupinus angustifolius*). With their choice using a primer

Table 3 CP values of samples using primers for α and δ-conglutine

Nr.	Sample	CP value α-conglutine	CP value δ-conglutine
1	Lupine (A)	**24.92 ±0.38**	**22,62 ±0.12**
2	Lupine (NL)	**25.64 ±0.57**	**23.15 ±0.49**
3	Lupine (SK)	**24.85 ±0.12**	**22.52 ±0.17**
4	Whole Soy chunks	not detected	39.60 ±0.59
5	Yellow pea flour milled	not detected	not detected
6	Chickpea flour milled	35. 94 ±0.45	35.88 ±0.47
7	Green pea flour milled	35.6 ±0.35	36.02 ±0.38
8	Bean flour brown milled	not detected	not detected
9	Defatted soybean powder	not detected	not detected
10	White bean flour milled	34.58 ±0.55	35.39 ±0.25
11	The whole pre-cooked lentils	not detected	not detected
12	Dried bean pods	not detected	not detected
13	Whole dried green peas	37.45 ±0.35	36.85 ±0.60
14	Freeze-dried green peas	not detected	not detected
15	Yellow peas cooked	not detected	not detected
16	The whole pre-cooked beans	not detected	>45
17	Soy lecithin powder	38.12 ±0.47	37.57 ±0.64

Table 4 Absorbance and concentration of lupine (ppm) in samples

Nr.	Sample	**Absorbance at 450 nm** (n=2)	**ppm (mg.kg^{-1})** (n=2)
1	Lupine (A)	>3	>30
2	Lupine (NL)	>3	>30
3	Lupine (SK)	>3	>30
4	Whole Soy chunks	>3	>30
5	Yellow pea flour milled	0.551	2.213
6	Chickpea flour milled	0.352	0.852
7	Green pea flour milled	0.5995	**2.650**
8	Bean flour brown milled	0.4055	1.186
9	Defatted soybean powder	0.482	1.669
10	White bean flour milled	1.1635	**22.554**
11	Pre-cooked lentils	0.5355	2.103
12	Dried bean pods	0.5585	**2.344**
13	Whole dried green peas	0.493	1.751
14	Freeze-dried green peas	0.55	2.214
15	Yellow peas cooked	0.469	1.571
16	Pre-cooked beans	0.489	1.760
17	Soy lecithin powder	0.877	**6.042**

pair for the detection of γ-conglutine it was possible to detect not only the sample to a concentration of 0.1%, equivalent to 1000 ppm, and so that we used the method of one, respectively two, logarithmic sensitive board.

At present, despite its relatively good detection capability the end point PCR reaction is not used very often. It is mainly used for the validation of specificity primer real-time PCR.

The disadvantage of this method is that in a single reaction we can look at more allergens at the same time and its elaborateness: the method requires a very high quality of DNA extraction, making the reaction mixture, producing an agarose gel and applying the PCR products

and the visualization by UV visualizer respectively, a computer program for figure processing. Therefore at present among the PCR reactions are the most common real-time PCR reaction. To speed up the analysis and of course, the number of samples analysed, we used duplexes **(Gomez-Galan et al., 2011)**, tetraplex **(Köppel et al., 2010)**, or other multiple access **(Waiblinger et al., 2014),** making it possible to analyze large amounts of allergens in real time. The most commonly used by real-time PCR reactions are Taq Man **(Gomez-Galan et al., 2011)**, SYBR Green I real-time PCR **(Scarafoni et al., 2009)**, methods based on FRET (fluorescence resonance energy transfer) **(Mair et al., 2014)** and others. Their advantage is to reduce the analysis time by about half compared with end-point PCR.

In the study by **Waiblinger et al. (2014)** interlaboratory tests were conducted (interlaboratory tests) between 17 participating laboratories, where using a single multiplex determined the presence of the following allergenic ingredients: sesame seeds, almonds, Brazil nuts and lupine. These tests were carried out and compared with each other. Using real-time PCR methods proved similarly as we detected in the sample, lupine, six of the seven PCR System at 10 ppm. Like us, most laboratories have reached the level of reliability equation R^2 = 0.99. Based on their work and results, we would like to note that our results, both the qualitative detection of lupine using real-time PCR results were closer to certified laboratories abroad. **Ecker et al. (2013)** used two ELISA tests in their work to detect and quantify lupine competitively. These products have two types of antibodies for the detection of lupine IgG antibody produced in rabbit and the body IgY antibodies produced in the body of a chicken. 32 plant samples were tested and found that both types of ELISA assays showed cross-reaction with pecans. Although the method used by us was not competitive with the ELISA method, but the sandwich ELISA method and kit according to the manufacturer set ELISA detected cross-reaction with chickpeas, lentils, soy flour, and a cooked and handled heat-treated flour showed greater cross-reaction by the manufacturer. Their ELISAs were able to detect the presence of *Lupinus albus*, *Lupinus luteus* and *Lupinus angustilofius* in the samples. We were able to detect the presence of *Lupinus albus* and *Lupinus angustifolius* as we had only these two types of lupines available. Through IgG ELISA they were able to detect 50 ppm of lupine in bread, vegetarian burgers and biscuits. The detection limit for IgY test was 50 ppm for vegetarian meatballs and bread, and 100 ppm for crackers. When we used the sandwich ELISA method we detected the presence of lupine ranging from 2.3 to 22.5 ppm.

CONCLUSION

Using end-point, we were able to detect the presence lupine at 100 ppm. Using Taq Man real-time, we were able to detect the presence of 10 ppm of lupine in the sample. Using a commercial ELISA kit, we were able to detect the presence of lupin in the range from 2 to 30 ppm in the sample. The most sensitive indirect method for the detection of lupine in our study was the method of real-time TaqMan PCR with a detection limit 10 000-10 ppm of lupine. The most sensitive direct method was ELISA with detection limit 2-30 ppm of lupine.

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DETERMINATION OF THE SPECIES SPECIFICITY OF THE PRIMERS FOR THE DETECTION OF CHICKEN AND TURKEY MEAT BY REALTIME PCR METHOD

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ABSTRACT

The aim of this work was to use TaqMan Real-Time PCR for quantitative authentication of chicken and turkey meat. To meet this purpose, a specific pair of primers and TaqMan probe was used. The test was aimed at identifying the reaction cycle of turkey and chicken meat using by two sets of primers. With first set of primer designed for chicken we obtained the following results: Cp = 16.18 for 100% chicken DNA Cp = 29, 18 100% turkey DNA It was also amplified DNA of pig that exceeded the detection threshold fluorescence intensities in the 31.07 cycle (Cp = 31.07). Using primers designed for turkey we obtained the following results Cp = 31.16 for 100% CHDNA, Cp =16.18 100% TDNA. It was also amplified the 100% DNA of rabbit in 31.63 cycle (Cp = 31.63) and deer in cycle 32 (Cp = 32). The DNA of all other animal species was amplificated after more than 35 cycles (Cp >35). It follows that the second detection primer pair is specific enough to unrelated species of animals by 30 cycles of the reaction. Species authentication based on DNA analysis from this perspective overcomes all the shortcomings of proteins. At present, DNA analysis use different types of PCR. Is the most progressive Real-time PCR, which is suitable for the specific use of detection (primers and TaqMan probe). The TaqMan Real-time PCR is within the sensitivity and specificity, clearly one of the best methods for identifying the species of chicken and turkey meat. The specificity of this method, however, depends primarily on the specificity of the primers and TaqMan probe. The 30 cycle reaction was chosen by us as the threshold for specificity using primers for authentication chicken and turkey meat.

Keywords: primer; chicken meat; turkey meat; PCR; TaqMan

INTRODUCTION

For food producers is particularly attractive for economic reasons to replace expensive components with cheaper. Therefore, they falsify especially expensive products or products that are produced in large volumes due to higher profits **(Lees, 2003; Peris and Escuder-Gilabert, 2009)**.

Popelka et al. (2002) the adulteration of food is associated with the deteriorating quality of food.

In Slovakia is the necessary verification of genuineness of certain products as a necessary part of a comprehensive examination of quality of goods with regard to consumer protection, together with the fight against counterfeiting of products in the package itself or directly for sales **(Takáčová and Bugarský, 2010)**.

At present is more and more used PCR method allowing the direct quantification of PCR products during the amplification reaction – Real-Time PCR. Quantification of nucleic acid molecules is important in determining the amount of target DNA in the samples analysed **(Šmarda et al., 2008; Špánová et al., 2005)**.

Haider et al. (2012), these techniques largely overcome the shortcomings of other methods, therefore, are promising and reliable tool for species identification of meat.

As an authentication marker, in this case, is use of nuclear genes or mitochondrial DNA. The DNA molecules are present in each cell, and in addition, in comparison with protein markers are more thermo stable **(Rojas et al. 2012)**.

Králová et al. (2007) indicate that a very important requirement for a successful reaction is to design appropriate primers so as to ensure the specificity of the reaction, the PCR is necessary to know the sequence of at least the border sections fragment to be amplified.

The method uses the properties of a DNA polymerase, such as the ability to synthesize the complementary strand by single-stranded template and primers need to initialize the polymerization **(Omelka et al., 2001)**.

The aim of this work is to evaluate the determination of species specificity of primers for detection of turkey and chicken.

MATERIAL AND METHODOLOGY

As biological material we used muscles of domestic fowl (*Gallus gallus*) and domestic turkeys (*Meleagris gallopavo*), since turkey is the most commonly counterfeited with chicken. In the next section assess the specificity of the first and second detection kits examining cross-reaction with other species. We used 100% of the DNA of different species: pig (*Sus scrofa ferus*), ducks (*Anas platyrhynchos*), moufflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), and rabbit (*Oryctolagus cuniculus*). DNA were isolated by phenol - chloroform extraction, preceded skiing individual samples (sample size was 1 mm) in 600 mL of lysis solution with

Table 1 Sequence of primers first detection kit reaction mixture 1

Primer	Bp	Sequence
Gallus F	27	5´-TCTCACTTACACTACTTGCCACATCTT-3´
Gallus R	23	5´-CGTGTGTGTCCTGTTTGGACTAG-3´
Gallus P	27	5´-(FAM) –CACTGCAACCTACAGCCTCCGCATAAC-(BHQ)-3

Table 2 Sequences of the primers of the second set of detection in the reaction mixture 2

Primer	Bp	Sequence
Mgal F	19	5' - CCGTAACCTCCATGCGAAT - 3'
Mgal R	22	5' - TAATATAGGCCGCGTCCAATGT - 3'
Mgal P	28	5' - (FAM)- CGCCTCATTCTTCTTCATCTGCATCTTC-(BHQ1) - 3'

the addition of 20 mL of the enzyme proteinase K. TaqMan Real-time PCR was carried out in the capillary reaction cycler LightCycler® 1.5 (Roche) and the results were evaluated with the help of the LightCycler software version 4.5 (Roche, Germany), which during the PCR reaction automatically creates a graph of the fluorescence intensity of the number of cycles.

Sets of primers and TaqMan probes first and second detection kit was designed according to **Jonker et al. (2008)** and all primers were synthesized by General Biotech (Czech Republic). Designed primers were derived from the sequences of a specific gene cyt b. The sequence of the primers and TaqMan probes of the first and second sets of detection are listed in Table 1 and Table 2.

The individual primers and TaqMan probes were supplied in lyophilized form. Dissolving the freeze-dried in ultrapure water (Milli-Q H_2O) were obtained 10x concentrated stock solutions of primers, which were stored at -20 °C. Primers from stock solutions were diluted working solutions so that their final concentration of 10 $pmol.\mu L^{-1}$. Working solutions were stored at 2-8 °C. Lyophilized TaqMan probe from a first and second detection kit was dissolved in ultrapure H_2O directly to a working concentration of 5 $pmol.\mu L^{-1}$. In a reaction mixture, we used the components necessary for optimum progress of the reaction: Colorless GoTaq® reaction buffer, $MgCl_2$, d NTP mix, individual primers and probes, and a dye ROX GoTaq ® Hot Start Polymerase. We used GoTaq® Hot Start polymerase having polymerase activity blocked. Restoring polymerase activity occurs at initiation denaturation at 94-95 °C for 2 minutes. This system eliminates nonspecific amplification and creating primer - dimer. Mastermix is added to the reference dye ROX, which is used for normalization of the reporter signal. The normalization of the signal is essential for the prevention of signal variations caused by the construction of the device frequently. Preparation of the reaction mixture was carried out in the UV-cleaner box (BioSan, Lithuania). Capillaries are adapted to the volume of the reaction mixture from 10 to 40 ml. After adding the desired amount of DNA we conclude capillaries and quickly spun on a centrifuge. After inserting the capillary into the rotary plate of thermo cycler (LightCycler 1.5) we recorded the intensity of the fluorescent signal after each cycle measured at a wavelength of 640 nm.

Temperature control PCR reactions for 1st and 2nd detection kit:

Initial denaturation	95 °C, 10 min.
Denaturation	95 °C, 10 sec.
Hybridzationa+ elongation	60 °C, 15 sec.
Cooling	40 °C, 30 sec.

Fluorescence intensity was measured at the end of each cycle of hybridization and elongation. After completion of the PCR results were evaluated in the LightCycler 5.4 using the tool "Absolute Quantification". This function determines the value of the threshold cycle (Cp), under which the samples were evaluated and used to determine the actual number of copies of the unknown sample in comparison to a standard curve. Of Cp value is based absolute quantification of the product, since it is inversely proportional to the logarithm of the initial template copy number **(Ciglenečki et al., 2008)**. Cp value is the lower, the higher the number of copies of template in the sample before the start of the reaction **(Yilmaz et al., 2012)**.

RESULTS AND DISCUSSION

We watched the specificity of the first and second detection kit for the screening and cross-react with other species. 100% of the DNA of different species: pig (*Sus scrofa ferus*), ducks (*Anas platyrhynchos*), mouflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), rabbit (*Oryctolagus cuniculus*).

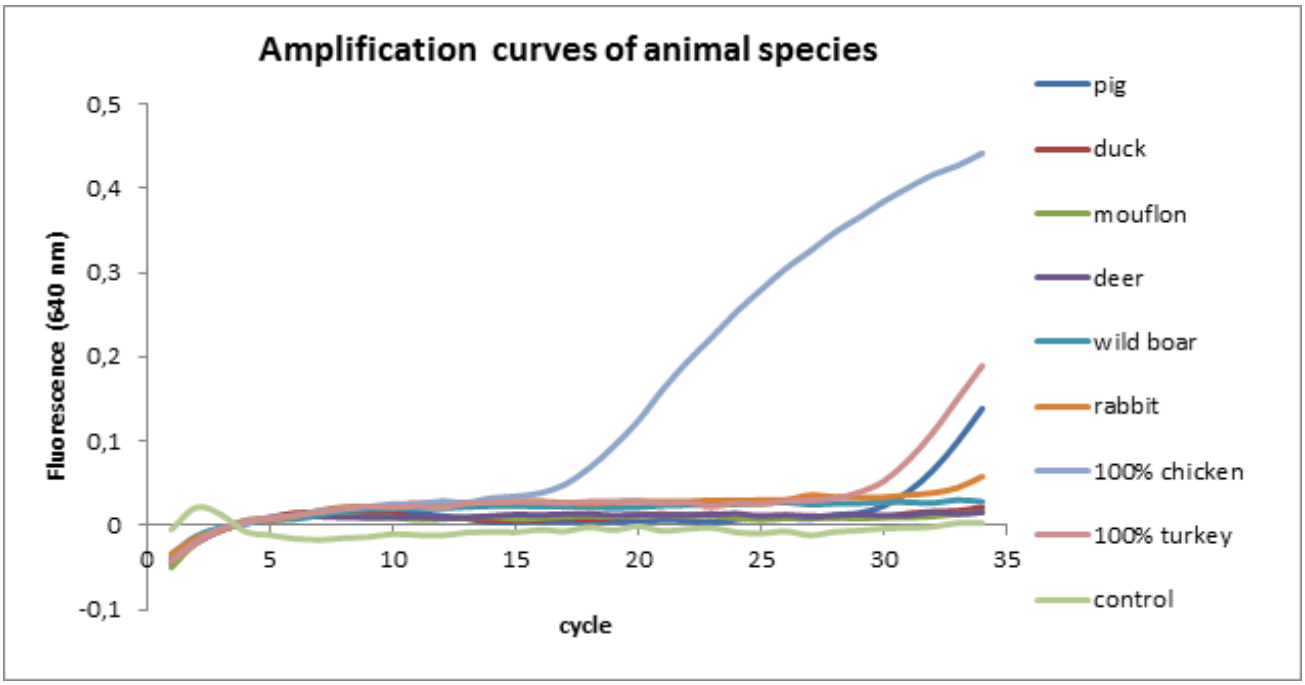

Figure 1 Amplification curves of species (first detection set): pig (*Sus scrofa ferus*), ducks (*Anas platyrhynchos*), mouflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), rabbit (*Oryctolagus cuniculus*) 100% chicken and 100% turkey DNA, using the first detection kit (Gal F, R primers, TaqMan probe)

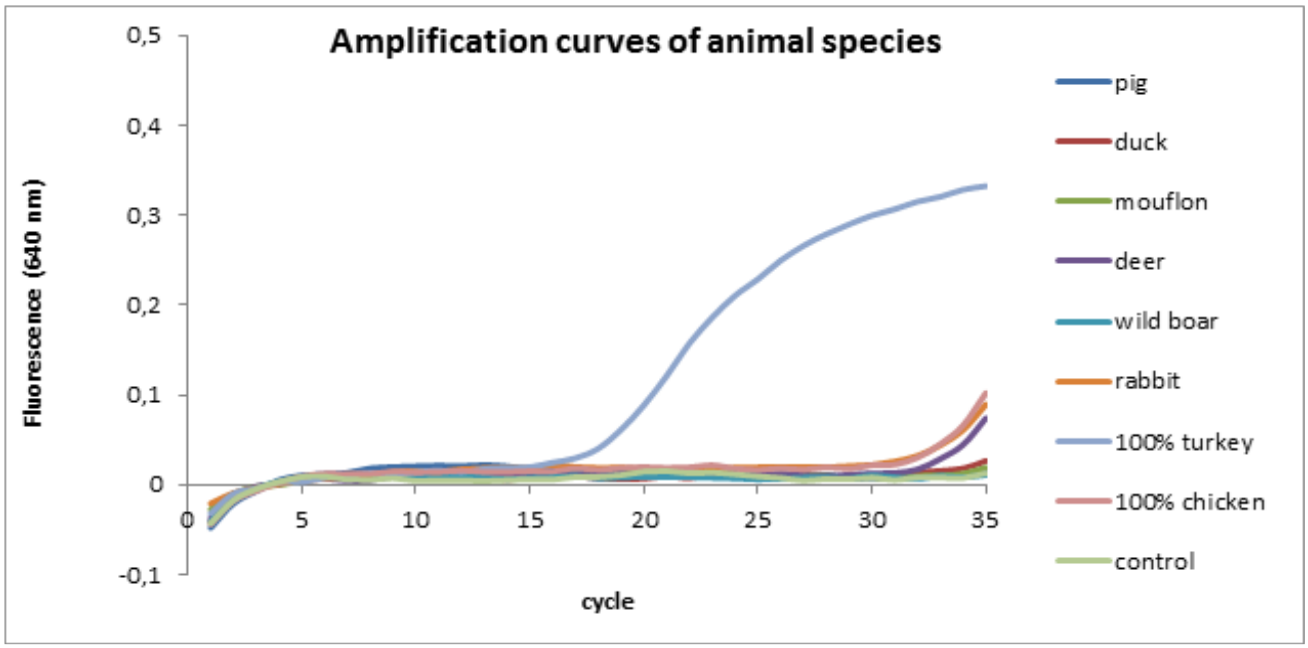

Figure 2 Amplification cures of species (second detection set): pig (*Sus scrofa ferus*), duck (*Anas platyrhynchos*), mouflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), rabbit (*Oryctolagus cuniculus*), 100% turkey a 100% chicken DNA, using the second detection kit (Mgal F, R primers, TaqMan probe)

In Figure 1 we follow the DNA amplification using by the first set of reaction kit with 100% chicken DNA at 16,18 cycle (Cp = 16.18) and 100% turkey DNA in cycle 29.18 (Cp = 29.18). It was also amplified DNA pig that exceeded the detection threshold fluorescence intensities in the 31.07 cycle (Cp = 31.07). Other species were amplified after more than 35 cycles, or at all. In this experiment was included as pig DNA in some products used, for example haemoglobin pork, pork fat, DNA boar as a close second to the pig and duck as another deputy of poultry. Also **Dooley et al. (2004)** developed a test for the detection of chicken, turkey, pork, beef and lamb. As authentication markers have chosen also species-specific regions of cyt b, which were amplified using appropriate species-specific primers. Detection of amplicons provides only two probes. The first was specific markers for mammalian meat and other markers for meat or birds. The test specifically targeting specificity chicken averages observed cross-

Table 3 Cross reaction of the first and second detection kits in various animal species.

	Concentration DNA (%)	Chicken primers (Gall)(Gall)		Turkey primers (Mgal)	
		Cp to 35 cycle of reaction	**Cross reaction to 30 cycle**	**Cp to 35 cycle of reaction**	**Cross reaction to 30 cycle**
Sus scrofa ferus	100	31.07	-	>35	-
Anas platyrhynchos	100	>35	-	>35	-
Ovis musimon	100	>35	-	>35	-
Red deer	100	>35	-	32	-
Sus scrofa vittatus	100	>35	-	>35	-
Oryctolagus cuniculus	100	>35	-	31.63	-
Gallus gallus	100	16.18	16.18	31.6	-
Meleagris gallopavo	100	29.18	29.18	16.85	-

reaction with DNA of all kinds. DNA was most intense with pork; amplification curve crossed the threshold of detection in 30.05 cycles. It's concluded that the threshold of 30 cycles is sufficient for qualitative authentication chicken. The gene for cytochrome b later used to authenticate the chicken also **Laube et al. (2007), Tanabe et al. (2007) and Jonker et al. (2008)**. **Kesmen et al. (2012)** point out the difficulty of developing a detection system to distinguish chicken from the turkey as it is a closely related species, which are characterized by a high degree of DNA homology.

Figure 2 display species specificity of the second screening kit. As can be seen there has been amplification of the 100% turkey DNA in 16.85 cycle (Cp = 16.85). Was also amplified a 100% chicken DNA 31.6 cycle (Cp = 31.6). Came amplification and the 100% rabbit DNA in 31.63 cycle (Cp = 31.63) and 32 deer in the cycleta (Cp = The DNA of all other animal species was amplificated after more than 35 cycles (Cp >35). It follows that the second detection primer pair is specific enough to unrelated species of animals by 30 cycles of the reaction. But we must take into account that it was not possible to determine the detection limit for a given set of primers.

As we shown in Table 3, using the two sets of detection kits occurring cross-reactions, but all up to 30 cycles of the reaction. The 30 cycle of reaction was chosen by us as the threshold for specificity using primers for authentication chicken and therefore can be considered a first screening set for species-specific and due to the presence of DNA species verified by us. A second set of screening can be considered a species-specific animal species verified by us but we have to take into account that it is not specific enough within the species. **Cammas et al. (2012)** summarized the results of studies published in 2012 that the generic authentication meat using Real-Time PCR using TaqMan probes. Found that many of them describe the cross-reactivity of primers and probes designed with other types of DNA, as well as the low efficiency of amplification of markers, especially in relation to the degradation of DNA in heat-treated foods. Effectiveness (efficacy) with the decline of efficiency, the number of amplicons generated in each cycle decreases, resulting in the generation of amplification curves of later cycles.

CONCLUSION

Currently, the use of TaqMan Real-Time PCR in food analysis focuses primarily on quantitative detection of materials of animal and plant origin in food with very complex texture. TaqMan real-time PCR is within the sensitivity and specificity clearly one of the best methods for identifying the species of chicken and turkey meat.

The specificity of this method, however, depends primarily on the specificity of the primers and TaqMan probes.

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EVALUATION OF SIGNIFICANT PIGMENTS IN GREEN TEAS OF DIFFERENT ORIGIN

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ABSTRACT

This work monitors flavonoid pigments (theaflavins and thearubigins), and chlorophyll in green teas from different growing regions (India, China, Russia, Vietnam). These pigments affect the quality of the finished tea infusion and their quantity is affected by the way of tea processing (fermentation). 15 samples of green loose teas were selected for the analysis. The content of these pigments was determined using UV-Vis spectrophotometry, the concentration of flavonoid pigments was measured at a wavelength of 665 nm (theaflavins) and 825 nm (thearubigins). Concentration of flavonoid pigments was determined using the standard conversion coefficients, which are 38.7 for TRs and 1.4 for TFs. Chlorophyll concentration was measured at 642.5 and 660 nm. Chlorophyll concentration was determined by the conversion using international standard conversion coefficients and calculation of linear regressions. The results were statistically processed and evaluated in the program of UNISTAT version 5.6. The total amount of chlorophyll ranged from 0.20 to 1.33 mg/L of tea. Concentrations of theaflavin ranged from 0.15 to 0.66 g/100 g of tea and TRs from 2.00 to 11.15 g/100 g of tea. The results showed that the amount of theaflavins, thearubigins and chlorophyll in green teas varied ($P < 0,05$), especially in teas from lowlands in Vietnam and Krasnodar (Russia). Statistically demonstrable difference ($P < 0,05$) in the content of pigments was also recorded in a group of Indian teas (from lowlands) and, from a group of Chinese teas, a statistical difference ($P < 0,05$) was demonstrated in the content of pigments in the mountain teas. On the basis of the results it can be concluded that the quality of green tea is mainly influenced by the processing method depending on the processing area (the factory), than by their country of origin.

Keywords: *Camellia sinensis* L.; theaflavins; thearubigins; chlorophyll; UV-vis spectrophotometry

INTRODUCTION

Green tea is produced of tea leaves (*Camellia sinensis L.*) that have not undergone the process of fermentation. Until recently, the world trade in tea focused almost exclusively on black tea. Nowadays, the situation in this respect has changed and tea fans can choose from many types of green tea **(Arcimovičová, Valíček, 2000)**. It is expected that global consumption of green tea will continue to rise, as the trend especially about its medicinal effects is spreading **(Mitscher, Dolby, 2006)**. World tea production had exceeded 4 million tons per year and is continuously growing. Around the world, green tea represents about 20 per cent of production **(Sang et al., 2011)**. Recently, green tea has been valued for its high content of antioxidants, which are positively applied in the prevention of many diseases, including cardiovascular diseases and some cancers.

The aim of this research is to determine the concentration of important pigments of green tea and thus to evaluate its sensory value (colour, taste) and quality of the analyzed teas, while determining the impact of the growing area on their concentration.

The green tea infusion has a pale yellow color, unlike that of black tea, which is reddish brown **(Owuor, 2003)**. The most numerous group of chemicals that significantly affect the color and sensory profile of green tea are components belonging to the group of polyphenols and chlorophyll. These compounds affect the sensory profile of tea, in particular its color and flavor **(Bernegg, 1991)**.

The polyphenolic compounds influencing the tea infusion color include generally defined flavonoid pigments, such as theaflavins (Figure 1) and thearubigins (Figure 2). Concentration of these pigments increases with the increasing degree of fermentation. Polyphenols (mainly catechins) of green tea leaves are oxidized during fermentation by oxidoreductases in enzymatic browning reactions to the respective o-quinones. These condense with the present catechins of green tea leaves to the pigments of black tea - flavonoid pigments. Therefore, the presence of these pigments in non-fermented tea can worsen its sensory quality; in particular indicate some degree of undesirable fermentation **(Velíšek, Hajšlová, 2009; Taylor, 2003)**.

Flavonoid pigments are responsible for the distinctive dark color and flavor of the tea **(Halder et al., 2005)**. Theaflavins give the tea infusion golden-yellow color and a characteristic brightness and freshness. Thearubigins produce red to brown infusion color and cause astringent taste **(Bhuyan et al., 2009)**. Thearubigins also negatively affect the brightness of tea. Although the concentrations of other chemicals contribute to the quality in the finished tea, it is the concentration of theaflavins and thearubigins that has a great effect on the brightness and color density, astringency, and the overall quality of tea **(Ghosh et al.,**

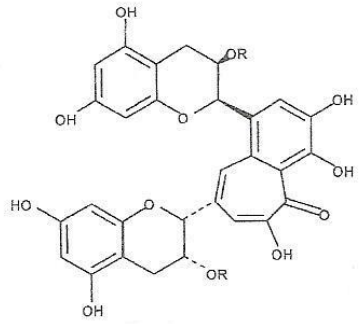

Figure 1 Structure of theaflavin

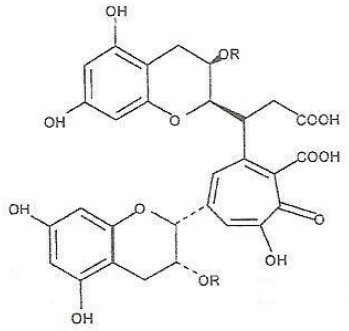

Figure 2 Structure of thearubigin

2012). Theaflavins (hereinafter TFs) are soluble dimeric flavonoids containing a seven-membered tropoline ring **(Velíšek, Hajšlová, 2009)**. TFs are formed by oxidative linking by dihydroxybenzene and trihydroxybenzene rings forming the respective pairs of flavan-3-ols (epicatechin and epigallocatechin respectively) in benzotropolone ring **(Wang, Ho, 2009)**. TFs cause bright red-orange color **(Menet, 2004)**. In black tea, the main theaflavin derivatives are theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3´-gallate (TF_2B), and theaflavin-3,3´digallate (TF_3) **(Liu et al., 2005)**. Thearubigins (hereinafter TRs) are a very heterogeneous mixture of soluble to insoluble oxidation products **(Velíšek, Hajšlová, 2009)**. TRs show a reddish-yellow, orange-brown, reddish-brown to dark brown color **(Menet, 2004)**. Unlike TFs, TRs have not been previously described and are continually under research **(Singh, 2011)**. TRs have a higher molecular mass (700-40000) than catechins and TFs, and are thus ill-defined in terms of chemical structure and biological activity **(Babich et al., 2006)**.

Besides these two pigments, colorless bisflavanols, red theaflavin acids, yellow theacitrins, and brown theafulvins are also formed. During fermentation, about 20 per cent of flavonoids in tea leaves turn into insoluble compounds (apparently they are covalently bound to proteins via their thiol groups) which remain in the remnants of tea leaves when the infusion is prepared **(Velíšek, Hajšlová, 2009)**.

Chlorophyll pigments (chlorophylls) are a group of green pigments which are found in the tissues providing photosynthesis. Among them chlorophyll are the most abundant pigments on the earth. It is a key component of photosynthesis necessary to absorb sunlight **(Hörtensteiner, Kräutler, 2011)**. Originally, chlorophyll only referred to green pigments involved in photosynthesis in higher plants. Later, this term was extended to all photosynthetic porphyrin pigments **(Velíšek, Hajšlová, 2009)**. From the viewpoint of food industry, the greatest importance is ascribed to chlorophylls (chlorophyll *a*, chlorophyll *b*) which are contained in higher plants in a ratio of approximately 3:1. Chlorophyll *a* is present in all plants, the occurrence of the other ones is limited. The difference between them is very small. Chlorophyll *a* is blue-green and it has a methyl group located at the second pyrrole ring. Chlorophyll *b* is yellow-green and it has an aldehyde (formyl) group at the said pyrrole ring **(Ustin et al., 2009; Heaton, Marangoni, 1996)**. Chlorophyll is a very important pigment in tea leaves and unfermented tea, because its concentration influences the final color of the infusion so typical for green (unfermented) tea. During tea fermentation, chlorophyll produces pheophorbines and phaeophytins that contribute to the dark color of black tea **(Harbowy, Balentine, 1999)**. Pheophorbines and phaeophytins are the main degradation products of chlorophyll **(Cartaxana et al., 2003)**.

MATERIAL AND METHODOLOGY

To compare the selected pigments, 15 types of loose green tea were selected from India, China, Vietnam, and Russia. All the analyzed samples are presented in Table No. 1.

Method for determination of flavonoid pigments - theaflavins and thearubigins

The principle of the method was extraction of theaflavins and thearubigins in aqueous solution for a period of 15 minutes and then measuring their absorbance at a wavelength of 665 nm for TFs and 825 nm for TRs compared to water as a blank sample **(Ošťádalová et al., 2010; AOAC, 2009; Perkampus, 1992)**. Concentration of flavonoid pigments was determined using the standard conversion coefficients, which is 38.7 for TRs and 1.4 for TFs. The conversed values of TFs and TRs were expressed as g per 100 g of tea **(USDA database; Bhagwat et al., 2010)**.

*Workflow***:** 3,0 g tea sample was weighed and bathed in 100 ml water at 85 °C. The samples prepared in this way were being extracted for 15 minutes. After cooling down to room temperature (20 °C), the absorption of the samples was measured. In all the cases, the measurement maximum absorption of the absorption spectrum was checked in a broader wavelength range **(Ošťádalová et al., 2010; AOAC, 2009; Perkampus, 1992)**.

Method for determination of chlorophyll

The principle of the method was the extraction of chlorophyll in diethyl ether for a period of 15 minutes and then measuring the absorbance at a wavelength of 500-900 nm in contrast to diethyl ether as a blank sample **(Ošťádalová et al., 2010; AOAC, 2009; Perkampus, 1992)**.

Table 1 Summary of all the analyzed samples of green tea

Sample Number	Tea Name	Origin
1	Darjeeling Green Soureni FTGFOPI	India (Darjeeling Province - Mirik; mountains)
2	Assam Green Tea OP	India (Assam Province; lowlands)
3	Ceylon Sencha	India - Sri Lanka (lowlands)
4	Darjeeling Green Hilton SFTGFOPI	India (Darjeeling Province - Ambiok; mountains)
5	En Shi Yu Lu "Green Dew"	China (Hubel Province; mountains)
6	Gunpowder Temple of Heaven	China (Zhejiang Province; mountains)
7	Yunnan green	China (Yunnan Province; mountains)
8	China Sencha	China (En Shi Province; mountains)
9	Green tea	Russia (Sochi region; lowlands)
10	Green tea "Class 1"	Russia (Krasnodar region; mountains)
11	Green tea "Extra"	Russia (Krasnodar region; mountains)
12	Green tea - hand made	Russia (Dagomis region; lowlands)
13	Vietnam Tea	Vietnam (lowlands)
14	Vietnam Ché ngon So	Vietnam (mountains)
15	Ché uôp hoa nhai with jasmine - hand made	Vietnam (lowlands)

Chlorophyll concentration was determined by the conversion using international standard conversion coefficients and calculation of linear regressions:

$ch_c = 7.12 \times A_{642.5} + 16.8 \times A_{660}$, where
ch_c….. concentration of total chlorophyll in tea infusion [mg/g of tea]
A_{660nm} … absorbance value measured at 660 nm
$A_{642.5nm}$ … absorbance value measured at 642.5 nm

Workflow: 1 g tea samples were weighed and bathed in 10 ml diethyl ether. The samples prepared in this way were being extracted for a period of 15 minutes and then their absorbance was measured. In all the cases, the measurement maximum absorption of the absorption spectrum was checked in a broader wavelength range (Ošťádalová et al., 2010; Britton et al., 1995).

Statistical analysis: All measurements were performed 3 times for each analyzed tea sample, and then average value and standard deviation were calculated. Parametric double-sided Student's test ($P < 0.05$) was used for the evaluation of the measured data. The calculations were computed by rational calculations using the UNISTAT software, version 5.6. Light.

RESULTS AND DISCUSSION

The average concentrations of flavonoid pigments, in particular theaflavins and thearubigins, in individual types of green teas analyzed, including standard deviations, are listed in the following Table No. 2. The lowest values of chlorophyll are highlighted therein.

As i tis shown in Tab. No. 2, theaflavin concentrations ranged from 0.15 to 0.66 g/100 g of tea and TRs from 2.00 to 11.15 g/100 g of tea. In accordance with **You et al. (2011)**, the average content of TFs in green teas should be about 0.34 per cent and, as stated by **Wang (2010)**, the concentration of TRs in green tea should not increase the average to 8 per cent, as this may impair the quality. **You et al. (2011)** further report that higher concentrations of TFs in green teas may indicate their partial fermentation, which points to their imperfect processing (heat treatment) or a long period of storage. This value in green tea is sensory acceptable, but the quality is not very good, as stated **Owuor (2003)**.

An interesting finding is that the highest values ($P < 0.05$) of both pigments were recorded in the same teas, namely in tea No. 3 originating from India and in Vietnamese tea No. 15. Compared to other samples of green tea, statistically proven ($P < 0.05$) higher TFs values were identified for the Chinese tea No. 7, Russian teas No. 9 and 11 (from the Russian region of Krasnodar), and Vietnamese tea No. 13. For all the above-listed tea samples, pigment content was found in higher values (then published in above mentioned literature, which is higher than 0.34 per cent for TFs and 8 per cent for TRs). According to proportional values of TFs and TRs, it can thus be assumed that undesirable fermentation had

occurred in these teas. Furthermore, the above can also be explained by the fact that both Russian and Vietnamese teas came from the same area and were processed in the same factory, therefore, under the same conditions, the so-called tea formulas.

As stated by **Obanda et al. (2001)**, the amount of flavonoid pigments depends on the original content of catechins in green tea leaves and subsequent fermentation. Higher concentrations of catechins in tea plants come from higher elevations and, as reported by **Brown et al. (2003)**, a higher concentration of flavonoid pigments may be caused by a higher content of catechins in the original green tea leaves. However, this is in contradiction to our results, because teas from mountain areas have a lower content of flavonoid pigments (approximately 3 g/100 g of tea; total amount TFs and TRs). According to our results, it cannot therefore be assumed that the contents of TFs and TRs is influenced by the tea growing regions, but by the processor.

In the further research, we focused on the analysis of chlorophyll as an important pigment of green tea. The results are presented in Table 3. The lowest values of chlorophyll are highlighted therein.

The total amount of chlorophyll ranged from 0.20 to 1.33 $mg.L^{-1}$ of tea. In his work, **Wolf (1959)** reported the average chlorophyll content in green tea in the amount of 1.39 mg per 1 g of dry leaves. Similar data have recently been published also in the works by **Wright (2005)** and **Lornaty et al. (2010)** who found in unfermented tea leaves an average of 1.2 mg of chlorophyll per 1 g of dry matter.

Except for samples No. 3 (India), 7 (China), 9, 11 (Russia), 13, and 15 (Vietnam) where the lowest chlorophyll amounts of all the analyzed green teas were found ($P < 0.05$), teas in our research corresponded to the above values.

The samples of green teas included in our analyses with the low total chlorophyll results rather correlated with the values of chlorophyll corresponding to partially fermented or fermented teas. Chlorophyll content in the fermented tea ranges from 0.9 mg to 0.1 mg per g of dry leaves, as stated by **Lornaty et al. (2010)**. Moreover, these are the same samples of green teas, in which statistically significantly higher values of flavonoid pigments were found (concentrations rather equivalent to fermented tea). Thus it can be assumed that these teas underwent a process of fermentation which is undesirable for green tea. Which can also be confirmed based on the results by **Khamessan, Kermasha (1995) and Daooda (2003)**.

Table 2 Average amounts of TFs and TRs [g/100g of tea] in individual samples of the analyzed green teas

Tea Samples	TFs	TRs
India		
1	**0.23** ±0.20	**2.34** ±0.75
2	**0.30** ±0.10	**4.31** ±0.88
3	**0.66** ±0.15	**10.49** ±0.05
4	**0.35** ±0.25	**5.86** ±0.09
China		
5	**0.21** ±0.12	**3.51** ±0.07
6	**0.45** ±0.07	**6.80** ±0.69
7	**0.50** ±0.16	**8.31** ±0.10
8	**0.34** ±0.12	**5.13** ±1.2
Russia		
9	**0.54** ±0.08	**10.20** ±0.33
10	**0.15** ±0.10	**2.00** ±0.09
11	**0.54** ±0.25	**10.49** ±0.21
12	**0.21** ±0.36	**3.27** ±0.29
Vietnam		
13	**0.58** ±0.22	**10.01** ±0.22
14	**0.25** ±0.21	**3.12** ±0.19
15	**0.64** ±0.11	**11.15** ±0.05

Table 3 Average amount of chlorophyll [mg.L^{-1}g of tea] in individual samples of the analyzed green teas

Tea Samples	Chlorophyll
India	
1	1.15 ±0.57
2	1.22 ±0.12
3	0.20 ±0.36
4	1.28 ±1.25
China	
5	1.28 ±1.01
6	1.18 ±0.99
7	0.68 ±0.12
8	1.33 ±0.33
Russia	
9	0.28 ±0.18
10	1.13 ±0.25
11	0.31 ±0.52
12	0.88 ±0.10
Vietnam	
13	0.58 ±0.08
14	1.22 ±0.09
15	0.62 ±0.02

Changes in chlorophyll concentration in different varieties of tea are explained by the fact that, after incomplete heat treatment of tea leaves and their subsequent fermentation, enzymes of chlorophyllase and chlorophyll degrading enzymes remain active, which results in chlorophyll degradation to pheophorbines and phaeophytins, their compounds play an important role in providing the typical dark color of fermented tea, during the processing.

In view of the growing area, its effect on the concentration of chlorophyll cannot be directly determined. No differences in chlorophyll content among the samples of green teas originating from lowlands and mountainous areas of the same origin were statistically demonstrated ($P < 0.05$). As stated by **Wei et al. (2011)**, although the amount of chlorophyll is affected by the cultivation conditions when its quantity increases in tea plants grown at lower temperatures and higher relative humidity, its production is, however, most affected by photosynthesis which is the most intense in young leaves **(Khamessan, Kermasha, 1995)**. Thus it can be concluded that the content of chlorophyll is mainly influenced by the way of tea leaves processing.

CONCLUSION

The aim of the study was to evaluate the content of major pigments (theaflavins, thearubigins, chlorophyll) in green tea affecting the sensory profile and quality of teas. It was found that teas of the same origin from different provinces differ statistically ($P < 0.05$) in their content of flavonoid pigments and chlorophyll. Specifically, it was the Russian tea from the mountainous region of Krasnodar and Vietnamese teas grown in the lowlands. Statistically demonstrable difference ($P < 0.05$) in the content of pigments was also recorded in a group of Indian teas (from lowlands) and, from a group of Chinese teas, a statistical difference ($P < 0.05$) was demonstrated in the content of pigments in the mountain tea En Shi Yu Lu "Green Dew". Other analyzed samples of green tea reached the values of pigments in similar concentrations typical for unfermented tea. Therefore, it cannot be directly determined whether the altitude of the growing region affects the final content of sensory important pigments. On the basis of the results it can be thus concluded that the quality of green tea is mainly influenced by the processing method depending on the processing area (the factory), than by their country of origin. Based on the total evaluation, it can also be noted that a tea from the retail market does not always match the quality standards and its sensory value (color and taste) does not always have to be constant.

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EFFECT OF CHAMOMILE SUPPLEMENTS TO FEEDING DOSES ON ANTIMICROBIAL PARAMETERS IN POULTRY

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ABSTRACT

Due to a ban of use of antibiotic growth promoters in the poultry industry it is necessary to look for alternative solutions. The use of some herbs showing antimicrobial effects can be one of such alternatives. In this experiment, effects of three different concentrations of chamomile (*Matricaria chamomilla)* extract, (0.3%; 0.6% and 1.2%) in feeding doses on the microbial population in the gastrointestinal tract of growing broiler chickens were studied. The main attention was paid to the population of *Clostridium perfringens* and to numbers of coliform microbes. Clostridia were cultivated under anaerobic conditions at 46 °C on the Tryptone Sulfite Neomycin (TSN) agar for a period of 24 hours. Coliform microbes were grown on the violet red bile lactose (VRBL) agar at 37 °C for a period of 24 hours. The experiment lasted 39 days and involved 80 chicks that were slaughtered in the course of their growth period at the age of 18, 25, 32 and 39 days; there were 5 chicks in each group. The obtained results indicated that increasing doses of chamomile in the feeding ration decreased numbers of coliform microbes in the digestive tract of chicks and also reduced the population of *C. perfingens*.

Keywords: poultry; chamomile; *Clostridium perfingens*; *Escherichia coli*

INTRODUCTION

C. perfringens is one of the most frequent etiological agents in both animals and humans **(Allaart, et al., 2013)**. In poultry industry, *C. perfringens* pathogens of the type A and/or C play an important role **(Shanmugavelu, et al., 2006)**. Microbes of the species *C. perfringens* are gram-positive, not-moving and spores producing bacteria that are present in soil, feedstuffs, litter and intestinal tract of both ill and healthy birds. Temperatures and pH values required for the growth and propagation of these bacteria range from 12 °C to 50 °C and from 6.0 to 7.0, respectively. Under optimum temperatures (i.e. 43 °C - 47 °C) bacteria of the species *C. perfringens* propagate extremely quickly: the generation interval is 8 - 10 min, and the growth is associated with gas production. Spores of these bacteria are very resistant against heat, desiccation, acids and many of chemical disinfectants **(Hafez, 2011)**.

While the acute form of this disease causes an increase in mortality, the symptoms of its subclinical (i.e. latent) form involve inhibited digestion and absorption of nutrients, lower weight gains and less efficient conversion of feedstuffs. As far as the poultry is concerned, bacteria of the species *C. perfringens* are considered to be a common part of their intestinal microflora. The manifestation of the necrotic enteritis requires the existence of several pre-disposing factors. The coccidiosis infection is one of the most important factors of this type **(Yegani, et al., 2008)**. Coccidia can damage intestines, cause inflammations and intensify the production of mucus. In this way favourable conditions for the propagation of *C. perfringens* bacteria can be created and these thereafter damage intestines more and more **(Cvikova, et al., 2006)**.

Escherichia coli is a commensal species belonging to the group of facultatively anaerobic microflora living in the large intestine. The pathogenic species *E. coli* is an important infective agent causing extraintestinal infections. Some pathotypes of *E. coli* cause infections in humans and some other in animals **(Kaper, 2005)**. In layers and broilers, avian pathogenic *E. coli* (APEC) cause above all infections of their reproductive organs. In chickens, the APEC infections develop probably independently on other microbes occurring in the intestinal reservoir. Although the inflammatory diseases of the oviduct are mostly of chronic nature, an acute sepsis may occur in 28% of infections **(Pires-dos-Santos, et al., 2014)**.

APEC stems cause colibacilloses in both domestic and wild birds. This infection plays an important role in the world poultry industry. Although the colibacillosis may be influenced by antimicrobial compounds, the most important problem represent residues of antibiotics in foodstuffs. Chances that we will find some successful alternative solutions are dependent on an exact characterisation of individual groups of pathogens (especially those that are infective also for humans) and also of possibilities and mechanisms of these infections **(Mora, et al., 2013)**. Plant extract, volatile oils as well been pollen are complex mixtures of many natural components in inexact proportions that have different structure and show different effects. The basic characteristics of plant additives and essential oils is their wide antimicrobial activity **(Opletal, et al., 2010, Omer et al., 2013)**. After the ban of application of antibiotics as

growth promoters in poultry the use of essential oils is more and more frequent. Basing on results of a literary survey it was possible to identify four different mechanisms of essential oils functioning of in the following categories: perception (i.e. interpretation of sensory information), metabolism, antioxidant activity and antimicrobial efficiency **(Brenes, et al., 2010, Liptaiová, et al., 2010)**.

The aim of this study was to assess effects of chamomile extract on two bacteria species (*C. perfringens* and *E. coli*) occurring in the digestive tract of growing broiler chickens.

MATERIAL AND METHODOLOGY

Experimental design

The experiment involved 80 male chicks of the hybrid combination Ross 308. All chicks were seven days old. There were altogether 4 groups of these birds, viz. control and three experimental groups receiving chamomile extract supplements in concentrations of 0.3%, 0.6% and 1.2%. In the course of the growing period of chicks, always five birds of each group were killed on Days 18, 25, 32 and 39 of age.

Birds and experimental conditions

Prior to the beginning of the experimental period, chicks were weighed, identified with wing tags, assorted into four groups and placed into metabolic cages. All birds received water and feed mixture *ad libitum*. The feed mixture consisted of following components: wheat (25%), maize (37%), soybean meal (28%), sunflower oil (6%), mineral-vitamin mixture without anticoccidial drugs (3%), monocalcium phosphate (0.8%) and finely ground limestone (0.2%). Chamomile extract was added into the feed mixture in concentrations of 0.3%; 0.6% and 1.2%.

The light regime was 6 hours of darkness and 18 hours of light. On the 7th day of age chicks were kept at the ambient temperature of 29.9 °C and relative humidity of 50%.

Preparation of samples

Diluted samples of excrements were homogenised in a Biosan (Latvia) multi-vortex. Samples of 1 ml were transferred by an automatic pipette into sterile Petri dishes and overflown with the corresponding agar (manufacturer the company Biokar, France). The violet red bile (VRBL) agar contained in 1 litre of medium: 7 g of peptic meat digest; 3 g of yeast extract; 10 g of lactose; 1.5 g of bile salts; 5 g of sodium chloride; 30 mg of neutral red; 2 mg of crystal violet and 12 g of bacteriological agar. The tryptone sulphite neomycin (TSN) agar contained in 1 litre of medium: 15 g of tryptone; 10 g of yeast extract; 1 g of sodium sulphite; 0.5 g of ferric ammonium citrate; 50 mg of neomycin sulphate, 20 mg of polymixin B sulphate and 13.5 g of bacteriological agar. Samples were prepared in two dilutions (*C. perfringens* 10^{-1} and 10^{-2} and of *E. coli* 10^{-7}, 10^{-8}).

Microbiological analysis

Microorganisms were assessed as follows: Petri dishes with coliform microbes inoculated on the violet red bile (VRBL) agar were placed into a thermostat and cultivated at the temperature of 37 °C for a period of 24 hours. Petri dishes inoculated with *C. perfringens* were placed at first into an anaerobic system (manufacturer the company Merc, Germany) with a generator of the anaerobic environment Anaerocult A (Merc, Germany). The anaerobiosis system was thereafter closed and placed into a thermostat and the sample was cultivated at 46 °C also for a period of 24 hours. Numbers of bacteria in 1 ml of sample were assessed on the base of characteristic colonies growing in Petri dishes and expressed as CFU (Colony Forming Units).

Statistical processing

Obtained results were analysed using the programme Microsoft Excel 2010 and the software Statistica 10 CZ.

RESULTS AND DISCUSSION

Chamomile (*Matricaria chamomilla* L.) belongs to a large group of cultivated medicinal plants. Chamomile plants contains a great number of therapeutically interesting active compounds. In general, sesquiterpens, flavonoids, coumarins and polyacetylens are considered as the most important components of the chamomile drug. Chamomile is used above all because of its antiphlogistic and antiseptic effects **(Singh, et al., 2011)**.

The aim of this study was to assess effects of different concentrations of chamomile extracts on the microbial population living in the small intestine and their propagation in the course of the growing period of broiler chickens of hybrid combination Ross 308. Attention was paid to *C. perfringens* and *E. coli* and the results were expressed in CFU.g^{-1}. Samples were taken from the small intestine in weekly intervals so that it was possible to monitor either the increase or the decrease in numbers of colonies in the small intestine in the course of the growing period of broiler chickens.

The highest numbers of clostridia were recorded in the control group (Fig 1). The maximum increase took place between Days 18 and 25 of age of birds ($P < 0.05$). The minimum increase was recorded in the group receiving chamomile extract in the concentration of 0.3%; in this group, there was only a slight increase in numbers of clostridia (Fig. 1).

The highest number of coliform bacteria were found out in the control group while the lowest one in the group C1 (i.e. with the concentration of 0.3% of chamomile extract) (Fig. 2). The observed increase in CFU was insignificant in all groups under study. To the end of the experimental period (i.e. between Days 25 and 32), the highest increase numbers of coliform bacteria was recorded in the control group C 0 (with the zero concentration of chamomile extract) and in the group C 3 (with the concentration of 1.2% of chamomile extract) (Fig. 2).

Abdoul-Latif, et al., (2011) demonstrated a high antimicrobial activity of *M. chamomilla* methanol extract and essential oil. Their results indicated that *M. chamomilla* could be used as a natural antimicrobial substance suitable either for the treatment of human infections or as a food preservative. An extended application of natural antimicrobial substances could also reduce negative environmental impacts of synthetic chemicals and drugs.

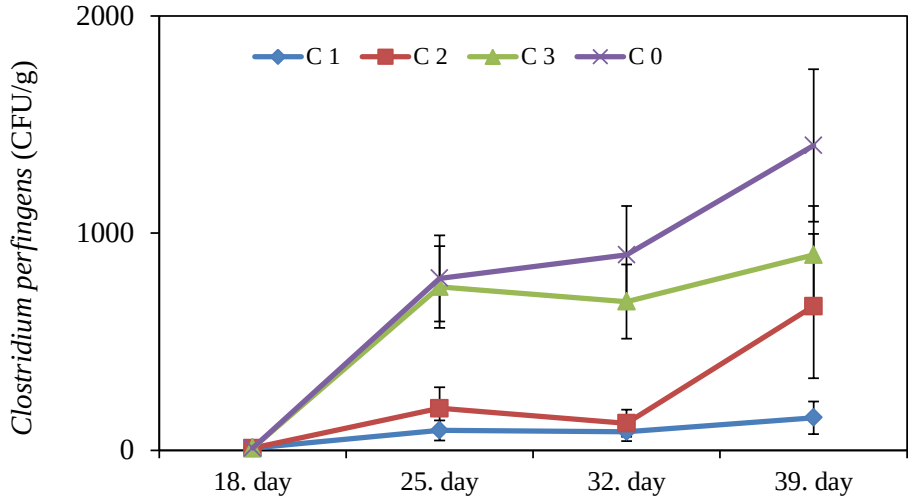

Figure 1 Changes in average numbers of *C. perfringens*.
C 1 - concentration 0.3% of chamomile extract, C 2 - concentration 0.6% of chamomile extract, C 3 - concentration 1.2% of chamomile extract, C 0 - concentration 0% of chamomile extract

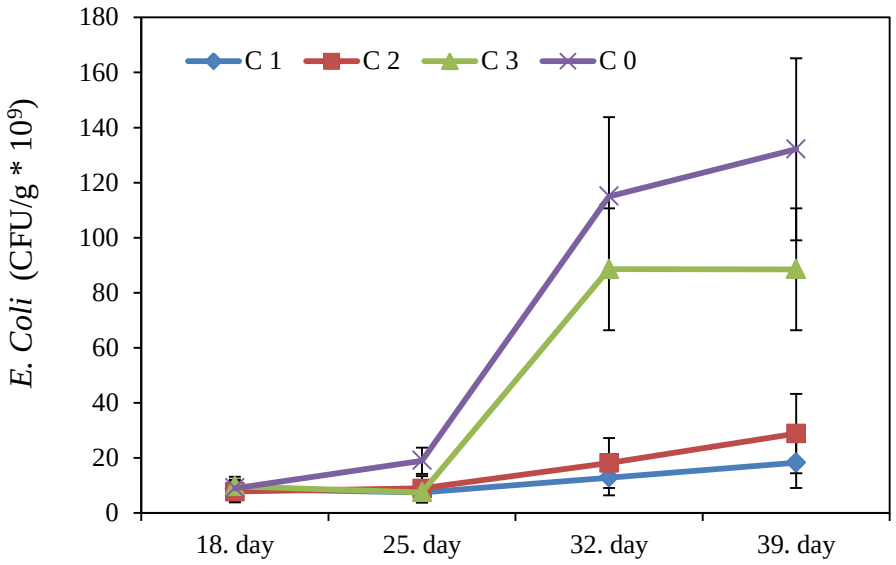

Figure 2 Changes in average numbers of *E. coli*.
C 1 - concentration 0.3% of chamomile extract, C 2 - concentration 0.6% of chamomile extract, C 3 - concentration 1.2% of chamomile extract, C 0 - concentration 0% of chamomile extract.

The effect of the addition of chamomile ethanol extract into drinking water on stress symptoms was already tested in broilers. It was found out that (as compared with the control group) the applied extract contributed to both a decrease in the level of cholesterol and to an increase in the level of the imunoglobulin complex in blood of experimental birds. The chamomile extract also contributed to an increase in the live body weight of broilers **(Skomorucha, et al., 2013)**. **Marques et al., (2010)** tested the effect of *M. chamomilla* on stress and growth of Japanese quails. In this experiment, tested concentrations of *M. chamomilla* did not show any effects on the growth, behaviour and physiological parameters of reared Japanese quail chicks.

Mitsch et al., (2004) demonstrated that specific blends of essential oils components can control the proliferation of *Clostridium perfringens* in the broiler intestine. In this study essential oils significantly reduced the number of *Clostridium perfringens* in the intestineand feces of broilers and therefore may have reduced the risk of necrotic enteritis. The fact that some essential oils have good potential in the control of *Clostridium perfringens* demonstrated in his study also **Si et al., (2008)**.

CONCLUSION

The obtained results indicate that increasing concentrations of chamomile extract showed a positive effect on the reduction of the number of coliform microbes in the digestive tract of experimental chickens and, above all, on microbes of the species *C. perfringens*. The highest and the most intensive increase in numbers of clostridia were observed in controls between Days 18 and 25 of the age ($P < 0.05$). The highest numbers of coliform bacteria were recorded also in the control group C 0 while the lowest one in the group C1 (i.e. with the concentration of 0.3% of chamomile extract). The increase in CFU was insignificant in all groups under study. The most pronounced inhibiting effect of chamomile extract of bacteria species *E. coli* and *C. perfringens* was observed in the group C1 with the supplement of 0.3% of chamomile extract into the feed mixture.

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CALCIUM IN EDIBLE INSECTS AND ITS USE IN HUMAN NUTRITION

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ABSTRACT

Calcium is one of the most problematic substances in human nutrition. Nutrition in the present population is not optimal, because of insufficient consumption of milk and dairy products. Due to the expanding interest of specialists and the general public about entomophagy, as well as increase of the EU interest in this type of food, there is a need to consider the use of edible insects as an alternative source of nutrition. From the perspective of edible insects as a source of calcium, edible insects could be considered as a possible source of calcium for enriching the diet and also as a substitute for people with lactose intolerance and allergies to other categories of foods rich in calcium. Of the six analysed species of edible insect, *Bombyx mori* had the highest calcium content, almost comparable to semi-skimmed cow's milk. *Gryllus assimillis* can also be a rich source of calcium as well as other analysed species. The lowest content of calcium was detected in *Zophobas morio*. Common meat (chicken, beef, pork) has lower calcium content comparing with all analysed species of edible insect (*Apis mellifera, Bombyx mori, Gryllus assimillis, Locusta migratoria, Tenebrio molitor, Zophobas morio*). Therefore, the selected species of edible insect could serve as an alternative source of calcium for people with lactose intolerance and allergies to soy. Phosphorus level in human body is closely related to calcium in the calcium-phosphate metabolism, therefore phosphorus level was detected in these samples too. *Bombyx mori* had the highest phosphorus content and the lowest content of phosphorus was measured in *Zophobas morio* samples.

Keywords: calcium; phosphorus; edible insects; nutrition; health

INTRODUCTION

Edible insects are part of the traditional cuisine of many nations. It is possible to consume more than 2,000 species of edible insects **(Bednářová et al., 2013)**. Although nowadays insects are not a traditional food in Western culture, increasing public interest gradually raises its importance as a source of nutrition **(DeFoliart, 1992)**. Edible insects reproduce rapidly, they are easy to breed, and have a low environmental footprint. Therefore they could become an important and valuable source of nutrition not only in the developing countries **(Kinyuru et al., 2013)**, but also a rich source of various nutrients, such as iron, zinc, or calcium as a part of a special diet.

Calcium is one of the most problematic substances in human nutrition. Considering the fact that the milk and dairy products are often the main sources of calcium, their insufficient consumption is quite unfavourable from a nutritional point of view **(Pánek et al., 2002; Habánová, 2005)**. Calcium is the main mineral constituent of the human body. It is in bones and teeth (99%) as calcium phosphate. The total content of calcium in the body is about 1500 grams **(Velíšek, 2002)**. Calcium effects metabolism of phosphorus, magnesium, manganese, zinc and copper. It plays an important role in maintaining homeostasis. Furthermore, it is necessary for blood clotting **(Horniaková et al., 2010)**. Calcium absorption occurs in the small intestine and depends on the chemical form of calcium and composition of the diet. Phytic acid and fibre reduces resorption, while its resorption increases with higher dietary protein intake **(Velíšek, 2002)**.

Body's ability to regulate the calcium level is relatively low. Therefore the organism is unable to compensate its level in low-income or reduced resorption, which depends mainly on vitamin D and parathyroid hormone, age, gender, pH of small intestine, and furthermore on the intake of an antacid and phosphorus. Also the reabsorption in the kidneys, the penetration of calcium from plasma into the bone tissue and from bone tissue into the blood, secretion and absorption in the intestine and renal excretion is important **(Pánek et al., 2002)**.

When inability to absorb calcium from the intestine occurs from various reasons, it leads to softening and deformation of the bones. In children this disease, which occurs mainly in the first two years of life, is called rachitis. In adults the main illness caused by the absence of calcium is osteoporosis, characterized by bone thinning and skeletal pain. Bones lose strength and risk of fractures increases **(Pánek et al., 2002)**.

Lack of calcium in the diet may manifest especially in women during the postmenopausal period with osteoporosis and senile osteoporosis. This problem affects not only women but also men. As stated by **Abraham (1991)**, manifestation of osteoporosis depends on many factors that can be affected. One of them is good and balanced nutrition. Especially in childhood and adolescence it is necessary to ensure an adequate intake of calcium to cover the loss of calcium in urine and faeces. Calcium deficiency leads to an increased risk of osteoporosis and bone fractures.

Calcium intake in the diet therefore becomes one of the major factors that have a direct impact on the quality of bone tissue and its structure. Excessive calcium mobilization from bones or poor kidney function lead to increasing saturation Ca-oxalate and Ca-phosphate in the urine and leads to hypercalciuria. Hypercalciuria is the main cause of calcium oxalate urolithiasis, which affects up to 80% of patients with urinary stones **(Prié, et al., 2001)**.

Calcium intake has decreased during recent years. It is lower by 12 - 15%, but its deficit during the childhood and adolescence may be up to 40%. According to health recommendations the optimal daily dose is 800 mg of calcium per day for adults. Special recommendations apply for women during pregnancy and lactation, 1200 mg per day. Increased calcium intake is important also for older children and adolescents (800 - 1200 mg/day). The recommended daily dose for children under one year is 400 - 500 mg/day **(Velíšek, 2002)**.

Major source of calcium in the common diet are milk and dairy products **(Table 1)**. Lactose intolerance and allergy to milk and dairy products are one of the most common problems of reduced calcium intake in the diet. One of possible alternatives to substitute calcium in milk and dairy products is poppy or fishes, and soybean among legumes **(Velíšek, 2002)**. However, these foodstuffs can also be allergenic or limiting for some dietary reasons **(Bednářová et al., 2013)**.

Edible insects could be used to eliminate the lack of calcium for people with special dietary requirements as well as a non-traditional source of calcium. The calcium content of edible insects is largely influenced by the feed. Diets high in calcium can increase the calcium content in insect 5 - 20 times **(Finke, 2002)**. The benefit of calcium intake in the form of edible insects is its better utilization comparing to plants **(Theobald, 2005)**, where the calcium is bound as calcium oxalate, phosphate and phytate **(Pánek et al., 2002)**.

The consumption of insects does not have only the positive aspects, but brings also various risk factors. Besides nutritionally beneficial ingredients, insects may also contain toxic or antinutritional substances. As with other foods of this kind (e.g. shellfish) allergic reactions may appear. Allergies can be caused by allergens injectant (bees, wasps, and ants) **(Koterba and Greenberger, 2012)**, contactant allergens or inhalant allergens (e.g. cast skins, excreta). Therefore, caution is recommended in case of handling material from edible insects and in case of the first consumption of edible insects **(Rumpold and Schlüter, 2013)**. Cases of botulism, food poisoning and parasites **(Belluco, et al., 2013)** may occur. E.g. steroids, pederin or cyanogenic and cardiac glucosides, as a chemical defence mechanism against insectivores, may be naturally contained in the insect.

Therefore, some of the insects are not edible, just like other kinds in vegetable or animal area **(Rumpold and Schlüter, 2013)**. Consumption of these insects can lead to visual disturbance, nausea, vomiting, edema, jaundice, hepatic carcinoma etc. **(Berenbaum, 1993, Belluco, et al., 2013)**. Furthermore, wild insects may contain pesticides. Therefore, eating normally safe insect species that were harvested in the open air, it may not be safe. However, the controlled breeding of edible insects in a non-toxic environment eliminates these risks **(Rumpold and Schlüter, 2013)**.

Phosphorus level in human body is closely related to calcium in the calcium-phosphate metabolism. The ratio of phosphorus and calcium in human nutrition should be around 1:1.5. The daily requirement of phosphorus is about 1.0 to 1.2 g. The Recommended Dietary Allowance

Table 1 The calcium content in various type of food **(Velíšek 2002)**.

Type of food	min. [mg.kg^{-1}]	max. [mg.kg^{-1}]
Cheese	1500	12000
Fishes	60	5200
Soy	1300	1800
Egg yolk	1300	1400
Semi-skimmed cow's milk	1100	1300
Pea	1100	1300
Beans	300	1800
Spinach	700	1250
Lens	400	750
Breast milk	250	310
Cauliflower	180	310
Strawberries	180	260
Tomatoes	60	140
Chicken	60	130
Beef	30	150
Pork	50	90

Table 2 Dry matter (DM) and ash of samples used for calcium and phosphorus content analysis **(Bednářová et al., 2013)**.

Type of edible insect	Dry matter [%]	Ash [g/100 g DM]
Bombyx mori	28.22	6.55
Apis mellifera	17.33	5.16
Locusta migratoria	31.56	8.32
Gryllus assimillis	33.28	4.26
Tenebrio molitor	37.45	3.84
Zophobas morio	40.61	3.61

(RDA) for adults is about 700 mg/day **(Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997)**. Phosphorus is an important component of bones and teeth, which give them their strength and is important for metabolism. Phosphorus with nucleotides ensures the transformation of energy (ATP) and with sugar participates in glycolysis. Phosphorus in the human body and in the diet is found in the phosphates form. A good source of phosphorus is milk, dairy products, meat, fish, eggs and pulses. Phosphorus bound in phytate (for example in cereals) is very little available, therefore plant foods are the worse source of phosphorus **(Horniaková et al., 2010)**.

A phosphor deficiency can be in case of pregnant and lactating women (RDA up to 1800 mg/day), adolescents (RDA to 1250 mg/day) **(Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997)**, people with digestive disorders, people with anorexia nervosa, alcoholics, etc. The lack of phosphorus causes deterioration of reproduction, later sexual maturation in children and malfunction ovarian cycle in women. Women with phosphorus deficiency give birth to babies susceptible to disease **(Horniaková et al., 2010)**. In the case of excess of phosphorus (high consumption of cola beverages, meat products and processed cheese), calcium intake is impaired **(Pánek et al., 2002)**.

The topic of this article describes the analysis of the calcium and phosphorus content in selected species of edible insects (*Apis mellifera* - AM, *Bombyx mori* - BM, *Gryllus assimillis* - GA, *Locusta migratoria* - LM, *Tenebrio molitor* - TM, *Zophobas morio* - ZM) and its comparison with conventional foods and assessment of its presumed potential for human nutrition. The selected species are accepted by the public in the Central Europe as species usable in the food industry **(Bednářová et al., 2013)**.

MATERIAL AND METHODOLOGY

All insect species used in this experiment were purchased from institutions and companies in the Czech Republic. All these institutions and companies have a long experience in breeding insects. Bee brood was purchased from the company Přidal Brno. BM caterpillars were purchased from farms at the Masaryk University in Brno. Other species were purchased from company Frýželka Brno.

Preparation of insect samples for analysis

GA and LM nymphs and TM and ZM larvae were purchased alive, left starving for two days without food, and consequently stored in the freezer. AM larvae and pupae were removed from honeycombs with tweezers before the analysis. Bee brood was purchased frozen.

BM caterpillars were purchased alive. They were stored under laboratory conditions (temperature 22 °C, humidity 42%) in sealed plastic containers without food.

Individuals who created the pupa were removed from the containers and immediately frozen. For entomophagy these individuals are optimal, because they have the largest size. Pupae were stored in a freezer and later used for nutritional analysis. All samples for calcium and phosphorus content analysis were randomly chosen from storage boxes from the freezer. Basic nutritional properties of the samples (dry matter, ash) are shown in Table 2 **(Bednářová et al., 2013)**.

Calcium and phosphorus content analysis

The concentrations of calcium and phosphorus nutritional parameters were determined after dry-ashing of the sample at 550 °C in a furnace. About 0.5 g of the sample was taken for the analysis. The ash was dissolved in 10% HCl, filtered and made up to standard volume with deionised water **(Omotoso, 2006)**. The analysis was carried out using the atomic absorption spectrophotometer (AAS, model Varian Spectra AA 300) **(Adeduntan, 2005)**.

RESULTS AND DISCUSSION

The largest amount of calcium had BM (min. / max. 1006 / 1149 $mg.kg^{-1}$), which is comparable to semi-skimmed cow's milk and to pea among the vegetable commodities. The disadvantage of this species is its low dry matter content **(Bednářová et al., 2013)**. Comparison of the calcium content in tested species of insects and some species of edible insects, which has been found by other authors, is shown in Figure 1.

GA had the second largest calcium content (min. / max. 677 / 782 $mg.kg^{-1}$) comparable to spinach and lentils. Among the observed species, GA has the lowest content of fiber **(Bednářová et al., 2013)**, so it is possible to assume a better utilization of calcium **(Hronek, 2004)**. The lowest calcium content was detected in ZM (min. / max. 151 / 179 $mg.kg^{-1}$). All analysed species had higher calcium content comparing to commonly eaten meat. For example very popular chicken meat has an average calcium content 95 $mg.kg^{-1}$. AM (min. / max. 460 / 540 $mg.kg^{-1}$), TM (min. / max. 196 / 501 $mg.kg^{-1}$)

and LM (min. / max. 265 / 305 mg.kg^{-1}) have an average calcium levels comparable with strawberries and cauliflower **(Velíšek, 2002)**. Their value is approximately twice comparing to that of meat.

Analysis of the calcium content in other surveyed edible insect species reported similar concentration of calcium. For example **Hyun et al. (2012)** reported for grasshopper 844 mg.kg^{-1}, **Banjo et al. (2006)** found in *Analeptes*

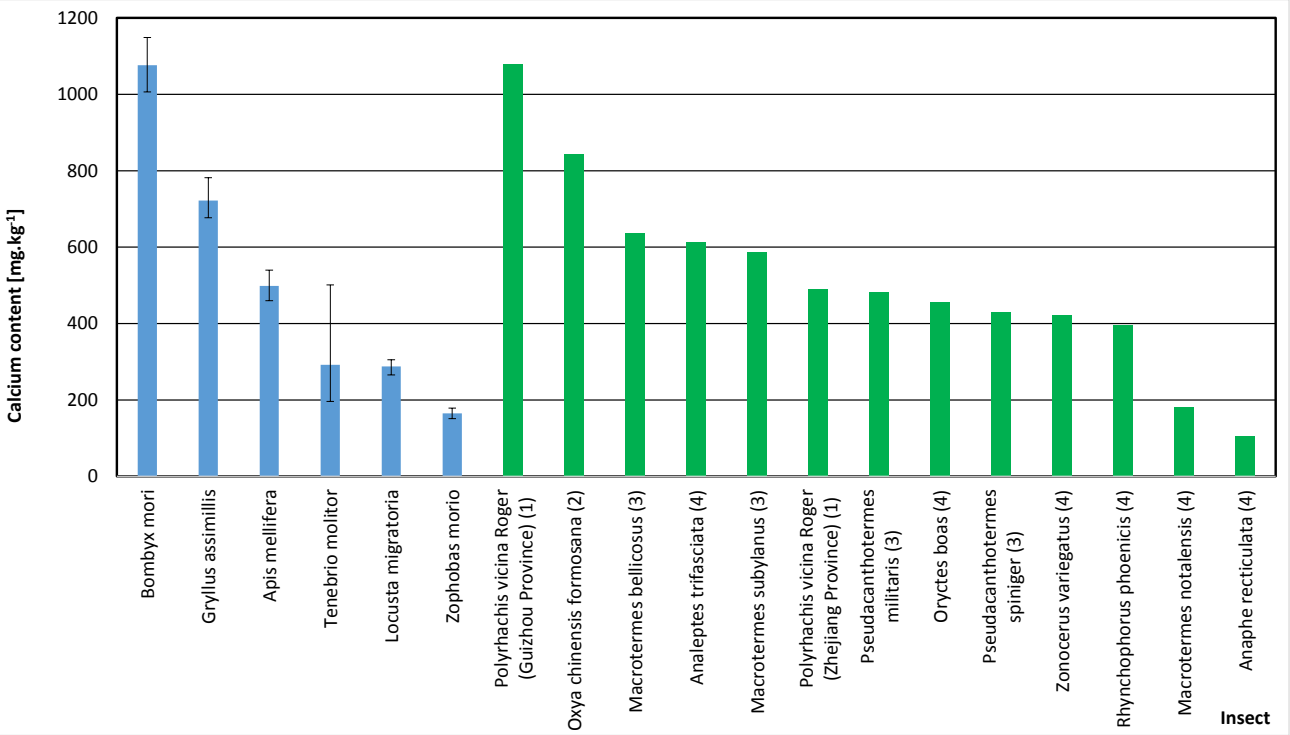

Figure 1 Comparison of the calcium content in tested species of insects (blue) and some species of edible insects, which has been found by other authors (green). Authors: (1) Bhulaidok et al., 2010; (2) Hyun et al., 2012; (3) Kinyuru et al., 2013; (4) Banjo et al., 2006.

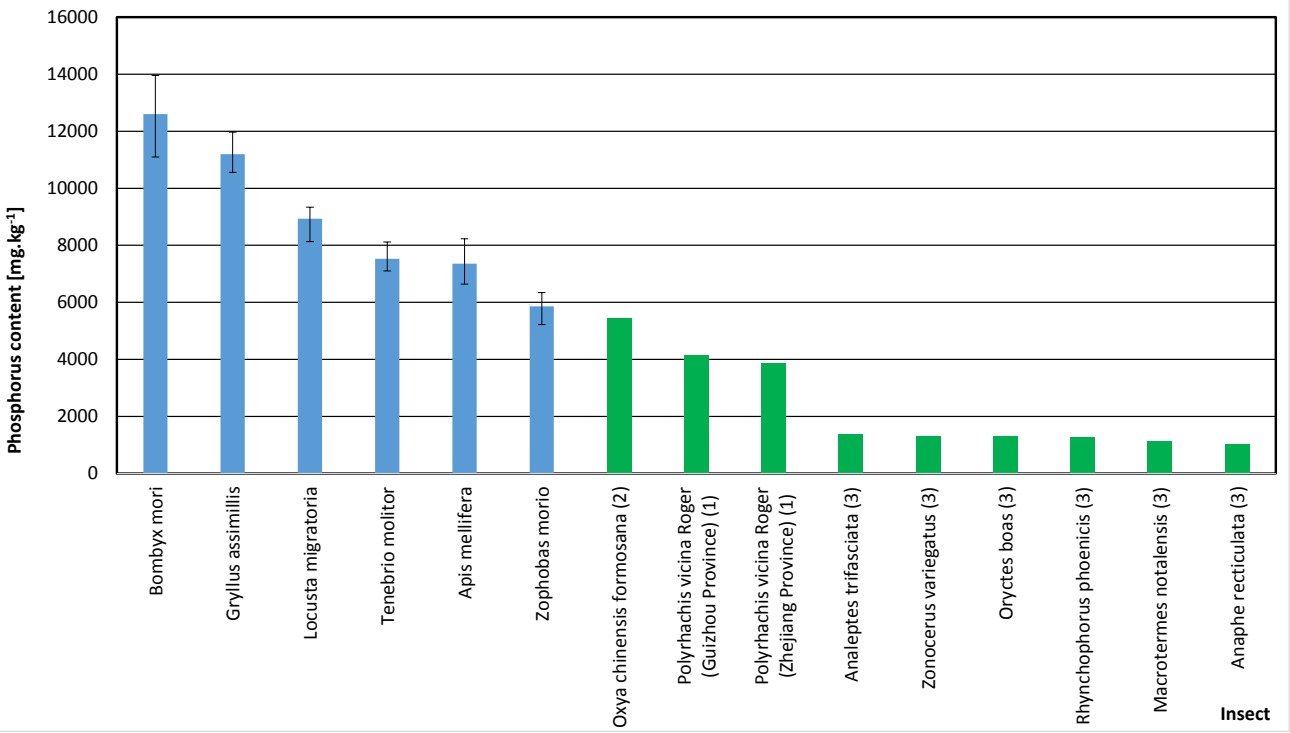

Figure 2 Comparison of the phosphorus content in tested species of insects (blue) and some species of edible insects, which has been found by other authors (green). Authors: (1) Bhulaidok et al., 2010; (2) Hyun et al., 2012; (3) Banjo et al., 2006.

trifasciata 613 mg.kg^{-1} and **Bhulaidok et al. (2010)** for edible black ants 1080 mg.kg^{-1}.

These results indicate that the selected species of edible insects could serve as an alternative source of calcium for people with lactose intolerance and for people with soy allergies.

Analysed insect species (BM, GA) rich in calcium are not suitable for patients with hypercalcemia. In this case the diet with a reduced content of calcium is recommended **(Kato et al., 2004)**. For these patients, it is possible to use bee brood as an alternative source of nutrition **(Finke, 2005)**.

Comparison of the phosphorus content in tested species of insects and some species of edible insects, which has been found by other authors, is shown in Figure 2. BM (min. / max. 11100 / 13960 mg.kg^{-1}) had again the largest amount of phosphorus among the samples. The second largest amount of phosphorus was detected in GA (min. / max. 10560 / 11970 mg.kg^{-1}). LM, TM and AM have a lower amount of phosphorus, which are comparable with cheese, soy beans and black tea **(Velíšek, 2002)**. The lowest phosphorus content was measured in samples of ZM (min. / max. 5220 / 6340 mg.kg^{-1}). Analysis of the phosphorus content in other surveyed edible insect species reported lower concentration of phosphorus. It is possible that this difference is caused by different environment of breeding and feed with varying amounts of phosphorus.

CONCLUSION

Due to the expanding interest of specialists and the general public about entomophagy, as well as increase of the EU interest in this type of food, there is a need to consider the use of edible insects as an alternative source of nutrition. From the perspective of edible insects as a source of calcium, edible insects could be considered as a possible source of calcium for enriching the diet and also as a substitute for people with lactose intolerance and allergies to other categories of foods rich in calcium. Of the six analysed species of edible insect, BM had the highest calcium content, almost comparable to semi-skimmed cow's milk. A rich source of calcium can also be GA, as well as other analysed species.

Phosphorus level in human body is closely related to calcium in the calcium-phosphate metabolism, therefore phosphorus level was detected in these samples too. BM and GA had again the largest content of phosphorus among the samples and the lowest content of phosphorus was measured in ZM samples.

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EFFECT OF SODIUM LACTATE / SODIUM DIACETATE IN COMBINATION WITH SODIUM NITRITE ON PHYSIOCHEMICAL, MICROBIAL PROPERTIES AND SENSORY EVALUATION OF COW SAUSAGE

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ABSTRACT

Sodium nitrite has been always considered as one of the common additives due to its antibacterial effects on *Clostridium botulinum* and meat products' color, however it produces cancer creating nitrosamine. Recently, organic acids and their salts such as lactates have been employed as antimicrobial compounds. Lactates also improve organileptic properties including color, texture and taste and antioxidant properties. Sodium lactate causes to more reduction of anaerobic spore former bacteria than nitrite, inhibits botulin produced by *Clostridium botulinum*. Sodium lactate produces a permanent reddish pink color through reduction of deoxymygloboline and producing deoxymyoglobuline. In this study, the decrease of sodium nitrite amount from 120ppm to 15ppm by adding sodium lactate / sodium diacetate led to achieve an acceptable product. The best results revealed through adding 3.0625% of sodium lactate / sodium diacetate in combination with 30ppm sodium nitrite. Results also exhibited more reduction of pathogens' growth than nitrite, enhanced flavor slightly, but unable to produce reddish pink color as produced by nitrite. Results also exhibited that sodium lactate / diacetate cause to retard in microbial growth, reducing chemical change, enhance sensory properties, partially improvement in taste and texture. Although inappropriate color demonstrated sodium lactate / diacetate's inability in red pink color production in 4th sample (contains 15 ppm nitrite), its synergy effect in combination with sodium nitrite on nitroso myoglobuline production has been proven, led to sodium nitrite reduction in sausages.

Keywords: Sodium lactate; sodium acetate; physiochemical properties; microbial properties; cow sausage

INTRODUCTION

Currently, demanding growth of fast food consuming has been increased due to their easy preparing. While most of microorganisms lives in low heated treatment, reproducing of microorganisms during storage lead to food spoilage and health risk. Nitrite and nitrate have positive effects on products' color and suitable antimicrobial effects on *Clostridium botulinum*, however they produce cancer creating nitrosamine Purchasing meat products base on color exhibits the important role of this factor as freshness and healthiness. Changing this factor can consider as a sign of the decrease of consumer acceptance and shelf life **(Bingöl and Boostan, 2007)**. Sodium lactate decreases M.O growth and pH, maintains taste in 7 - 10 days in comparison with control, inhibits *Clostridium botulinum* and *Listeria monocytogenes* growth as well **(Brewer et al., 1991)**. Adding of antimicrobial and antioxidant compounds like sodium lactate **(Choi and Chin, 2003)** to increase shelf life is one way to replace nitrite in meat products. Sodium lactate which is an associated acid penetrates across from microbial membrane, acidifies inner part of cell, but unable to produce red color like nitrite **(Choi and Chin, 2003)**. While lactate uses as an antimicrobial compound in meat products, there are a few studies on lactate's effect on color development. This study aims to investigate the affection on nitrose myoglobuline, minimizing sodium nitrite in cow sausage in order to increase shelf life, keeping sensory properties and products' color. The results come from measuring of bacterial growth in storing time, color assessment, oxidative rancidity and sensory evaluation **(Hunter and Segel, 1973)**.

MATERIAL AND METHODOLOGY

Production procedure

Frozen beefsteak minced into small pieces by Magurit guillotine, extruded by KramerGrebe extruder (model ww 160, 3 mm mesh), mixed by salt, sodium nitrite, sodium lactate / diacetate in Seydelmann cutter model K120 Ras 60090. This method causes to a better extraction and solution of myofibril protein which creates an emulsion like structure and sticky membrane around meat pieces. After adding sodium polyphosphate, half of ice and water added, then filler compounds after 30s paste cutting, finally the rest of ice added, paste cutting continued to produce a homogenous mixture. Table 1 shows the sodium actate / diacetate in 4 samples.

Cow sausage ingredients are including 12 kg of beefsteak meat with 10% fat, soybean oil 1.6 kg, non-skinned fresh garlic 0.8 kg, wheat flour 0.8 kg, ice 3 kg, gluten 1 kg, potato starch 0.2 kg, season 0.06 kg,

Table 1 Sodium lactate/diacetate and nitrite sodium in 4 produced sample.

Samples	**Sodim choloride (gr)**	**Sodium lactate / diacetate(%)**	**Sodium nitrite (ppm)**
Sample 1	0.3	3.0625	15
Sample 2	0.27	2.625	30
Sample 3	0.2475	1.75	60
Sample 4	0.239	0	120

0.08 kg of sodium poly phosphate 60%, ascorbic acid 0.02 kg, soybean protein isolate 0.2 kg.

And then paste carried to filler Vemag model Robot DP10C, filled in 5 layers poly amid casings produced by Mashahad Arta Co (diameter 2.7 cm, length 30 cm), cooked at 75 °C for 45 min in 6 wagons cooking room (produced by wimeg company in dimension 3 × 2 m, finally kept at 5 ±1 °C to evaluate their shelf life.

Total count

Plate count agar (Fluka) prepared, sterilized in autoclave at 121 °C for 15 min, put in water bath at 47 °C, then one ringer Merk tablet dissolved in 500 ml distilled water, autoclaved at 121 °C 15 min.

Samples extruded to small pieces by steel and sterilized extruder (in oven at 180 - 200 °C for 2 hr). 10 g of sample added to 90 mL ringer solution, shook and kept in station for 15 min, 9 mL of ringer sterilized solution poured in test tube (in oven at 180 - 200 °C for 2 hr), adding 1 mL of last dilution achieved 0.01 dilution. To achieve 10^{-5} follow this method again.

Samples carried to plates by sterile pipet after dilution, 12-15 ml of PCA added to plates, shook to produce a homogenous mixture, after setting put and kept in incubator upside-down at 30 ±1 °C for 72 ±3 hr. Consecutive plates contain 15-300 colonies counted and the colony amount calculated based on the following formula:

$$N = \frac{\sum C}{V(n_1 + 0/1n_2)d}$$

In which:

$\sum C$: Sum of selected colonies
V: Injected volume in each pipet
n_1: Counted plates in 1st dilution
n_2: Counted plates in 2nd dilution
d: Dilution coefficient base on 1st selected dilution

Color measurement

Cow sausage's color Measured using Hunter lab Color flex spectrophotometer model No45/0, s.ncx2547. The average of triplicate color measurements was recorded, and the results were expressed in terms of lightness (L*), redness (a*), and yellowness (b*). 3 Random sausage rolls selected in each replicate, cut their head and tail, filled in cell (D=6.35 cm), putting black cover on it, light radiated in 6 different and random angels, recorded the resultant N.Os.

Texture profile ynalysis

A texture analyzing TPA Lloyd model TA Plus MTM S/NO. 108059 Version 3/64 Issue 1 and software NEXYGENPlus Materials Testing and Data Analysis Software, version 2.1, copyright 2009, Ametek, inc. was used to conducted to texture profile analysis. Samples placed in ambient temperature 15 min at 25 mL, cut into pieces (diameter 2.7 cm and height 2 cm) placed under a cylindrical probe. Samples compressed by a 3 inches aluminum sheet twice to 50% of their original height (load cell and rate were 500 N and 1 mm/s respectively), factors including hardness, stickiness, springiness, brittleness, deformation force, rough ness and sticky force measured **(Yang et al., 2007)**.

Thiobarbitoric acid measurement

50 - 200 mg of sample weighed, poured in Volumetric flask 25 mL, dissolved by 1-butanol and reached to volume, 5mL of solution carried to test tube by pipet, indicator added, shook and placed in thermostatic bath at 95 °C for 2 hr, then cooled by cool water for 10 min to room temperature, its absorption recorded using spectrophotometer model shimadzoo UV 1700 in 530 nm wavelength, TBA calculates based on the following formula:

$$\text{TBA value} = \frac{50 \times (\text{A} - \text{B})}{\text{m}}$$

In which:

A: Absorption of experimented solution
B: Absorption of control solution
M: Mass at mg

And number 50 is correction factor when volumetric flask is 25 mL and cell width was 10 mm.

2.5 Cow sausage sensory evaluation

A six group of penalist described sensory evaluation including flavor, tatse, appearance, texture. Skilled panelist quantifying these factors, then quality factors determined based on a 15 cm accurate scale (Table 2).

Chemical properties analysis

Protein percent measurement

1 g of sample weighed on paper, poured on a 25 mL digestion flask, concentrated sulfuric acid added, 8 g catalyzer mixture including 96% potassium sulfate (7.68 g), 3.5% copper sulfate (0.28 g), 0.5% selenium oxide (0.04 g), snappy flask contains sodium hydroxide 30% placed on digestion flask, heated 15min not to foam while observing green color, experiment continued 1 hr. (According to Iran National Standard N. O. 924). Compounds in flask carried to distillation flask by 200-250 mL distilled water, some glass Perl added, placed under a condenser which contains 50 mL buric acid 4% and some droplets of bromochrosol green poured in it, then flask heated, 70 - 80 mL sodium hydroxide 5% poured by funnel, flask heated to collected 200 - 250 mL distilled solution containing ammoniac, titrated by chloridric acid and protein calculated by the following formula:

$$\%\text{P} = \frac{\text{V} \times 0/000114 \times 100 \times 6/25}{\text{m}}$$

Table 2 Evaluated factors of formulated cow sausages

Taste	Mouth fell	Basic flavor	Smell	Appearence	Traits
chemical sourness season meat cow taste unfavorable smell and taste	Strength frangibility juiciness brittleness	Sourness bitterness	Aroma meat rancidity	Color homogenous	Traits features

Table 3 Analysis of 4 cow sausage produced samples

samples	Features (%)			
	humid	fat	ash	protein
Sample 1	62.80	11.42	2.71	16.41
Sample 2	62.93	11.50	2.72	16.52
Sample 3	62.70	11.41	2.72	16.90
Sample 4	62.75	11.44	2.68	16.41

pH measurement

pH meter calibrated by buffers N.O 4 and 7. Sausages extruded twice, mixed and pH measured.

Ash measurement

Crucible heated in furnace at 550 ±25 °C for 20 min, cooled in desiccator and weighed, then 1 g of sample poured in it, heated on flame up to no smoke observed, heated in furnace at 550 ±25 °C for 4 - 6 hr, cooled in desiccator and weighed, ash calculates based on the following formulation (in accordance with national Iran standard N. O. 1028):

$$\text{Ash } \% = m2 - m0 / m1 - m0 \times 100$$

In which:

m2: Contain ash weight

m1: Weight of crucible contain sample

m0: Wight of empty crucible

Humidity measurement

Plate contain lab sand dried in oven at 103 ±2 °C for 60 - 90 min, cooled in desiccator and weighed. 5 g of sample carried to plate, mixed by sand and ethanol uniformly, dried at the same conditions cooled in desiccator and weighed (in accordance with national Iran standard N. O. 745).

Fat measurement

5 g of sample weighed, poured in flask, heated by 50 mL hydrochloric acid 50% for 30 min (digestion stage), cooled, filtered through filter paper and washed by distilled water up to no acid trace observed in out water, then filter paper dried in oven at 103 ±2 °C. Soxhelet flask also dried at the same condition for 1 hr, placed in desiccator, cooled and weighed.

Then filter paper rolled, placed in extraction cartouche, fat absorbed by hexane inoculated cotton to cartouche, then cartouche placed in extraction part and hexane added.

Soxhelt set up and heated by electric heater, this continued to 8 - 10 hr., cartouche removed and the most part of hexane removed, then separated and solvent vapored by water bath, put in oven for 90 min at 103 ±2 °C dried and cooled in desiccator and weighed.

Statistical Analysis

Statistical analysis performed based on complete randomized block design with factorial and Minitab 16th software. Average comparison performed by Duncan experiment and SPSS 20th software ($\alpha < 0.05$).

RESULTS AND DISCUSSION

Basic experiments

To insure that all produced samples are similar in chemical properties, 4 produced samples tested (2 times) in 1st week, with respect to no significant differences ($p < 0.05$) between samples, other experiments tested (Table 3).

Microbial experiment results

Total microbial count

Sodium lactate is a salt which acts as associated acid, passes microbial cell membrane, and acidifies inner part of cell **(Carpenter and Broadbent, 2009)**. Denaturation can lead to quick pH reduction which causes to cell death **(Lamkey et al., 1991)**. No significant differences observed in 1st week, sample 4th which contained 120 ppm nitrite displayed more microbial count reveals that adding sodium lactate / diacetate has been better proficiency in preventing microbial growth in other samples, the best result observed in sample 1 with respect to its slowest microbial growth between samples. No significant differences observed in 1st week, sample 4th which contained 120 ppm nitrite displayed more microbial count reveals that adding sodium lactate / diacetate has been better proficiency in preventing microbial growth in other samples, the best result observed in sample 1 with respect to its slowest microbial growth between samples (Table 4).

Table 4 Cow sausage total count (CFU/g) during 21 days storing time at 5 °C.

samples	Days			
	0	7	14	21
Sample 1	$2.5\times10^{2\ a,A}$	$1.5\times10^{2\ a,A}$	$9\times10^{2\ a,B}$	$9\times10^{2\ a,B}$
Sample 2	$2\times10^{2\ a,A}$	$7.5\times10^{2\ a,A}$	$2\times10^{3\ b,B}$	$2.5\times10^{3\ a,b,B}$
Sample 3	$3\times10^{2\ a,A}$	$7.5\times10^{2\ a,A,B}$	$2\times10^{3\ b,C}$	$3.5\times10^{3\ b,D}$
Sample 4	$1.5\times10^{2\ a,A}$	$4\times10^{3\ b,B}$	$4.5\times10^{3\ c,B}$	$6.5\times10^{3\ c,C}$

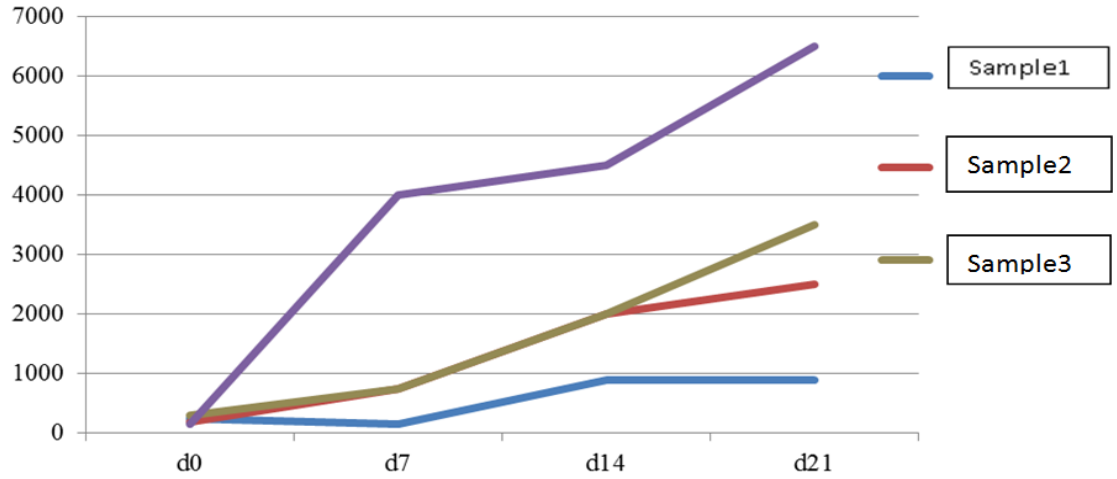

Figure 1 Cow sausage total count (CFU/g) during 21 days storing time at 5 °C.

Adding 3.0625% sodium lactate / diacetate in combination with 30 ppm nitrite to 2nd sample reduced microbial growth more than 4th sample and displayed better antimicrobial effects, no changes in sensory properties, improved quality, however its only defect related not to create red pink color which observed by increasing sodium lactate / sodium diacetate % and reducing sodium nitrite to 15 ppm in sample 1 (Figure 1). High level amounts of lactate can change pirovate reaction to lactate, prevents anaerobic growth, therefore adding sodium lactate causes to reduce microbial growth by aw reduction **(Meng and Genigeorgis, 1993)**. Number of *Clostridium botulinum* trace found, more reduction of anaerobic spore former bacteria number than nitrite.

Findings report that species including *Debaryomycess hunseni* and *Candida* separated from meat products **(Houtsma et al., 1993)** can tolerate sodium lactate, this is 10 times stronger in separated yeasts, while sodium lactate displays no tangible effect on yeast, postpones mold growth 30 days **(Bingöl and Bostan, 2007)**.

***Staphylococcus aureus* and *Clostridium perfringenes* count**

None of the samples displays these bacteria, no assumption can present about them, however in other researches inhibiting effects of sodium lactate / diacetate against former bacteria have been reported.

Chemical experiment results

pH measurement

Buffering capacity in meat seems to be effective on keeping low pH of meat, the same results in comparison of other research, no significant differences between 1 and 3% sodium lactate / diacetate (Table 5) in pH changing. **(Gonzalez-Fandos et al., 2009)**.

Sodium lactate / diacetate keep pH during storing time due to its buffer capacity.

Number of significant differences observed in 4 samples during 21 days which showed the pH stability during storing at 5 °C, the same result in comparison with **Brewer et al. (1991)** in which adding 2 - 3% sodium lactate in pig sausage caused to postpone pH reduction, besides fidnings about keeping pH by adding 3% sodium lactate during 30 days storing at 20 °C, additionally in another study keeping pH in 4 - 12 weeks storing in (Figure 2).

Oxidative rancidity measurement (TBA index)

Oxidative rancidity is known as the second most important factor in quality reduction in meat products. Rancidity occurs through unsaturated fatty acid, oxygen or other oxidizing compounds such as iron which is influenced by peroxidant compounds, heat and light **(Cheng et al., 2007)**. Lactates minimize fatty acids reduction that effects on color stability as fat and myoglobuline oxidation are dependent reactions lead to reduction of meat's color lifetime **(Mancini et al., 2009)**. Thiobarbituric acid showed no significant differences in the beginning of storing time (Table 6), however 3rd sample (containing 60 ppm sodium nitrite and 1.75% sodium lactate / diacetate) and 1st sample (containing 15 ppm sodium nitrite and 3.0625% sodium lactate / diacetate) displayed the most and the least thiobarbituric acid respectively. Results also showed that sodium lactate / diacetate has more prominenance than sodium nitrite (120 ppm) in 4th sample.

Table 5 pH results during 35 days of storing at 5 °C.

Samples	Days					
	0	7	14	21	28	35
Sample 1	5.64	5.65	5.65	5.63	5.65	5.64
Sample 2	5.65	5.66	5.66	5.65	5.63	5.61
Sample 3	5.72	5.74	5.78	5.78	5.77	5.76
Sample 4	5.75	5.92	5.88	5.93	5.90	5.87

Table 6 TBA amounts of sausages during 21 days of storing at 5 °C.

Samples	Days			
	0	7	14	21
Sample 1	$0.051^{a,B}$	$0.059^{b,C}$	$0.08^{c,D}$	$0.031^{b,A}$
Sample 2	$0.049^{a,A}$	$0.059^{c,B}$	$0.11^{d,D}$	$0.082^{d,C}$
Sample 3	$0.049^{a,D}$	$0.069^{c,C}$	$0.036^{b,B}$	$0.015^{a,A}$
Sample 4	$0.053^{a,B,C}$	$0.051^{a,B}$	$0.028^{a,A}$	$0.059^{c,C}$

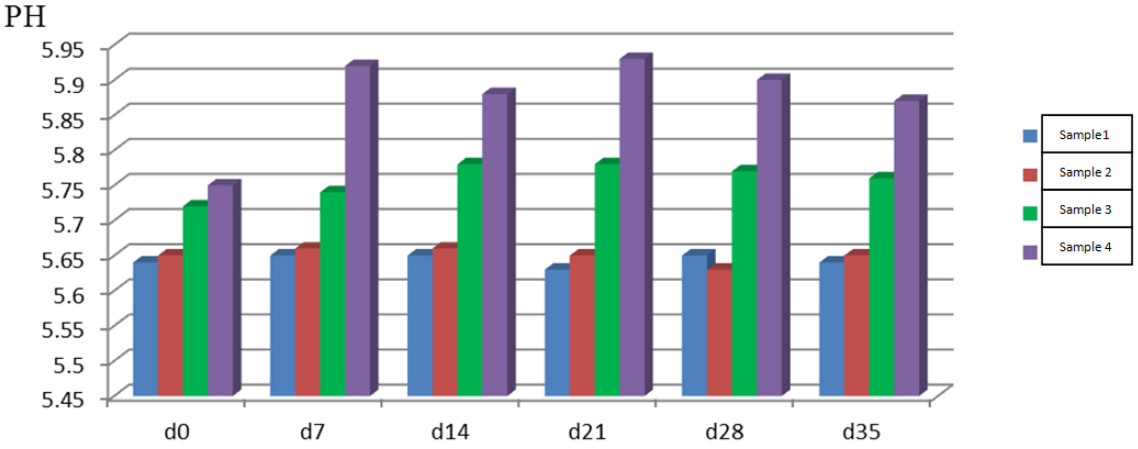

Figure 2 pH results during 35 days of storing at 5 °C.

Sensory evaluation

No significant differences observed between 2nd and 3rd sample which contained 3.02652% and 2.625% sodium lactate / diacetate respectively in comparison with 4th sample contained 120 ppm sodium nitrite thus the only decision made by panelists about quality reduction related to 1st sample's color.

Texture analysis

Increasing sodium lactate / diacetate reduced texture properties including springiness, hardness, roughness and stickiness in samples, however these factors were more in 4th sample which revealed the frangibility property by adding sodium lactate / diacetate in other samples, clearly showed in 1st and 1nd samples containing 2.625% and 3.0625% respectively. Changing in samples' tecture and color which contained 0.6% and 1.2% sodium lactate / diacetate observed after 45 and 60 days (Table 7) (in agrrement with **Bingöl and Bostan (2007)**).

Color measurement

Nitrosmyoglobuline which is the most important meat pigment transforms to a pink pigment called nitrosyl miocorgen. L factor varied during storage. While b factor increased in 4th sample, decreased in other samples, yet 2nd sample's "a index" was more than for in 1st and to the end of 21st storing day, but 1st sample (contains 15 ppm sodium nitrite, 3.6025% sodium lactate / diacetate) displayed the least "a index". These results were in agreement with **Brooke et al. (2011)** results (Table 8).

Adding lactate reduced rest nitrite (base on **Brooke et al., 2011** results), both experiments support this assumption that lactate produce NADH, which reduced metmyglobuline to deoxymyoglobuline or lactate myoglobuline reaction. More lactate also reduces myoglobuline, subsequently react nitrite produces more nitric oxide and reduces nitrite concentration **(Brooke et al, 2011)**.

Table 7 Sensory evaluation results of sausages during 21 days of storing at 5 °C.

Property	Sample	Days		Property	Sample	Days	
		0	21			0	21
Basic taste	Sample 1	$4.25^{a,A}$	$^{a,A}4.25$	Total quality	Sample 1		
Sourness	Sample 2	$3^{b,A}$	$^{b,A}3$		Sample 2		
	Sample 3	$^{b,A}3$	$^{b,A}2.75$		Sample 3		
	Sample 4	$2^{c,A}$	$^{c,A}2$		Sample 4		
Saltiness	Sample 1	$6^{a,A}$	$^{a,A}5.25$	Appearcane			
	Sample 2	$^{a,A}5.25$	$^{a,A}4.75$	Color homogenity	Sample 1		
	Sample 3	$4.5^{b,A}$	$4^{b,A}$		Sample 2		
	Sample 4	$4.5^{b,A}$	$^{b,A}4.5$		Sample 3		
Taste and smell	Sample 1	$13.75^{a,A}$	$^{a,B}12.5$		Sample 4		
Meat flavor	Sample 2	$13.25^{a,b,A}$	$^{a,b,B}11.5$	Mouth feel			
	Sample 3	$11.75^{b,A}$	$^{b,B}10.75$	Hardness	Sample 1	$8.25^{a,A}$	$6.75^{a,A}$
	Sample 4	$10.25^{c,A}$	$^{c,B}9.25$		Sample 2	$10^{b,A}$	$^{b,A}9.75$
Fat complex	Sample 1	$^{a,A}6.25$	$^{a,A}7.5$		Sample 3	$11.5^{c,A}$	$^{c,A}12.75$
	Sample 2	$^{a,A}4.75$	$^{a,A}5.5$		Sample 4	$13.75^{d,a}$	$^{d,a}13.5$
	Sample 3	$^{a,A}4.75$	$^{a,A}4.25$	Frangibility	Sample 1	$13^{a,A}$	$^{a,A}13$
	Sample 4	$^{a,A}3.5$	$^{a,A}3.25$		Sample 2	$11.75^{b,A}$	$^{b,A}10.5$
Meat Taste	Sample 1	$14^{a.A}$	$^{a.A}12.5$		Sample 3	$^{b,A}11.75$	$^{b,A}10.25$
	Sample 2	$^{a.A}13$	$^{a.A}11.5$		Sample 4	$9.5^{c,A}$	$^{c,A}7.25$
	Sample 3	$10.25^{b,A}$	$^{b,A}10.5$	Juiciness	Sample 1	$12.5^{a,A}$	$^{a,A}13.75$
	Sample 4	$^{b,A}9$	$^{b,A}9.75$		Sample 2	$11.75^{b,A}$	$^{b,A}10.75$
Brittleness	Sample 1	$8^{a,A}$	$^{a,A}6.75$		Sample 3	$^{b,A}10.5$	$^{b,A}9.5$
	Sample 2	$9^{b,A}$	$^{b,A}8.75$		Sample 4	$^{c,A}7.5$	$^{c,A}6.5$
	Sample 3	$10^{c,A}$	$^{c,A}10$				
	Sample 4	$12.75^{d,A}$	$^{d,A}13$				

a,b,c,d : Least squares means with different superscripts in the same row differ significantly ($p < 0.05$).
A,B,C,D : Least squares means with different superscripts in the same row differ significantly ($p < 0.05$).

Nitrite reaction rate influenced by meat properties, surrounded conditions which change processing conditions such as adding sodium erisorbate or sodium ascorbate and pH reducing products, dramatically increase nitrite reduction to nitric oxide **(Honikel, 2008)**.

It can assume that adding lactate increase reduction capacity of myoglobuline, more producing deoxymyoglobuline, therefore more rest nitrite reduction and more nitric oxide production predicts **(Brooke et al., 2011)**.

CONCLUSION

Results indicated that sodium lactate / diacetate cause to: retard in microbial growth, reducing chemical changes, enhance sensory properties and partially improve taste and texture. Although inappropriate color demonstrated sodium lactate / diacetate's inability in red pink color production in 4th sample (contains 15 ppm nitrite), its synergy effect in combination with sodium nitrite on nitroso myoglobuline production has been proven and led to sodium nitrite reduction in sausages. Using these cheap materials can consider as an evolution in meat industry, in other words more studies need to be done on suitable additives in order to develop products, because of more complication in this field related to animal genetics, chemical structure of muscles, pre and post mortem condition, manufacturing, packaging distribution, storing and etc.

With respect to the results of this study, future studies can focus on factors effecting meat properties in combination with sodium lactate / sodium diacetate, removing nitrite by replacing it with other compounds and other factors affecting nitrosomyoglobuline formation. Sodium lactate / diacetate postpones microbial growth depends on its concentration, cause no pH change during storing but effects on microbial growth. This study revealed that not only adding Sodium lactate / diacetate improve microbial quality depends on its concentration, but improve shelf life with better effects than sodium nitrite, however meat science must focus on development in the field of color improvement in relation with no sodium nitrite consuming. Future trends can answer to these questions.

Table 8 TPA results of sausages during 21 days of storing at 5 °C.

Index	sample	Days			
		0	7	14	21
Hardness	Sample 1	6.68 ^{a,A}	6.44 ^{a,A}	6.18 ^{a,A}	6.66 ^{a,A}
	Sample 2	6.78 ^{a,A}	6.36 ^{a,A}	6.31 ^{a,A}	6.43 ^{a,A}
	Sample 3	10.68 ^{b,A}	10.35 ^{a,A}	10.25 ^{a,A}	10.48 ^{a,A}
	Sample 4	11.39 ^{c,A}	11.39 ^{a,A}	11.51 ^{a,A}	11.22 ^{a,A}
SEM		0.113	0.113	0.113	0.113
Stickiness	Sample 1	0.149^{a,A}	0.153 ^{a,A}	0.149 ^{a,A}	0.155 ^{a,A}
	Sample 2	0.166 ^{b,A}	0.164 ^{b,A}	0.145 ^{b,A}	0.162 ^{b,A}
	Sample 3	0.213 ^{c,A}	0.213 ^{c,A}	0.206 ^{c,A}	0.216 ^{c,A}
	Sample 4	0.233^{d,A}	0.233 ^{d,A}	0.199 ^{d,A}	0.232 ^{d,A}
SEM		0.871	0.871	0.871	0.871
Springiness	Sample 1	5.80^{a,A}	5.53 ^{a,A}	5.61 ^{a,A}	5.76 ^{a,A}
	Sample 2	6.35 ^{a,A}	5.56 ^{a,A}	5.54 ^{a,A}	5.99 ^{a,A}
	Sample 3	7.17 ^{b,A}	7.04 ^{b,A}	7.22 ^{b,A}	7.36 ^{b,A}
	Sample 4	7.46^{b,A}	7.46 ^{b,A}	7.25 ^{b,A}	7.37 ^{b,A}
SEM		0.201	0.201	0.201	0.201
Brittleness	Sample 1	5.56 ^{a,A}	5.47 ^{a,A}	5.19 ^{a,B}	5.97 ^{a,A}
	Sample 2	6.27 ^{a,A}	5.20 ^{a,A}	5.10 ^{a,A}	6.27 ^{a,A}
	Sample 3	15.50^{b,A}	15.59 ^{b,A}	15.33 ^{b,A}	16.75 ^{b,A}
	Sample 4	19.82^{c,A}	19.82 ^{c,A}	19.61 ^{c,A}	19.20 ^{c,A}
SEM		0.542 ^{a}	0.542	0.542	0.542
Refract force	Sample 1	0.416 ^{a,A}	0.419 ^{a,A}	0.419 ^{a,A}	0.421 ^{a,A}
	Sample 2	0.417 ^{a,A}	0.413 ^{a,A}	0.419 ^{a,A}	0.417 ^{a,A}
	Sample 3	0.434 ^{a,A}	0.422 ^{a,A}	0.425 ^{a,A}	0.422 ^{a,A}
	Sample 4	0.419 ^{a,A}	0.419 ^{a,A}	0.422 ^{a,A}	0.417 ^{a,A}
SEM		0.838 ^{a}	0.838 ^{a,A}	0.838	0.838
Rigidity	Sample 1	1.21^{a,A}	1.12 ^{a,A}	1.14 ^{a,A}	1.18 ^{a,A}
	Sample 2	1.14 ^{a,A}	1.14 ^{a,A}	1.13 ^{a,A}	1.13 ^{a,A}
	Sample 3	1.73^{b,A}	1.66 ^{b,A}	1.74 ^{b,A}	1.73 ^{b,A}
	Sample 4	1.94 ^{b,A}	1.94 ^{b,A}	1.98 ^{b,A}	1.85 ^{b,A}
		0.745	0.745	0.745	0.745

a,b,c,d : Least squares means with different superscripts in the same row differ significantly ($p < 0.05$)
A,B,C,D : Least squares means with different superscripts in the same row differ significantly ($p < 0.05$)
ABBREVIATION :SEM Standard Error Mean

Table 9 TPA results of color sausages during 21 days of storing at 5 °C.

Index	Sample	Days			
		0	7	14	21
L	Sample1	54.5^{d,A}	^{d,B} 54.8	^{d,C} 55.4	^{d,D} 55.7
	Sample 2	53.4 ^{c,A}	^{c,B} 53.9	^{c,C} 54.1	^{c,D} 54.8
	Sample 3	52.7 ^{a,A}	^{a,B} 53.3	^{a,B} 53.6	^{a,B} 53.6
	Sample 4	^{b,A} 53.1	^{b,B} 53.6	^{b,C} 53.8	^{b,D} 54.2
SEM		0.0252	0.0252	0.0252	0.0252
	Sample1	^{a,D} 6.9	^{a,C} 6.7	^{a,B} 6.2	^{a,A} 6
	Sample 2	^{d,D} 12.3	^{d,C} 12.2	^{d,B} 12.1	^{d,A} 11.9
	Sample 3	^{b,A} 11.6	^{c,B} 11.8	^{c,C} 12	^{c,A} 11.6
	Sample 4	^{c,C} 11.8	^{b,A} 11	^{b,B} 11.1	^{b,B} 11.1
SEM		0.0711	0.0711	0.0711	0.0711
	Sample1	^{d,A} 12.2	^{d,B} 12.5	^{d,D} 12.8	^{c,C} 12.6
	Sample 2	^{b,C} 11.5	^{a,B} 11.4	^{a,C} 11.5	^{a,A} 11.3
	Sample 3	^{c,B} 12.1	^{b,A} 11.8	b,A 11.8	b,A 11.8
	Sample 4	^{a,A} 11.4	^{c,B} 12.2	^{c,C} 12.5	^{c,D} 12.6
SEM		0.0115	0.0115	0.0115	0.0115

a, b, c, d: Least squares means with different superscripts in the same row differ significantly ($p < 0.05$)
A, B, C, D: Least squares means with different superscripts in the same row differ significantly ($p < 0.05$)
ABBREVIATION: SEM Standard Error Mean

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CHEMICAL COMPOSITION OF BUCKWHEAT PLANT PARTS AND SELECTED BUCKWHEAT PRODUCTS

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ABSTRACT

Chemical composition plant parts (roots, stalks, leaves, blossoms) of common buckwheat (*Fagopyrum esculentum* Moench) and selected products made from its seeds (peels, whole seed, wholemeal flour, broken seeds, crunchy products Natural and Cocoa, flour, and pasta) was determined. Samples were dried and ground to a fine powder. All analyses were performed according to the Commission Regulation no. 152/2009, while rutin concentration was determined by the modified HPLC method. The lowest content of moisture was found in roots (4.3%) and in peels (almost 8%) and the highest moisture (nearly 11%) was discovered in seeds. The lowest amount of crude protein (3.5%) was found in peels, the highest crude protein amount (>13%) in both flours and leaves (23%). The starch content (>50% in dry matter) differs from one sample to another. Only in peels the content of starch was about 3.5%. From all examined samples, the lowest content of fat was found in crunchy products Cocoa, 1.7%. The lowest amount of histidine was determined in all studied samples, except peels, the highest content of glutamic acid was determined in almost all samples, except peels. Whole-meal flour is very rich source of Ca and Fe. The content of these elements was 1172 $mg.kg^{-1}$ and 45.9 $mg.kg^{-1}$, respectively. On the other hand, the highest content of Pb (>1 $mg.kg^{-1}$) was found in broken seeds. The greatest concentration of rutin was determined in blossoms and leaves (83.6 and 69.9 $mg.g^{-1}$), respectively. On the other hand, the lowest concentrations of rutin were found in buckwheat products (generally less then 1 $mg.g^{-1}$, i.e. in wholemeal flour, 702 $\mu g.kg^{-1}$, the lowest (almost 10 $\mu g.kg^{-1}$) in pasta.

Keywords: chemical composition; buckwheat products; *Fagopyrum esculentum* Moench; moisture

INTRODUCTION

Buckwheat is one of the traditional crops cultivated in Asia, Central and Eastern Europe **(Wijngaard and Arendt, 2006)**. Common buckwheat (*Fagopyrum esculentum* Moench) is the most commonly grown species. Buckwheat is categorized as a pseudocereal, so it shows both differences and similarities with cereals. It is an annual, dicotyledonic plant from the family of *Polygonaceae* **(Skrabanja et al., 2004)**. Buckwheat does not have a large root system, but its physiological activity is significant. Buckwheat roots excrete formic, acetic, citric and oxalic acids which help the plant to take nutrients, mainly phosphorus, from hard available forms. The stalks are hollow and their colour is green to red. Leaves alternate on the both sides of the stalk. Buckwheat inflorescence is formed by 7 to 9 blossoms. They are tiny of white, pink or red colour **(Janovská et al., 2009)**. Its seeds are edible and have triangular shape. The pericarp has a hard fibrous structure and surrounds the seed coat, endosperm and embryo tightly. The endosperm consists mainly of starch. The buckwheat fruit contains proteins, saccharides, lipids, fibre, vitamins and minerals as basic components. It is a source of dietary minerals like zinc, copper and manganese **(Ikeda and Yamashita, 1994)**. It is also rich in dietary fibre, which has a positive physiological effect in the gastrointestinal tract and also significantly influences the metabolism of other nutrients. Buckwheat seeds do not contain any gluten proteins so they are safe for people with celiac disease.

Buckwheat can act in the prevention and treatment of hypertension and hypercholesterolemia and colon cancer. The preventive effect can be connected with the content of dietary fibre in buckwheat **(He et al., 1995)**. Similar effects are associated with the inclusion of resistant starch in the diet. Buckwheat seeds contain an important amount of resistant starch **(Skrabanja et al., 2001; Kreft and Skarabanja, 2002)**.

Rutin is a secondary plant metabolite that antagonizes the increase of capillary fragility associated with hemorrhagic disease or hypertension in humans. It also decreases the permeability of the blood vessels and has an anti-oedema effect, reduces the risk of arteriosclerosis and has antioxidant activity. Rutin is a flavonol glycoside synthesized in higher plants as a protection against ultraviolet radiation and diseases **(Rozema et al., 2002)**. It was firstly detected in *Ruta graveolens*, which gave the common name to this pharmaceutically important substance. Among fruits, vegetables and grain crops, grapes and buckwheat are the most important rutin containing food **(Hagels, 1999)**. Most rutin is accumulated in the inflorescence, stalks and upper leaves **(Kreft et al., 2006)**.

This study has been carried out to determine the basic chemical composition of plant parts (roots, stalks, leaves,

blossoms) of common buckwheat (*Fagopyrum esculentum* Moench) cultivated in the Czech Republic and selected products made from its seeds (peels, whole seed, wholemeal flour, broken seeds, crunchy products Natural and Cocoa, flour, and pasta).

MATERIAL AND METHODOLOGY

Sample preparation

Harvested plants of common buckwheat (*Fagopyrum esculentum* Moench), cultivated in the region of Slezské Rudoltice, Czech Republic, were washed several times in tap water to remove all soil and finally in distilled water. Plants were divided into four parts (roots, stalks, leaves, blossoms) and dried at the ambient temperature. Buckwheat products (peels, whole seed, wholemeal flour, broken seeds, crunchy products Natural and Cocoa, flour, and pasta) were from Pohankový mlýn Šmajstrla s.r.o., Frenštát p. R., Czech Republic. All samples were ground to a fine powder and sieved through 1 mm mesh. All analyses were realized according to the Official Journal of the European Union **(EC, 2009)** at the laboratory temperature 21 ±2 °C in triplicate. All used reagents were of the analytical grade.

Basic chemical analyses

First of all, the moisture content was determined using drying at 103 ±2 °C to the constant loss of the weight. Ash content was determined by burning of sample in the muffle-furnace at 550 °C for 5 hours. The energy was determined in an automatic bomb calorimeter PARR 1281 (Parr Instrument Company, Moline, IL, USA). The fat content was determined gravimetrically by a Soxhlet method using n-hexan as an extractant. Crude protein content was determined according to the Kjeldahl method using the Pro-Nitro 1430 apparatus (BIO PRO, Prague, Czech Republic). Tashiro indicator (Fluka, Germany) was used for the final titration with hydrochloric acid solution. Results were recalculated to the sample weight and by multiplying it with the factor 5.7, thus, the percentage of crude protein was obtained. Samples for starch determination were treated with dilute hydrochloric acid (Penta, Chrudim, Czech Republic). After clarification and filtration the optical rotation of the solution was measured by polarimetry (OPTIKA Microscopes, Ponteranica, Italy). Total fibre in legumes and buckwheat products was determined using the apparatus Ancom220 Fibre Analyzer (ANCOM Technology, New York, NY, USA). For the analysis, filter bags F57 with pore size 50 μm were used.

Amino acids

An amino acid analyzer AAA 400 (INGOS, Prague, Czech Republic) with spectrophotometric post column derivatization with ninhydrine was used for total amino acids determination after their release from proteins and peptides by acid and oxidative hydrolysis **(Buňka et al., 2004; Lazárková et al., 2011)**.

Minerals

Samples (0.3 to 0.5 g) were decomposed in a microwave device Ethos SEL (Milestrone, Sorisole, Italy) using concentrated HNO_3 (5 ml conc. HNO_3 + 5 ml of deionised H_2O) at a temperature of 210 °C for 30 min. The final mineralizate was transferred into 25 ml volumetric flasks after cooling to 80 °C. The flasks were refilled to the mark after cooling to a room temperature. Mineralizates were analyzed on an atomic absorption spectrometer AA 30 (Varian A.G., Australia, see Table 1 for wavelengths).

Na, K, Ca, Mg, Fe, Zn and Cu were determined by flame AAS (acetylene-air). Strontium nitrate at a concentration of 1000 $mg.L^{-1}$ was used as a spectral buffer to suppress the flame emission in the case of Ca, Mg. Cu, Fe, Zn, Ca and Mg were measured in absorption mode while Na and K in emission mode. Pb, Cd and Cr were measured in absorption mode with electrothermial atomization in the graphite tube. For protection, the N_2 gas in a purity of 5.0 was chosen. A matrix modifier (10 $g.L^{-1}$ solution $NH_4H_2PO_4$ + 10 $g.L^{-1}$ solution of $Mg(NO_3)_2$ (Sigma Aldrich, USA) and a deuterium lamp background correction was used in the case of Pb and Cd. A 10 $g.L^{-1}$ solution of ascorbic acid (reduced formation of CrO_2Cl_2) was selected as a matrix modifier for Cr determination. Concentrations of all elements were determined by the calibration curve method and the integration of peak area.

Rutin concentration

Rutin was determined using a modified HPLC method according to **Deineka et al. (2004)** and **Gokarn et al. (2010)** using an HPLC 10 AVP system equipped with a SCL-10 AVP control unit with a control software Class-VP 5.02, two LC-10AVP pumps, a GT-154 degasser, a CTO-10ASVP column thermostat, a Rheodyne 7120 injector valve, and a SPD-M10AVP diode array detector (all from Shimadzu, Tokyo, Japan). Two grams of the sample (rutin hydrate was from Dr. Ehrenstorfer GmbH, Augsburk, Germany) were extracted with methanol:acetic acid:water (100:2:100). After sonication and shaking, test-tubes were centrifuged at 4000 rpm for 5 minutes and filtrated through 0.45 μm filter (Millipore, Bedford, MA, USA). The subsequent HPLC analysis was provided using Waters C18 column (75 mm x 4.6 mm, 5 μm pore size; all from Waters Corp., Milford, MA, USA). Used mobile phase consisted of 2 % acetic acid:acetonitrile:methanol (75:15:10), the flow rate was 1 ml per minute, and the detection was performed at 355 nm.

Statistical evaluation

All results were statistically evaluated using the variation statistics (ANOVA, StatSoft, Prague, Czech Republic) according to **Snedecor and Cochran (1967)** using the statistical package Unistat, v. 5.5 (Unistat Ltd, England).

Table 1 Wavelengths for particular elements [nm].

Element	Na	K	Ca	Mg	Zn	Cu	Fe	Pb	Cd	Cr
Wavelength	589.0	766.5	422.7	285.2	213.9	324.7	248.3	217.0	228.8	357.9

RESULTS AND DISCUSSION

Chemical composition of buckwheat plant parts (Table 2) and buckwheat products (Table 3) was studied. Firstly, moisture content of all samples was determined. It was subsequently recalculated to the dry matter content.

Basic chemical composition

The lowest content of moisture was found in roots (4.3%). As can be seen from Table 2, the highest crude protein amount was determined in leaves (22.7%) and in blosssoms (19.1%). Very low contents of starch were found in all parts, especially in blossoms they were undetectable. Contrarily, the highest content of fat was found in roots (4.3%). The greatest concentration of rutin was determined in blossoms and leaves (83.6 and 69.9 $mg.g^{-1}$, respectively).

Results for buckwheat products are presented in Table 3. The lowest content of moisture is in peels, almost 8%. On the other hand, the highest moisture was discovered in seeds, nearly 11%. The ash content ranges from 1 to 3%. The lowest content of fat was found in crunchy products Cocoa, 1.68%. The highest content of fat was determined in wholemeal flour, 7.49% in average. **Edwardson (1996)** reported the amount of fat in buckwheat seeds, dark and light flour as 3.2, 3.5 and 2.5%, respectively. These values are lower than those obtained in our experiment. The greatest crude protein content was determined in both flours, almost 14%, the decreased content of crude protein is in peels, about 3.5%. **Edwardson (1996)** presents the protein content in seeds as 16.8% and in light flour 11.7%. His value for seeds is higher than the one determined in our laboratory experiment, the second one, for the light flour, is lower than the value presented in Table 3.

The starch content (Table 3). In studied products its content is in the range of 50-70% in dry matter, except peels. In them, the starch amount was only about 3.62 %. Table 3 also shows the amount of fibre in particular samples. This component was detected only in peels and products which contain peels as whole seeds and wholemeal flour. Peels contain more than 65% of fibre. The energy values of examined samples range from 16 to 18%.

Amino acid composition

Amino acid composition of all analyzed buckwheat products is presented in Table 4. All products contain all amino acids. The lowest amount of histidine was determined in all studied samples, except peels, where the lowest content of methionin was found; the highest content of glutamic acid was determined in almost all samples, only in peels, the greatest content of glycine was ascertained. Also the arginine content is quite high.

Table 2 Average concentration of moisture, crude protein, starch, fat in % (mean ±S.D.) and rutin contents (mean ±S.D.) in mg.g-1.

Sample	Moisture	Crude protein	Starch	Fat	Rutin
Roots	4.3 ±0.02	5.6 ±0.16	0.0 ±0.01	4.3 ±0.01	3.6 ±0.12
Stalks	7.7 ±0.01	6.5 ±0.03	1.1 ±0.01	2.6 ±0.01	0.5 ±0.09
Leaves	7.5 ±0.02	22.7 ±0.26	6.0 ±0.01	3.1 ±0.01	69.9 ±2.7
Blossoms	6.5 ±0.02	19.1 ±0.10	n.a.	5.7 ±0.01	83.6 ±3.1

Table 3 Content of moisture, ash, fat, crude protein, starch, fibre in % (mean ±S.E.) and energy values (mean ±S.E.) in $MJ.kg^{-1}$.

Sample	Moisture	Ash	Fat	Crude protein	Starch	Fibre	Energy
Peels	7.98 ±0.03	1.57 ±0.05	4.61 ±0.00	3.48 ±0.23	3.62 ±0.18	65.9 ±1.17	18.2 ±0.18
Whole seed	10.4 ±0.03	2.00 ±0.01	7.34 ±0.01	10.2 ±0.23	53.3 ±0.36	14.8 ±1.04	16.9 ±0.03
Seeds	10.7 ±0.06	2.36 ±0.03	3.97 ±0.00	12.8 ±0.26	53.5 ±0.36	6.65 ±0.19	16.7 ±0.10
Broken seeds	9.11 ±0.04	1.59 ±0.01	5.95 ±0.02	9.16 ±0.02	69.0 ±0.35	ND	16.4 ±0.10
Crunchy Natural	7.53 ±0.02	0.82 ±0.00	2.44 ±0.00	6.77 ±0.33	77.8 ±0.18	ND	16.6 ±0.12
Crunchy Cocoa	6.53 ±0.03	0.98 ±0.01	1.68 ±0.01	6.50 ±0.17	72.9 ±0.18	ND	16.7 ±0.12
Flour	10.1 ±0.01	2.18 ±0.01	3.06 ±0.01	13.8 ±0.53	66.8 ±0.18	ND	16.8 ±0.08
Wholemeal flour	9.46 ±0.07	2.81 ±0.05	7.49 ±0.01	13.9 ±0.00	61.7 ±0.00	ND	17.3 ±0.05
Pasta	9.35 ±0.03	0.94 ±0.00	3.51 ±0.01	8.06 ±0.37	75.5 ±0.36	ND	16.4 ±0.11

S.E. - standard error; ND - fibre was not detected

Table 4 Amino acid content in buckwheat products (mean ±S.D.) in mg.g^{-1}DW.

AA	Peels		Whole seed		Seeds		Broken seeds		Crunchy Natural	
	AA content mg.g^{-1}	CV %	AA content mg.g^{-1}	CV %	AA content mg.g^{-1}	CV %	AA content mg.g^{-1}	CV %	AA content mg.g^{-1}	CV %
Lys	0.99 ±0.04	4.0	6.01 ±0.64	11.0	4.10 ±0.37	9.0	2.63 ±0.11	4.0	2.19 ±0.05	8.0
His	0.70 ±0.04	6.0	2.71 ±0.35	13.0	2.37 ±0.14	6.0	1.21 ±0.12	10.0	1.16 ±0.03	3.0
Arg	0.97 ±0.08	9.0	11.5 ±0.81	7.0	8.82 ±0.61	7.0	4.57 ±0.16	4.0	4.21 ±0.59	14.0
Glu	1.71 ±0.06	4.0	14.1 ±1.60	11.0	14.1 ±0.12	1.0	7.04 ±0.34	5.0	6.70 ±0.67	10.0
Asp	1.82 ±0.12	7.0	9.83 ±0.26	3.0	7.61 ±0.18	2.0	4.43 ±0.21	5.0.	4.21 ±0.43	10.0
Thr	0.92 ±0.08	9.0	3.27 ±0.17	5.0	3.39 ±0.18	5.0	1.85 ±0.06	3.0	1.75 ±0.14	8.0
Ser	0.95 ±0.08	9.0	3.89 ±0.43	11.0	3.90 ±0.03	1.0	2.12 ±0.04	2.0	2.02 ±0.18	9.0
Pro	0.99 ±0.07	7.0	4.02 ±0.42	10.0	3.07 ±0.25	8.0	2.05 ±0.11	6.0	1.97 ±0.10	5.0
Gly	1.90 ±0.13	7.0	6.35 ±0.15	2.0	4.58 ±0.24	5.0	2.94 ±0.13	4.0	2.83 ±0.32	11.0
Ala	1.02 ±0.08	8.0	4.36 ±0.48	11.0	3.62 ±0.27	7.0	2.31 ±0.07	3.0	2.18 ±0.18	8.0
Val	1.06 ±0.07	6.0	5.36 ±0.63	12.0	4.44 ±0.40	9.0	2.65 ±0.07	3.0	2.52 ±0.16	6.0
Ile	0.68 ±0.04	6.0	3.43 ±0.14	4.0	2.75 ±0.02	1.0	2.03 ±0.07	4.0	1.92 ±0.02	1.0
Leu	1.15 ±0.10	8.0	5.77 ±0.25	6.0	4.70 ±0.26	6.0	3.35 ±0.05	1.0	3.18 ±0.11	3.0
Met	0.57 ±0.01	3.0	2.93 ±0.16	6.0	1.90 ±0.17	9.0	1.90 ±0.08	3.0	1.44 ±0.17	12.0
Tyr	0.71 ±0.06	9.0	3.09 ±0.35	11.0	2.29 ±0.07	3.0	1.44 ±0.11	8.0	1.35 ±0.09	6.0
Phe	0.90 ±0.08	9.0	6.09 ±0.30	7.0	3.59 ±0.27	8.0	2.39 ±0.11	4.0	2.35 ±0.24	10.0
Cys	0.44 ±0.03	7.0	4.45 ±0.07	5.0	3.07 ±0.05	2.0	1.51 ±0.05	4.0	1.84 ±0.13	7.0

Table 4 (Continue) Amino acid content in buckwheat products (mean ±S.D.) in mg.g^{-1} DW.

AA	Crunchy Cocoa		Wholemeal flour		Pasta	
	AA content mg.g^{-1}	CV %	AA content mg.g^{-1}	CV %	AA content mg.g^{-1}	CV %
Lys	6.18 ±0.32	5.0	7.57 ±0.07	9.0	3.05 ±0.06	2.0
His	2.78 ±0.23	8.0	3.20 ±0.37	12.0	1.66 ±0.06	4.0
Arg	11.9 ±0.50	4.0	14.0 ±1.47	11.0	5.00 ±0.35	7.0
Glu	16.4 ±1.07	6.0	19.0 ±1.99	10.0	8.23 ±0.80	10.0
Asp	9.06 ±0.88	10.0	11.0 ±0.89	8.0	5.44 ±0.60	11.0
Thr	3.80 ±0.32	8.0	4.77 ±0.61	13.0	2.25 ±0.22	10.0
Ser	4.62 ±0.34	7.0	5.73 ±0.71	12.0	2.52 ±0.27	11.0
Pro	4.03 ±0.25	6.0	4.96 ±0.38	8.0	2.20 ±0.17	8.0
Gly	6.24 ±0.27	4.0	8.18 ±0.77	9.0	3.96 ±0.38	10.0
Ala	4.51 ±0.26	6.0	5.45 ±0.56	10.0	2.66 ±0.27	10.0
Val	5.45 ±0.38	7.0	6.48 ±0.65	10.0	3.08 ±0.32	10.0
Ile	4.11 ±0.50	12.0	4.52 ±0.30	7.0	1.95 ±0.18	9.0
Leu	6.71 ±0.59	9.0	7.33 ±0.71	10.0	3.43 ±0.31	9.0
Met	2.91 ±0.08	3.0	5.58 ±0.12	2.0	2.31 ±0.15	7.0
Tyr	3.01 ±0.03	1.0	4.06 ±0.37	9.0	1.81 ±0.20	11.0
Phe	4.94 ±0.35	7.0	6.09 ±030	5.0	2.70 ±0.22	8.0
Cys	4.32 ±0.03	1.0	4.45 ±0.07	2.0	2.42 ±0.19	8.0

S.D. - Standard deviation; CV - coefficient of variability; DW – dry weight

Table 5 Content of mineral elements (mean ±S.D.) in 1000 g DW.

Element	Concentrations of elements in 1000 g DW								
	Peels	Whole seed	Seeds	Broken seed	Crunchy natural	Crunchy cocoa	Flour	Wholemeal flour	Pasta
Pb[a]	428 ±4.27	510 ± 5.10	222 ±2.15	1049 ±10.5	194 ±1.94	422 ±4.22	412 ±4.12	831 ±8.31	384 ±3.84
Cd[a]	44.0 ±0.40	78.0 ±0.77	73.0 ±0.72	53.0 ±0.52	67.0 ±0.66	44.0 ±0.40	108 ±1.06	130 ±1.30	54.0 ±0.05
Cr[a]	64.0 ±0.64	49.0 ±0.49	69.0 ±0.69	477 ±4.77	109 ±1.09	111 ±1.10	144 ±1.42	149 ±1.49	113 ±1.13
Zn[b]	5.56 ±0.05	17.6 ±0.18	27.9 ±0.28	16.7 ±0.17	11.9 ±0.12	17.6 ±0.17	32.6 ±0.32	35.4 ±0.17	10.1 ±0.12
Cu[b]	4.71 ±0.05	7.32 ±0.07	6.73 ±0.07	5.03 ±0.05	4.78 ±0.05	5.01 ±0.05	7.82 ±0.08	11.6 ±0.11	5.71 ±0.01
Na[b]	8.56 ±0.09	6.80 ±0.07	5.56 ±0.06	1.62 ±0.02	10.9 ±0.11	15.9 ±0.05	2.11 ±0.02	5.29 ±0.05	5.87 ±0.06
Fe[b]	16.5 ±1.65	24.3 ±0.24	28.7 ±0.29	16.9 ±0.17	11.7 ±0.12	20.0 ±0.20	30.1 ±0.30	45.9 ±0.45	15.3 ±0.15
Ca[b]	999 ±4.99	533 ±2.66	148 ±0.74	113 ±0.56	246 ±1.23	87.9 ±0.87	266 ±1.33	1171 ±5.85	123 ±1.22
Mg[c]	1.08 ±0.05	1.71 ±0.01	2.20 ±0.01	1.44 ±0.01	0.87 ±0.00	0.94 ±0.01	2.16 ±0.01	2.38 ±0.01	0.99 ±0.05
K[c]	5.76 ±0.03	4.79 ±0.02	4.76 ±0.02	3.16 ±0.01	2.02 ±0.01	2.65 ±0.13	4.61 ±0.02	6.08 ±0.03	2.47 ±0.01

[a] $\mu g.kg^{-1}$; [b] $mg.kg^{-1}$; [c] $g.kg^{-1}$; ; DW – dry weight

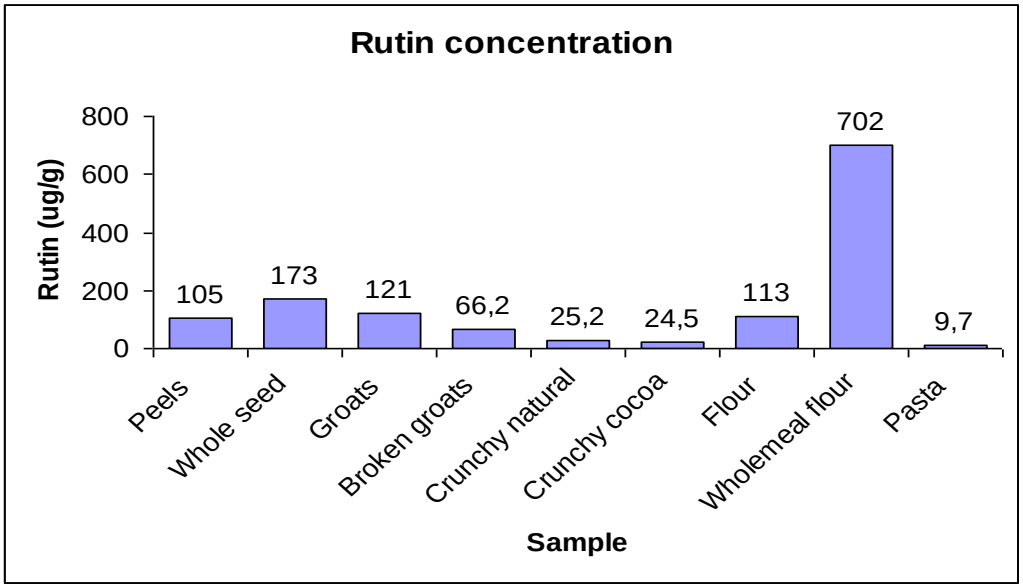

Figure 1 Concentration of rutin in $\mu g.g^{-1}$ DW.

Minerals

The mineral composition of examined buckwheat products is presented in Table 5. Wholemeal flour is very rich source of Ca and Fe. The content of these elements is 1172 $mg.kg^{-1}$and 45.9 $mg.kg^{-1}$of dry matter, respectively. Peels are also good source of Ca. The lowest content of Ca was determined in crunchy Cocoa, 87.90 $mg.kg^{-1}$, maybe because of the processing. On the other hand, the highest content of Pb was found in broken seeds, more than 1 $mg.kg^{-1}$.

Rutin concentration

From Figure 1, it can be concluded, that the greatest concentration of rutin in buckwheat products was determined in wholemeal flour, 702 $\mu g.g^{-1}$. On the other hand, the lowest concentration of rutin (nearly 10 $\mu g.g^{-1}$) was found in pasta. **Kreft et al. (2006)** in their study presented the value of rutin concentration in buckwheat seeds as 0.2 $mg.g^{-1}$ in dark flour. The value of rutin concentration in light flour is close to the value obtained in our experiment. Other values, when compared to our experiment, are a little bit different. In wholemeal flour, the concentration of rutin is more than three times higher than the value reported by **Kreft et al. (2006)**, probably due to the traditional mechanical milling.

CONCLUSION

Buckwheat is a plant from the family of Polygonaceae. It contains potassium, phosphorous, calcium, iron, zinc, vitamins B, E and rutin. Rutin is a bioflavonoid which is used for blood vessels treatment. Obtained results confirm that the highest concentration of rutin is accumulated in leaves and blossoms of the buckwheat plant. Usually, they are the main part of tea mixtures used for vessel diseases treatment.

On the other hand, lower concentration is in buckwheat products. Used buckwheat seeds, were mechanically

peeled. These seeds then have light colour and the preparation does not require any long-time cooking.

Buckwheat flour is processed by milling of seeds or by broken seeds and the value of dry matter/moisture is depending on the quality of storage. Total crude protein content is influenced genetically, by the area of growing, by weather and soil. Dry weather during buckwheat seeds´ creating causes premature ripening; a part of sugars stays in stalks and the amount of crude protein in seeds is increasing. Buckwheat for human nutrition is used in many forms. People use mainly seeds for making a meal.

Nowadays, people can buy a broad range of buckwheat products, such as pasta, crunchy products, flour etc.

It is difficult to compare obtained results with literature sources because not all products have been studied. All experiments could be influenced by many factors, e.g. the variety of the plant, different climatic conditions, processing of the seed, laboratory conditions, reagents, modification of the method, etc. Also the processing of seeds to buckwheat products can influence their chemical properties.

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ORGANIC AND CONVENTIONAL PRODUCTION IN SLOVAKIA: COMPARISON OF SELECTED MANAGERIAL AND FINANCIAL INDICATORS

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ABSTRACT

The paper deals with comparison of organic and conventional agriculture from two points of view. Firstly the managerial point of view examing number of employees and the personal costs per hectare. Secondly, the owners` point of view examing profitability of organic farming. Both views result from the specifics of organic farming. Organic production usually generates higher employment, higher personal cost and results in lower yields. We evaluated differences between organic and conventional agriculture in Slovakia over period of years 2009-2012 on a sample of more than 1050 farms in each year. The share of organic farms was 15%. Using t-test as evaluation method we found no significant differences from the managerial point of view (measured by employees per hectare and personal costs per hectare). From the owner` s point of view (measured by return on costs, return on equity and total assets per hectare) we conclude that the subsidies successfully compensate the lower revenues of organic farms (there was no significant difference in ROE and ROC). Our results are based on the sample of organic farms with Average utilised area of 850 hectares which reflects the farm structure in Slovakia. We can conclude that large organic farms do not support employment in rural areas and generate comparable profit when compared to conventional farms.

K**eywords**: organic farming; conventional farming; labour imput; ROE; ROC

INTRODUCTION

Organic Agriculture is a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved **(IFOAM, 2008)**. Organic agriculture is based on four fundamental principles (which also constitute the main organic research areas):

1. The Principle of Health - organic agriculture should sustain and enhance the health of soil, plant, animal, human and planet as one and indivisible.
2. The Principle of Ecology - organic agriculture should be based on living ecological systems and cycles, work with them, emulate them and help sustain them.
3. The Principle of Fairness - organic agriculture should build on relationships that ensure fairness with regard to the common environment and life opportunities.
4. The Principle of Care - organic agriculture should be managed in a precautionary and responsible manner to protect the health and well-being of current and future generations and the environment.

The definition and the fundamental principles are based on environmental and social aspects of an entrepreneurship. An increasing number of papers have recently started to explore the relevance of social and moral concerns when focusing on environmental sustainability and environmental compliance (**van den Bergh et al., 2000; Schwartz, 2002; Venkatachalam, 2008**). **Frey and Stutzer (2008)**, argue that individuals might contribute to a public environmental good because of an "intrinsic motivation to act according to one' s values". On the other hand, farmers (including organic farmers) are still entrepreneurs, which manage their business with the goal to generate profit. Despite the fact that such a pragmatic point of view is not outlined in official statements (for example FAO/WHO „Guidelines for the Production, Processing, Labeling and Marketing of Organically Produced Foods", U.S. National Organic Program Standards, Codex Alimentarius) it is actually becoming a point of interest for steadily increasing number of authors.

The increasing consumer demand for organic products caused that the organic food market has expanded in all continents of the world. Organic foods represent a specific segment of the food market **(Kozelová et al., 2013)**. Organic farming research is wodwide provided primarily by FiBL – Research Institute of Organic Agriculture. They are focusing mainly on the general characteristics of the system and its development in individual countries. **(Willer et al., 2010, 2013)**. Apart from the evaluation of various organic aspects, authors often examine the conversion process from conventional to organic farming **(Kerselaers et al., 2007; Acs, 2007; Stolze and Lampkin, 2009; Damgaard et al., 2014)** and the comparison of organic and conventional farming systems from different perspectives, such as soil, energy use, sustainability, social responsibility or risk **(Gündoğmuş, 2006**; **Berentsen et al., 2012; Ubrežiová et al., 2013; Arnhold et al., 2014; Patil et al., 2014)**. Comparison of

both systems is also reflected in the economic research. In this area, attention is drawn primarily to support of organic agriculture (in form of subsidies) **(Lesjak, 2008; Palšová et al., 2014)** and its impact on farm efficiency **(Breustedt et al., 2011; Argyropoulos et al., 2013)**.

In Central and Eastern Europe (CEE) countries economic marketing and safety issues of organic farming and food processing is examined by: **Wožniak, 2002; Wolcz and Pummer, 2004; Wachter et al., 2005; Jánský, 2005; Březinová, 2008; Moschitz and Stolze, 2010**; **Živělová et al., 2010; Bujna et al., 2013, Rozman et al., 2013**. Mentioned authors primarily focus on income and expenses of organic food production and organic farm economy. Their research results clearly shows that organic production generates increased costs (material and labour). On the other hand, this production system depends less on expensive technology and chemicals widely used in conventional farming.

While some authors **(**e.g., **Nieberg and Offermann, 2003)** argue that organic production allows relatively high price premiums, others argue that organic farming is not more profitable than conventional farming **(Klonsky and Greene, 2005)**. Thanks to a review of the literature on profitability of organic farming, **Greer et al. (2008)** report that the profitability of organic and conventional farms in the EU and the US has generally been found to be similar. Interestingly, **Acs et al. (2007)** report higher income for organic farming. However, after taking into account some factors likely to influence conversion, namely, extra depreciation costs, hired labour availability, organic market price uncertainty and minimum labor income requirement, organic farming may become less profitable than staying conventional **(Mzoughi, 2011)**.

Both conventional and organic agriculture are considerably subsidized. Theoretical studies suggest that subsidies may have a positive impact on farm production and at the same time a negative impact on farm productivity **(Hennessy, 1998; Ciaian and Swinnen, 2009)**. According **Rizov et al. (2013)** the impact of subsidies on productivity is a net effect of allocative efficiency losses and the investment-induced productivity gains caused by the interaction of market imperfections with the subsidy. As a response to the agrarian crisis, both national as well as state governments introduced measures like loan waivers, subsidiesand policies favoring sustainable agricultural practices including organic farming **(Patil et al., 2014)**. This support has common rules under „direct support schemes for farmers" in all EU member states as a part of Common Agriculture Policy (CAP). With a yearly budget of € 40 billion **(EC, 2014)**, direct payments form a significant part of the EU budget. Direct payments are payments granted directly to farmers under certain direct support scheme (Single Payment Scheme - SPS, Single Area Payment Scheme - SAPS, coupled schemes and/or specific support) listed in Annex 1 of Council Regulation (EC) No 73/2009. Payments under direct support scheme can be required by each farmer (who declare min. 1 ha of agriculture land) in Slovakia. This is financed by *European Agricultural Guidance and Guarantee Fund* (*EAGGF*). For organic farmers there are Agroenviromental payments financed by European Agricultural Fund for Rural Development (EAFRD). Mentioned supports are in Slovakia administred by Agricultural Payment Agency (APA) - Part Direct and Agroenvironmental Support, which is subordinated by the Ministry of Agriculture and Rural Development.

In Slovakia there are authors who examined the economy of organic agriculture **(Šimčák, 2005; Paška, 2006; Kozáková, 2011)**. Market of organic products and consumer's opinion in Slovakia are analysed by **Kozelová et al. (2010)**. From this analysis it can be concluded that even though consumers have some idea about bio - food and trust them more compared to other conventional food, they think that their market supply is not sufficient. This article builds on their results and compares organic and conventional agriculture from two points of view. Firstly, the managerial point of view examing the number of employees and personal costs, secondly the owners view examing his motivation to focus on organic agriculture as a business through selected financial indicators. Based on the literature and previous research we formulated following research hypotheses:

H1: Number of employees per hectare is higher in the case of organic producers when compared to conventional producers.

H2: Personal Costs per hectare are higher in the case of organic producers when compared to conventional producers.

H3: Total assets per hectare are lower in the case of organic producers when compared to conventional producers.

H4: Return on Costs per hectare is higher in the case of organic producers when compared to conventional producers.

H5: Return on Equity per hectare is higher in the case of organic producers when compared to conventional producers.

MATERIAL AND METHODOLOGY

For calculations we used the data from database of the Slovak Ministry of Agriculture and Rural Development (IL MoARD, 2013), over the period 2000-2012. The database consists of individual farm data, including balance sheets and income statements. Data submission is obligatory for all agricultural farms. For our analysis, data were selected according to the type of production to subset of the conventional producers (farmers) and the subset of the organic producers (farmers). We included only active organic producers (those enterprises generating sales from organic farming) into subset of organic producers. Only data for production cooperatives and companies (Ltd., JSC) were avialable. There were no data for family farms and soleholders.

We calculated following indicators for each farm. These indicators are commonly used to evaluate managerial and financial aspects of efficiency and profitability **(Rábek and Čierna, 2012; Klieštik and Valášková, 2013; Krechovská and Taušl Procházková, 2014)**:

$$PCH = \frac{personal\ costs\ (EUR)}{UAA\ (ha)} \quad (1)$$

$$ROC = \frac{net\ profit\ after\ taxes\ (EUR)}{total\ costs\ (EUR)} \times 100 \quad (2)$$

$$ROE = \frac{net\ profit\ after\ taxes\ (EUR)}{UAA\ (EUR)} \quad (3)$$

$$TAH = \frac{total\ assets\ (EUR)}{UAA\ (ha)} \quad (4)$$

We had to do data adjustment **(Klocoková, 2011**; **Munk et al., 2013)**. In order to assess the personal costs per hectare (PCH), return on costs (ROC), return on equity (ROE) and total assets per hectare (TAH), the data of the following farms were excluded from the dataset:

- farms with negative equity (liabilities exceeding total assets),
- farms with return on equity (ROE) exceeding +/- 100% (average profit or loss exceeds equity) over the observed period,
- another outliers (obvious mistakes in filling the financial statements).

After the necessary adjustment there remained 1150 farms in 2009 (1037 conventioanl farmers and 113 organic farmers), 1086 farms in 2010 (970 conventioanl farmers and 116 organic farmers), 1159 farms in 2011 (1021 conventioanl farmers and 138 organic farmers) and 1169 farms in 2012 (1029 conventioanl farmers and 140 organic farmers).

To evaluate the significance of differences in selected indicators over the period of years 2009-2012 (for each

Table 1 Employees per hectare (2009-2012).

Year	Category	Mean	Median	Percentile 25	Percentile 75
2009	conventional farmers	0.036	0.025	0.016	0.038
	organic farmers	0.030	0.023	0.014	0.032
2010	conventional farmers	0.035	0.023	0.014	0.035
	organic farmers	0.031	0.020	0.014	0.029
2011	conventional farmers	0.032	0.021	0.013	0.034
	organic farmers	0.031	0.021	0.013	0.028
2012	conventional farmers	0.033	0.021	0.013	0.032
	organic farmers	0.025	0.019	0.011	0.028

Source: own calculation based on data from the Information Letters of the MoARD SR (2013)

Table 2 Personal Costs per hectare (2009-2012).

Year	Category	Mean	Median	Percentile 25	Percentile 75
2009	conventional farmers	340.111	224.859	127.489	358.793
	organic farmers	247.975	191.600	101.500	290.926
2010	conventional farmers	340.266	221.489	124.954	352.782
	organic farmers	251.992	196.249	112.172	275.175
2011	conventional farmers	324.012	214.661	114.213	364.048
	organic farmers	285.931	192.299	109.673	290.458
2012	conventional farmers	319.993	212.566	119.911	365.924
	organic farmers	264.844	181.975	93.866	285.006

Source: own calculation based on data from the Information Letters of the MoARD SR (2013)

Table 3 Total assets per hectare (2009-2012)

Year	Category	Mean	Median	Percentile 25	Percentile 75
2009	conventional farmers	3011.854	1695.306	999.878	2620.712
	organic farmers	2550.479	1340.016	894.423	2501.909
2010	conventional farmers	2905.651	1671.518	1000.765	2597.653
	organic farmers	2521.328	1325.729	833.147	2142.107
2011	conventional farmers	2959.783	1686.976	1014.125	2586.478
	organic farmers	2970.407	1364.298	938.057	2913.860
2012	conventional farmers	3030.367	1698.094	1029.533	2654.888
	organic farmers	2879.030	1331.135	974.626	2426.586

Source: own calculation based on data from the Information Letters of the MoARD SR (2013)

year separatedly) we used t-test for independent samples and the statistical software IBM SPSS v.20 for calculation.

RESULTS AND DISCUSSION

As a first indicator we evaluated the number of employees per hectare separately for each year and each farming system. The results are summarized in table 1. We found out that in each year the average number of employees per hectare is higher in the case of conventional farmer when compared to the organic farmers (0.036 to 0.030 in 2009; 0.035 to 0.031 in 2010; 0.032 to 0.031 in 2011 and 0.033 to 0.025 in 2012). The differences in median are even lower (Table 1). This finding is further confirmed by the 25 and 75 percentiles.

We evaluated the existence of statistically significant differences using t-test for independent samples. As the descriptive statistics indicated there is no significant difference in the number of employees per hectare in organic and conventional production (Table 5). We conclude the hypothesis H1 was not confirmed.

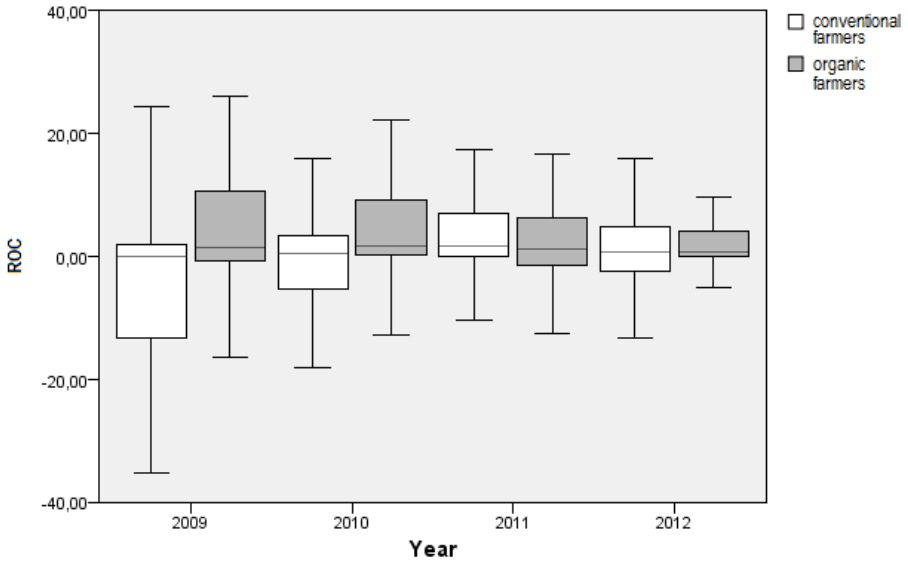

Figure 1 Return on Costs – Boxplot (2009-2012).
Source: own calculation based on data from the Information Letters of the MoARD SR (2013)

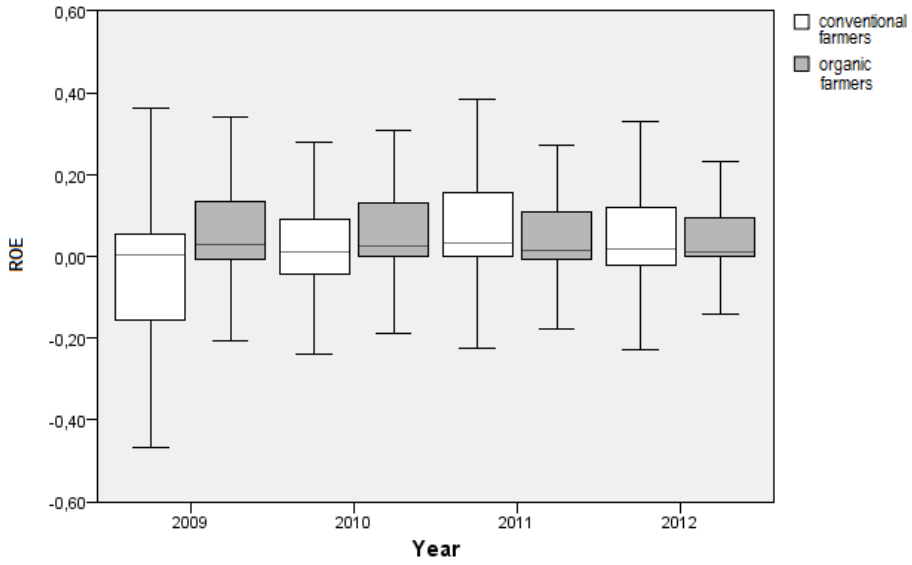

Figure 2 Return on Equity – Boxplot (2009-2012).
Source: own calculation based on data from the Information Letters of the MoARD SR (2013)

Table 5 Table of Contrasts (2009-2012) – Results of t-test.

Year	EH	PCH	NPH	ROC	ROE	TAH
2009	no	yes	yes	yes	yes	no
2010	no	no	yes	yes	yes	no
2011	no	no	no	no	no	no
2012	no	no	no	no	no	no

Source: own calculation based on data from the Information Letters of the MoARD SR (2013)
no - there is no statistically significant difference in evaluated indicator between the conventioanal and organic farming,
yes - there is statistically significant difference in evaluated indicator between the conventioanal and organic farming,

Obtained result directly contradicts the theory that the organic farming system is characterized by higher amount of manual work which leads to the higher number of employees. One of the possible explanations is the average acreage of the Slovak farms. The majority of the land is utilized by farms with the acreage over 500 hectares **(Rábek et al., 2014)**. In this aspect the Slovak Republic is unique when compared to other European Union member states. To be able to utilize such acreages the farm needs to rely heavily on the technology and not on the manual work.

As a next indicator we analysed personal costs per hectare. Theory says **(Offerman and Nieberg, 2000)** that personal costs in organic farming are higher than in conventional system because of higher requirements for manual work. However, theories differ from praxis in the Slovak Republic. Average personal costs per hectare are lower in organic farming than costs in conventional system (Table 2) in every year of the observed period (the difference changes from approximately 93 EUR in 2009 to 55 EUR in 2012). Based on the obtained results we conclude the hypothesis H2 was not confirmed (Table 5). Lower personal costs in organic farming are connected with lower number of employees per hectare in organic farming (Table 1). In 2009 personal costs in organic farming represented 73% of personal costs in conventional system. In 2012 this share was 83 %.

The third evaluated indicator were total assets per hectare. In respect to theory **(Paška, 2009)** the organic farmers need lower assets when compared to conventional farmers (in form of equipment and machines) because the higher share of the manual work in case of organic farmers. In three years of four observed years (2009, 2010, 2012) the total assets per hectare were indeed lower in farms with organic farming system (Table 3). Total assets per hectare (mean value) of organic farmers were 2550 EUR (conventional farmers 3011 EUR) in 2009; 2521 EUR (conventional farmers 2905 EUR) in 2010; 2879 EUR (conventional farmers 3030 EUR) in 2012. In 2011 the total assets per hectare of organic farmers were 2970 EUR while the total assets per hectare of conventional farmers were 2959 EUR. According to results of t-test these differences were not statistically significant (Table 5). We conclude the hypothesis H3 was not confirmed.

Return on costs (Figure 1) and return on equity (Figure 2) are without doubt important factors from the financial and economic point of view. There are statistically significant difference between organic and conventional farmers in 2009 and 2010 in both indicators (Table 5). The organic farmers were more profitable in comparison to the conventional farmers (they generated higher return on equity and higher return on costs). However, in 2011 and 2012 there are no significant differences. Based on these results hypotheses H4 and H5 were not confirmed.

CONCLUSION

Organic farming and organic food products are nowadays preffered by individuals and society. Also public funds in form of subsidies are supporting this farming system. Higher subsidies are underlining the benefits of organic farming for the whole society. According to the theory this type of farming results in higher number of employees per hectare, lower caital needs and higher costs (material and labour). In the paper we focused on these specifics in Slovak farms. The structure of farms in Slovakia is different in comparison to other EU member states mainly in Utilised agricultural area (UAA) per farm. Farms with more than 500 hectares manage almost 80 % of the total UAA.

Our aim was to measure the differences in the specifics of organic farming on a sample of more than 1050 large farms in Slovakia. Based on the results we can conclude there are no statistically significant differences between conventional and organic farms over the whole observed period. The fact that organic farms have higher number of employees was confirmed in none of the years observed. Also higher personal costs of organic farms could not be confirmed. Organic farming in Slovakia does not generate increased labour input. The financial benefit for the owner was evaluated by ROC and ROE. The statisticaly significant differences between organic and conventional farming were confirmed only in two out of four years. According to the theory organic farms are less profitable and therefore the production should be more subsidized when compared to conventional farms. In respect to our results we conclude that organic farms in our sample generate results comparable with conventional farms in sense of profitability. Higher subsidies of organic farms successfully compensate lower revenues and therfore the motivation for the owner to focus on organic farming is not lower than to focus on conventional farming. In some years of the observed period organic farms were even more profitable than conventional ones.

Further research should be focused on the differences between organic farms with respect to UAA. The average UAA of organic farms in our sample was 850 hectares. The absence of above described general specifics in Slovak organic farms included in our sample might be influenced by this fact.

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LUTEIN IN FOOD SUPPLEMENTS AVAILABLE ON THE MARKETS OF THE VISZEGRAD COUNTRIES

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ABSTRACT

RP-HPLC method with UV-VIS detection was implemented for determination of contents of lutein in food supplements available on the markets in the Czech Republic, Slovakia, Poland, and Hungary. Altogether, 48 samples of food supplements in three dosage forms (22 samples of tablets, 18 samples of soft capsules, and 8 samples of hard capsules) were analysed. The amounts of lutein specified by the producer complied with their real contents only in 7 samples of the food supplements. Lutein in soft capsules showed the highest stability against oxidation; lutein in tablets was more prone to oxidation and lutein in hard capsules was most susceptible to oxidation process. Out of 21 Czech products, only four fell into the category of satisfactory products, three of them were soft capsules and one was a tablet. Out of 27 products manufactured abroad, only three were evaluated as satisfactory products, all of them were soft capsules, out of 48 analysed food supplement samples just seven fell into the category of satisfactory preparations, eight were evaluated as less satisfactory preparations, five were found inadequate products and 28 samples were labelled unsatisfactory. Only one in six analyzed samples contained the amount of lutein specified by the manufacturer, almost 60% of monitored lutein containing food supplement samples fell into the unsatisfactory product category.

Keywords: Carotenoids; RP-HPLC; marigold flower; tablets; capsules

INTRODUCTION

Lutein (systematically named (3R,3´R,6´R)-β, ε-carotene-3,3´-diol or 3,3´-dihydroxy-α-carotene, see Figure 1), is a yellow plant pigment that belongs to the carotenoid family, namely to xanthophylls. It occurs in many kinds of fruits and vegetables, especially in leafy vegetables. It is also found in yolk and eye tissues **(Calvo, 2005; Čopíková et al. 2005)**. Pure lutein is a red-orange crystallic substance soluble in fats and organic solvents, but insoluble in water.

Lutein (luteus means yellow in Latin) acts as an effective antioxidant. It is also able to stop degenerative changes of macula lutea resulting in blindness. Lutein plays an important role in perception phenomenon called Heidinger´s brush that enables human's determination of plane or direction of polarized light rotation.

Moreover, lutein protects the organism against heart diseases and cancer. It is soluble in fats, therefore it is transported by a form of cholesterol, namely by low density (LDL) lipoproteins. Lutein protects vitamin E against oxidation and, furthermore, it most likely improves function of immune system **(Calvo, 2005)**. It also protects both eyes and skin against strong sun radiation and against effects of air pollutants and products of smoking.

Moreover, lutein prevents fat peroxidation that widely occurs both in blood serum and eyes. High doses of lutein decrease risk of cervical cancer **(Calvo, 2005; Evans and Johnson, 2010)**.

All over the world, interest in healthy life-style has been increasing recently and the conceptions of disease prevention have been adopted intensively. Human organism does not show ability to synthesise lutein, which is why humans can acquire it solely by consumption of fruits, vegetables and food supplements **(Calvo, 2005)**. Due to relatively low biological utilizability of lutein contained in natural resources, consumption of lutein enriched food or intake of food supplements are suggested **(Calvo, 2005)**. Recommended daily dose is 6 - 10 mg of lutein. Some researchers suggest even higher daily intake up to 20 mg of lutein **(Garti et al., 2003; Abdel-All et al., 2007; Bernstein et al., 2010; Cerón-García et al. 2010; Evans a Johnson, 2010; Li et al., 2011)**.

Lutein containing food supplements have become integral part of our common everyday nourishment. They

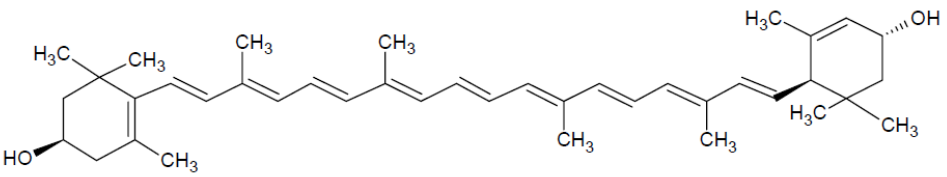

Figure 1 Structural formula of lutein.

are widely available in Czech pharmacies, supermarkets or in E-shops. Many of lutein containing products available on the market declares high contents of lutein, which calls their real lutein levels, in question. Such products are sold at relatively low prices. Lutein content is usually determined by common spectrophotometric methods that are not specific, though, to find lutein content using HPLC, a chromatographic column with C18, or C30 sorbents is commonly applied; it is also suitable for separation of individual trans- and cis- isomers of lutein. To analyse substances with identical molecular mass such as lutein and zeaxanthin, cyanopropyl column with silica sorbent containing -$(CH_2)_3$-CN end group is recommended.

Our study had three goals: i) determination of lutein contents in products available on Czech, Polish, Hungarian and Slovak markets; ii) comparison of lutein quality in various dosage forms like tablets and soft or hard capsules; iii) comparison of quality of lutein containing food supplements manufactured by various producers.

MATERIAL AND METHODOLOGY

Sample selection

Selected food supplements available on Czech, Slovak, Polish and Hungarian markets were subjected to study. Contents of lutein ranging between 0.25 to 25 mg in one tablet or capsule were declared in food supplements manufactured by the producers from the Czech Republic, Denmark, Finland, Canada, Hungary, Germany, Poland, Austria, Slovakia, Sweden, Switzerland, and USA. We monitored samples of food supplements produced in the form of tablets, soft capsules or hard capsules. Their characteristics are listed in Table 1. All the food supplement samples were analysed before expiration date stated by their producers.

Lutein standard

Standard solution of lutein was prepared by dissolution of accurate amount (0.50 ±0.01 mg; Extrasynthese, France) of lutein standard in acetone-methanol solvent (50 ml; 1:1 v/v). Calibration curve was plotted based on signals of various volumes of standard lutein solution injected into an HPLC column: 1, 2, 3, 5, 8, and 10 μL. Calibration was always implemented on the day of analysis.

HPLC determination of lutein content

Contents of food supplement samples in the form of a tablet or a capsule with average mass of 1 g was dissolved in 50 ml of acetone-methanol solvent (1:1 v/v). For 10 minutes, the sample was treated in an ultrasound

Table 1 Dietary supplements: lutein content (mg per tablet or mg per capsule) and their percent rate found in tablets (D1-D22), soft capsules (D23-D40) and hard capsules (D41-D48).

Sample	Product expiration	Lutein content		%	Sample	Product expiration	Lutein content		%
		Declared	Determined mean ±S.D.				Declared	Determined mean ±S.D	
D1	II/2013	0.25	0.01 ±0.00	4.0	D25	V/2013	10.0	12.5 ±0.09	125
D2	IX/2013	2.0	3.07 ±0.03	15.4	D26	VI/2012	10.0	9.45 ±0.05	94.5
D3	IX/2012	3.8	0.02 ±0.00	5.3	D27	III/2013	12.0	11.4 ±0.12	95.0
D4	I/2013	5.0	0.08 ±0.01	1.5	D28	VIII/2012	12.0	6.13 ±0.07	51.1
D5	III/2013	5.0	0.04 ±0.00	0.8	D29	VII/2013	12.0	10.2 ±0.11	85.0
D6	V/2013	5.5	0.08 ±0.00	1.5	D30	III/2013	15.0	13.4 ±0.13	89.3
D7	II/2013	15.0	0.04 ±0.00	0.3	D31	V/2014	15.0	14.8 ±0.14	98.7
D8	III/2013	3.0	0.09 ±0.00	3.0	D32	VI/2012	15.0	5.66 ±0.20	3.7
D9	IV/2014	3.0	2.44 ±0.07	81.3	D33	IX/2012	6.0	0.10 ±0.01	2.2
D10	VIII/2012	6.0	5.71 ±0.09	95.2	D34	IX/2013	4.0	6.52 ±0.09	161
D11	II/2013	6.0	0.02 ±0.00	0.3	D35	I/2014	4.0	7.64 ±0.11	190
D12	V/2013	10.0	0.02 ±0.00	0.2	D36	IX/2012	8.0	4.52 ±0.09	56.8
D3	III/2013	12.0	1.19 ±0.00	9.9	D37	V/2014	6.0	2.33 ±0.07	38.2
D14	IX/2012	0.3	0.01 ±0.00	3.3	D38	II/2013	10.0	1.22 ±0.04	12.2
D15	III/2013	6.0	0.05 ±0.01	0.8	D39	IX/2012	10.0	0.99 ±0.06	9.9
D16	VI/2013	6.0	0.04 ±0.00	0.7	D40	II/2014	20.0	23.3 ±0.18	116
D17	VIII/2013	12.0	10.0 ±0.11	83.3	D41	III/2014	3.0	0.02 ±0.00	0.7
D18	IV/2013	0.5	0.12 ±0.01	24.0	D42	V/2013	6.0	0.05 ±0.00	0.8
D19	II/2013	1.0	0.16 ±0.01	16.0	D43	IV/2014	6.0	0.10 ±0.01	1.7
D20	VIII/2012	6.0	0.16 ±0.01	2.7	D44	I/2014	11.0	0.07 ±0.01	0.6
D21	VII/2012	3.0	0.02 ±0.00	0.7	D45	IX/2012	5.0	0.20 ±0.01	4.0
D22	V/2014	0.25	0.11 ±0.01	44.0	D46	X/2012	25.0	0.09 ±0.01	0.4
D23	VI/2014	3.0	6.44 ±0.08	205	D47	IV/2013	0.8	0.09 ±0.00	11.3
D24	III/2013	3.0	4.10 ±0.10	137	D48	III/2013	20.0	0.05 ±0.00	0.3

shaking apparatus and then spun at 6,000 g for 5 minutes. Separation was performed using an HPLC 1100 instrument with UV-VIS DAD detector at 30 °C (all instruments produced by Agilent Technologies, Waldbronn, Germany) with linear gradient elution (0 min 30% A, 10 min 0% A and 15 min 30% A) on a ZORBAX SB CN (75 mm x 4.6 mm, 3.5 μm) column with mobile phase flow of 0.7 ml/min (3.153 g/L of ammonium formate in water - A; and methanol - B). The signal was recorded at 446 nm with bandwidth of 16 nm upon the injection of analysed samples (1-10 μL). The reference signal was monitored at λ = 600 nm with bandwidth of 100 nm. Figure 2 illustrates an example of a chromatogram of a lutein containing food supplement sample.

HPLC Metod validation

Accuracy, precision, and recovery were evaluated (n = 6 - 10) with model solutions and samples spiked with lutein standards (concentrations varying from 0.5 to 3.0 μg/g). Intraday and interday repeatability were verified by analyzing standard solutions and lutein samples using the same procedure as in **Šivel et al. (2015)**.

The limit of detection (LOD, S/N = 3) was 0.22 μg/g and the limit of quantification (LOQ, S/N = 10) was 0.73 μg/g. Two independent sample solutions were always prepared. The HPLC analysis of each of them was determined in triplicate. The recorded results were processed by ANOVA variance analysis using both statistical Unistat 5.1 software and Office Excel®Microsoft program **(Snedecor and Cochran, 1967)**.

RESULTS AND DISCUSSION

Lutein content in food supplements

Altogether, the following 48 lutein containing food supplement samples were analysed: 22 supplements produced in the form of tablets (D1 - D22 samples), 18 food supplements manufactured in the form of soft capsules (D23 - D40), and 8 food supplements produced in the form of hard capsules (D41 - D48). The declared and detected contents of lutein in samples are specified in Table 1; their percent rate found in tablets is shown in Figure 3.

Out of seven tablet samples of Czech food supplements, only one (D2) demonstrated lutein level higher than declared by the producer. We found that the D2 sample contained 3.07 mg/tablet; the manufacturer stated that it included just 2.00 mg/tablet. The contents of lutein in the other tablet products (D1, D3 - D7) were below 10% of the level declared by the producers (Figure 3). None of 15 tablet samples produced abroad (D8 - D22) showed lutein content given by the producers. In three samples, the detected contents of lutein were almost the same as the levels declared by the producers (D9, D10, and D17). The other analyzed tablet samples contained less than 50% of lutein level indicated by their producers (Figure 3).

The structure of tablets in four food supplement samples (D2, D9, D10, and D17) that contained more than 80% of declared lutein amount suggests the use of encapsulated form of lutein in the manufacture. For soft capsule samples, Figure 4 depicts the percent proportion of detected lutein contents to levels declared by producers (Table 1).

Out of ten Czech soft capsule samples, three (D23 - D25) contained lutein levels higher than indicated by producers. Other five Czech samples (D26, D27, D29 - D31) included above 85% of lutein amounts specified by producers (see Figure 5). Out of eight soft capsule samples produced abroad, three (D34, D35 and D40) showed higher contents of lutein than declared by producers.

The D23 sample contained 6.14 mg/capsule, which corresponds to almost 205% of the amount specified by its producer, that is 3.00 mg per soft capsule. Figure 5 plots percent proportion of lutein contents detected in hard capsules to levels declared by producers (values stated in Table 1).

Four hard capsule samples produced by the Czech manufacturers (D41 - D44) included less than 2% of the declared lutein contents, out of which three even less than 1%. Four samples of hard capsules made abroad (D45 - D48) contained less than 12% of the specified lutein amounts; two samples showed even contents lower than 1%.

Quality of lutein in food supplements

To compare quality of lutein in various dosage forms of food supplements (tablets; soft and hard capsules),

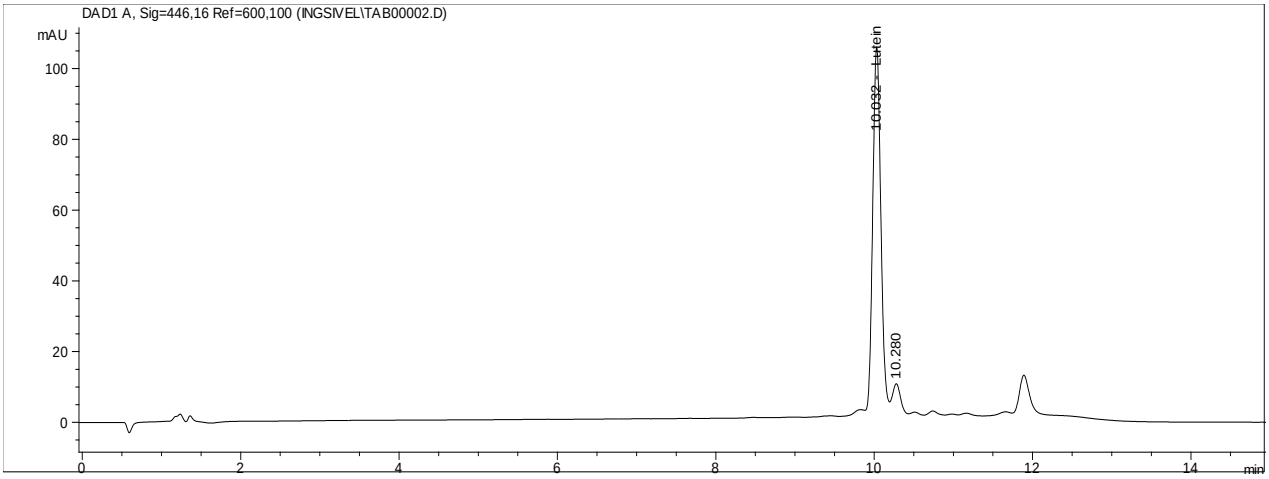

Figure 2 Chromatogram of a dietary supplement sample D2 containing lutein.

categorization according to proportion of detected to declared lutein contents was employed: i) satisfactory (~ 100%), ii) less satisfactory (75 - 100%), iii) inadequate (25 - 75 %), and iv) unsatisfactory (<25%).

Three samples of soft capsules and one sample of tablets of Czech producers were evaluated as satisfactory products. Five samples of soft capsules were labelled less satisfactory; six tablet samples and all four samples of hard capsules were found unsatisfactory goods. Three samples of soft capsules of food supplements produced abroad were evaluated as satisfactory; three samples of tablets were evaluated as less satisfactory preparations; eleven samples of tablets, three samples of soft capsules and all four samples of hard capsules were found unsatisfactory goods.

Comparing three dosage forms such as tablets, soft or hard capsules in reference to lutein resistance against oxidation by air oxygen, we can conclude that soft capsules are the most resistant dosage form followed by tablets and hard capsules, the least resistant ones.

Quality of lutein containing food supplements

Out of all 21 Czech product samples, four were classified as satisfactory preparations, five samples fell into the less satisfactory product category, two products were found inadequate, and ten were evaluated as unsatisfactory products. Out of all 27 products manufactured abroad, three samples fell into the satisfactory preparation

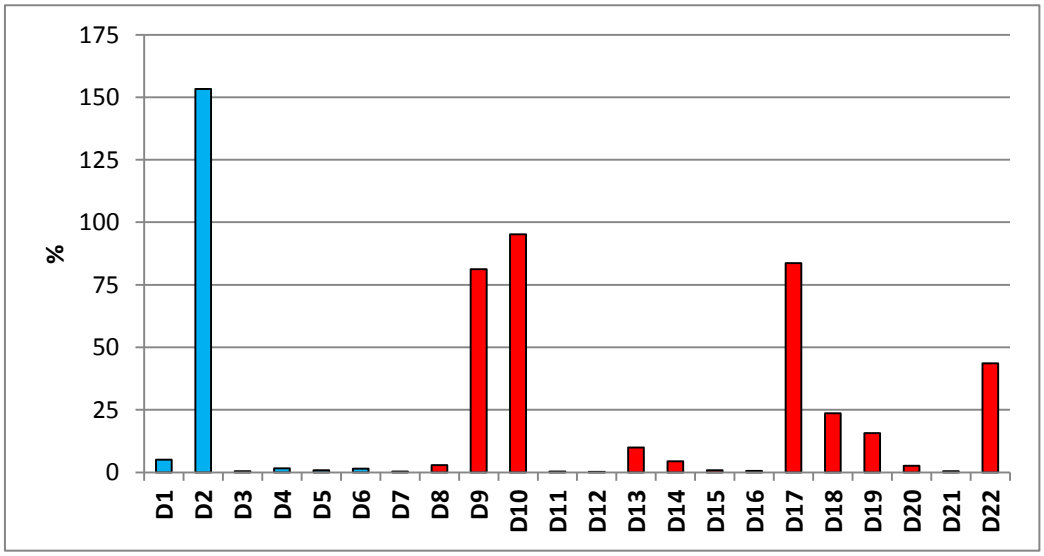

Figure 3 The percent proportion of lutein contents detected in samples to levels declared by producers in tablets.

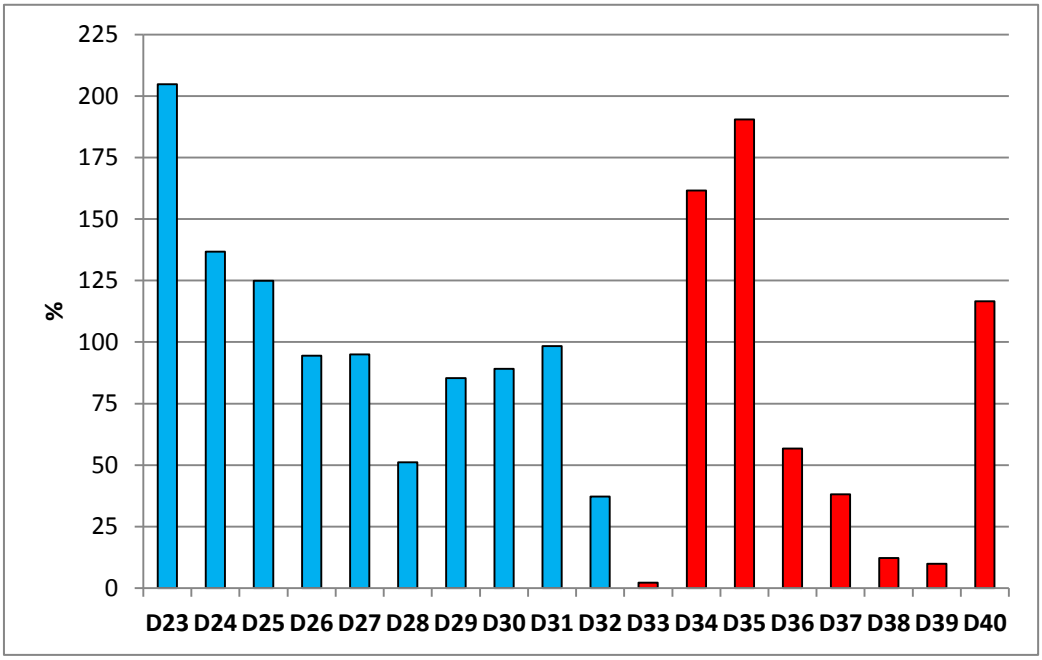

Figure 4 The percent proportion of lutein contents detected in the sample to levels declared by producers in soft capsules.

category, three samples were found less satisfactory preparations, three samples were labelled inadequate, and 18 samples were included in the unsatisfactory products category.

Comparing the quality of Czech and foreign products, the Czech ones ranked higher; 19% of them were evaluated as satisfactory products, while just 11% of the products made abroad fell into the same category. Moreover, only 48% of Czech products fell into the unsatisfactory category compared to 67% of products manufactured abroad.

CONCLUSION

Before manufacture, producers of lutein containing food supplements have to implement a thorough selection of raw materials. Reliable and trustworthy supplier might rank among the most important manufacturing factors. Suppliers are supposed to provide high quality lutein and to employ reliable analytical methods used for detection of its content; thereby they guarantee declared content of lutein in the given marigold plant extract.

The food supplement manufacturers should implement input analyses of ingredients and output analyses of products. Using cheap ingredients like lutein containing extracts, that are widely available on the market, they should also consider possible negative effects.

The worldwide trend leads food supplement producers to make products with 5 - 15 mg of lutein per a tablet or a capsule; the above amount corresponds to recommended daily intake of lutein. Using HPLC method, some researchers **(Aman et al., 2004; Young et al., 2007; Kroll et al., 2008; Thomas et al., 2012)** tested 20 samples of lutein containing food supplements. To conclude, we can recommend production of soft gelatine capsules with declared content of lutein ranging between 3 - 15 mg per one capsule that is the dosage form that enables the highest protection of lutein against oxidation.

Based on the analyses of ingredients and food supplements, the following findings can be arrived at: i) out of 22 samples of food supplements produced in the form of tablets, only one showed the qualities of a satisfactory product; ii) out of 18 samples of food supplements produced in the form of soft capsules, six samples fell into the category of satisfactory products; iii) out of eight samples of food supplements produced in the form of hard capsules, no sample was evaluated as a satisfactory preparation; iv) concerning stability of lutein in all three dosage forms, we arrived to the following conclusions: lutein contained in soft capsules showed the highest stability against oxidation; lutein in tablets was more prone to oxidation and lutein in hard capsules was most susceptible to oxidation process; thus soft capsules proved to be the most suitable application form followed by tablets and hard capsules; v) out of 21 Czech products, only four fell into the category of satisfactory products, three of them were soft capsules and one was a tablet; vi) out of 27 products manufactured abroad, only three were evaluated as satisfactory products, all of them were soft capsules; vii) out of 48 analysed food supplement samples just seven fell into the category of satisfactory preparations, eight were evaluated as less satisfactory preparations, five were found inadequate products and 28 samples were labelled unsatisfactory; viii) only one in six analyzed samples contained the amount of lutein specified by the manufacturer; ix) almost 60% of monitored lutein containing food supplement samples fell into the unsatisfactory product category.

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DETERMINING THE PRESENCE OF CHICKEN AND TURKEY MEAT IN SELECTED MEAT PRODUCTS USING REALTIME PCR METHOD

Miloš Mašlej, Jozef Golian, Lenka Maršálková

ABSTRACT

The one of the most convenient method for the identification of animal species in raw and processed meat products is the examination of DNA sequences. Real-Time PCR are particularly suitable because even small fragments of DNA formed during heat processing of the meat can be amplified and identified. TaqMan Real-Time PCR is a rapid, convenient and sensitive assay for meat identification. For chicken and turkey meat identification we were using species-specific primers and TaqMan probe designed on the mitochondrial cytochrome b. The intensity of the fluorescence signal has risen at a variety of different samples. We analysed sixteen the samples of turkey meat products and we found the incidence of chicken at nine samples in the range of the detection range of the reaction0.1 to 100%. Sample 8 fluorescence intensity exceeded the detection threshold in the 22.11 cycle (Cp = 22.11); Sample 6, (Cp = 23.19); Sample 1 in 27.08 cycle (Cp = 27.08); Sample 7 in 31,7 cycle (Cp = 31.7) and sample 5 in 32.32 cycle (Cp = 32.32). All Cp values for these samples fluorescence intensity exceeded the detection threshold in earlier cycles as sample the 100% turkey DNA. It follows that in the samples no. 8, 6, 1, 5, and 7 is in the range of chicken DNA detection range of the reaction, from 0.1 to 100%. Sample 11 in the cycle 27,08 (Cp = 27.08); Sample 10 in the cycle 27.8 (Cp = 27.8); sample 16 in 28.03 cycle (Cp = 28.03) and sample 13 in the cycle of 29.18 (Cp = 29.18). In recognition of the results of the monitoring of the content of chicken meat in meat products it is appropriate to further verification and testing detection kits used to work for possible use in practice since it has been found to be sufficient sensitivity and specificity to 30 cycle reaction.

Keywords: chicken; turkey; meat products; Real-Time PCR

INTRODUCTION

Meat products usually contain different kinds of meats, each of these must be declared on the label. These include hot dogs and sausages, cold meats and a variety of products containing, respectively containing pork (halal) and products with a defined share of several different kinds of meat. Prices of products vary greatly depending on the region and the current market situation **(Köppel et al., 2009)**. Authentication of meat products is currently focused mainly on demonstrating the replacement of more expensive meat cheaper, to show the presence of undeclared type of meat and use vegetable proteins because they are much cheaper compared to meat protein **(Soares et al., 2010)**. Commercial immunoassay kits are available for meat speciation, but do not normally differentiate chicken from turkey. Several electrophoretic techniques are also now available for species identification, including isoelectric focusing, sodium dodecylsulfate polyacrylamide gel, 2D and capillary zone electrophoresis. These techniques are, however, not reliable for resolving mixtures of meat species, or identifying species in highly processed meat products. The protein profiles of a single species produces a complex banding pattern, and even small amounts of protein from other species will often overlap the species-specific bands making interpretation of the resulting profile equivocal. This is compounded by the problem that heat treatments denature proteins, destroying the profile of water-soluble proteins **(Hird et al., 2003)**.

Counterfeiting of meat and supervision of food has their roots in the distant past. Since ancient times, some people are willing to adulteration of food illegally enriched at the expense of financial loss and other health risk. Each company felt the need for independent oversight over the quality and wholesomeness of food, to protect consumer-focused attention of civil, social and civil authorities. Food adulteration remains a serious problem and present **(Obrovská et al., 2002)** The Slovak Republic is a need for verification of genuineness of certain products as a necessary part of a comprehensive investigations on the quality of the goods in terms of consumer protection, together with the fight against counterfeit products in the package itself or directly for sales **(Takáčová, Bugarsky et al., 2010)**. To detect the type of meat in the composite sample were discovered many different methods, for example high performance liquid chromatography **(Espinoza et al., 1996)**, electrophoresis **(Ozgen-Arun, Ugur, 2000)** and enzyme analysis **(Hajmeer et al., 2003)**. One of the most specific methods for detecting food adulteration PCR is a method allowing precise identification of materials of biological origin **(Lepešková, 2002)**.

The aim of this work is to evaluate the determination of the presence of chicken and turkey meat in selected meat products using Real-Time PCR.

MATERIAL AND METHODOLOGY

We analysed 16 different meat products specified percentage of turkey meat purchased on the Slovak market (Tab 1). DNA were isolated by phenol - chloroform extraction, preceded skiing individual samples (sample size was 1 mm) in 600 ml of lysis solution with the addition of 20 ml of the enzyme proteinase K. TaqMan Real-time PCR was carried out in the capillary reaction cycler LightCycler® 1.5 (Roche) and the results were evaluated with the help of the LightCycler software version 4.5 (Roche, Germany), which during the PCR reaction automatically creates a graph of the fluorescence intensity of the number of cycles.

Sets of primers and TaqMan probes were designed according to **Jonker et al. (2008)** and all primers were synthesized by General Biotech (Czech Republic).

Designed primers were derived from the sequences of a specific gene cyt b. The sequence of the primers and TaqMan probes of the first and second sets of detection are listed in Table 2.

The individual primers and TaqMan probes were supplied in lyophilized form. Dissolving the freeze-dried in ultrapure water (Milli-Q H_2O) were obtained 10x concentrated stock solutions of primers, which were stored at -20 °C. Primers from stock solutions were diluted working solutions so that their final concentration of 10 pmol.μl^{-1}. Working solutions were stored at 2 - 8 °C. Lyophilized TaqMan probe from first and second detection kit was dissolved in ultrapure H_2O directly to a working concentration of 5 pmol.μl^{-1}. In a reaction mixture, we used the components necessary for optimum progress of the reaction: Colorless GoTaq® reaction buffer, $MgCl_2$, dNTP mix, individual primers and probes, and a dye ROX GoTaq® Hot Start Polymerase. We used GoTaq® Hot Start polymerase having polymerase activity blocked. Restoring polymerase activity occurs at initiation

Table 1 Analyzed meat products with percentage content of turkey meat.

no.	Product	Type and % of the declared meat content
1.	*Admirál turkey ham*	Turkey breast 64 %
2.	*Turkey breast ham, exclusive*	Turkey breast 90 %
3.	*Turkey ham*	Turkey breast 88 %
4.	*Turkey ham for children*	Turkey breast 83 %
5.	*Milled turkey meat product*	Turkey breast 92 %
6.	*Turkey breast ham*	Turkey breast 80 %
7.	*Mortadella with turkey meat*	Turkey breast 45 %
8.	*Turkey ham*	Turkey breast 71 %
9.	*Premiér ham specialty*	Turkey breast 63 %
10.	*Turkey ham*	Turkey breast 83 %
11.	*Admirál turkey ham*	Turkey breast 64 %
12.	*Turkey ham*	Turkey breast 83 %
13.	*Admirál turkey ham*	Turkey breast 64 %
14.	*Turkey ham*	Turkey breast 88 %
15.	*Turkey ham for children*	Turkey breast 83 %
16.	*Turkey breast ham*	Turkey breast 80 %

Table 2 Sequence of primers **(Jonker et al., 2008)**.

Primer	Bp	Sequence
Gallus F	27	5´-TCTCACTTACACTACTTGCCACATCTT-3´
Gallus R	23	5´-CGTGTGTGTCCTGTTTGGACTAG-3´
Gallus P	27	5´-(FAM) –CACTGCAACCTACAGCCTCCGCATAAC-(BHQ)-3

denaturation at 94 - 95 °C for 2 minutes. This system eliminates nonspecific amplification and creating primer - dimer. Mastermix is added to the reference dye ROX, which is used for normalization of the reporter signal. The normalization of the signal is essential for the prevention of signal variations caused by the construction of the device frequently. Preparation of the reaction mixture was carried out in the UV-cleaner box (BioSan, Lithuania). Capillaries are adapted to the volume of the reaction mixture from 10 to 40 ml. After adding the desired amount of DNA we conclude capillaries and quickly spun on a centrifuge. After inserting the capillary into the rotary plate of thermo cycler (LightCycler 1.5) we recorded the intensity of the fluorescent signal after each cycle measured at a wavelength of 640 nm.

RESULTS AND DISCUSSION

We determined the incidence of chicken meat in 16 selected product with the declared percentage of turkey meat (45 - 92%) without such additives of chicken meat.

In Figure 1, we can follow the fluorescence signal of DNA product of samples 1-8 and 100% chicken and 100% turkey DNA. The intensity of the fluorescence signal has risen at a variety of different samples. Sample 8 fluorescence intensity exceeded the detection threshold in the 22.11 cycle (Cp = 22.11); Sample 6, (Cp = 23.19); Sample 1 in 27.08 cycle (Cp = 27.08); Sample 7 in 31,7 cycle (Cp = 31.7) and sample 5 in 32.32 cycle (Cp = 32.32). All Cp values for these samples fluorescence intensity exceeded the detection threshold in earlier cycles as sample the 100% turkey DNA

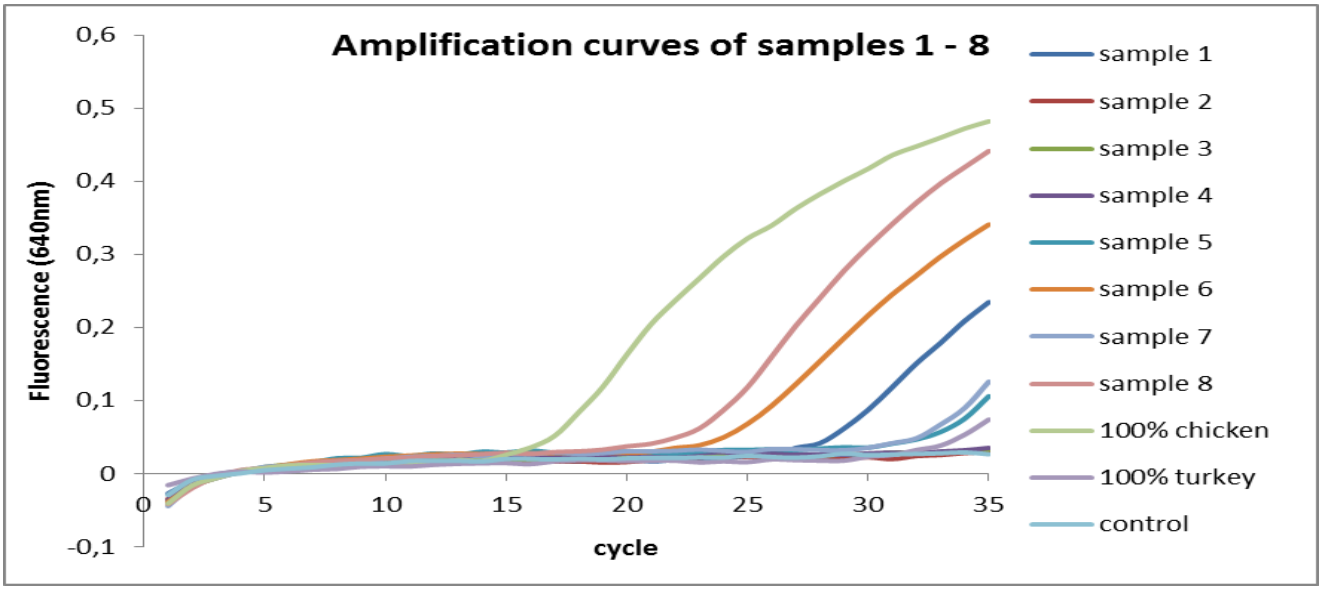

Figure 1 Amplification curves of samples 1 – 8.

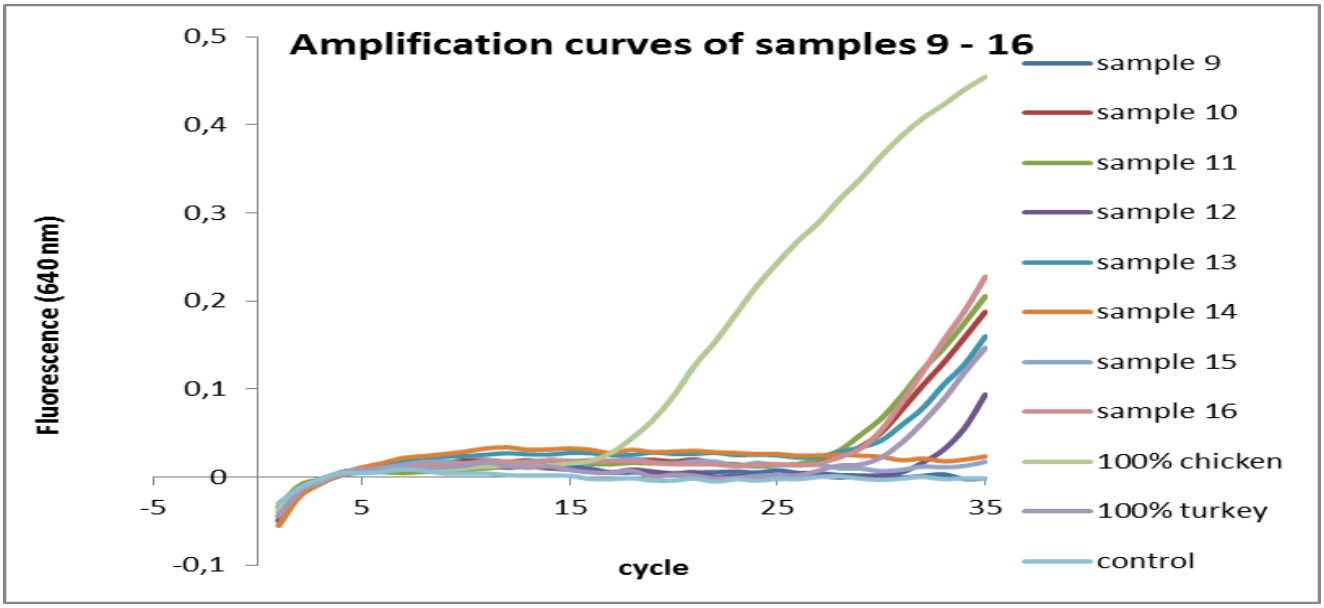

Figure 2 Amplification curves of samples 9 – 16.

(Cp = 32.94). It follows that in the samples no. 8, 6, 1, 5, and 7 is in the range of chicken DNA detection range of the reaction, from 0.1 to 100%. In samples 8, 6, 1 we observed a high incidence of chicken, which is not listed on the label in the form of impurities. With a decreasing concentration of chicken in the sample increases the number of cycles required to detect the DNA. Samples 7 and 5 are close to the lower limit of detection reactions such extent. Samples 2, 3 and 4, the fluorescence intensity exceeded the detection threshold for more than 35 cycles. For these samples, we can exclude the presence of chicken in connection with counterfeiting turkey meat on the packaging. Firgure 2 shows the amplification curves of the samples 9 - 16, the 100% chicken and 100% turkey DNA. The fluorescence intensity in the four samples exceeded the detection threshold has been exceeded rather than at the 100% turkey DNA. Sample 11 in the cycle 27,08 (Cp = 27.08); Sample 10 in the cycle 27.8 (Cp = 27.8); sample 16 in 28.03 cycle (Cp = 28.03) and sample 13 in the cycle of 29.18 (Cp = 29.18). Cp value at 100% strength turkey DNA was 29, 24 Fluorescence intensity when the sample 12 exceeded the detection threshold in the cycle 31,38. If we consider that we have established a detection range of up to 30 cycles of the reaction and sample 12 is out of the detection range of the reaction. The fluorescence intensity of the samples 9, 14 and 15 exceeded the detection threshold for more than 35 cycles, hence are also located outside the detection range of the reaction.

Using primers designed to identify chicken DNA, we were detected in chicken DNA unknown samples representative products of the stated percentage of turkey meat (45 - 92%) without the additives of chicken, in the four samples in the range of 0.1 - 1 %. The fluorescence intensity in the two samples exceeded the detection threshold for 30 cycles of reaction and therefore for these samples we cannot confirm the presence of chicken. A lower Cp value means a larger amount of initial target DNA **(Laube et al., 2006).** In the study of **Cheng et al. (2014)** succeeded in blood products successfully detected 1% strength addition of various types of blood. **You et al. (2014)** using a detection system based on cytochrome b to identify the 2% share of chicken. In studies of **Cheng et al. (2014)** and **You et al. (2014)** cross-reactivity was observed. **López-Andreo et al. (2005**) reported the detection of DNA in excess of 10%, efficiency of detection of 5 - 10% content was reduced to below 80% and 5% were able to detect species but was not effectively quantified.

CONCLUSION

Real-time PCR is a technique particularly suitable for its ability amplification and identification of small fragments of DNA resulting from thermal treatment of meat. The fluorescence intensity is measured directly during the reaction, which reduces the number of operations needed to evaluate the samples and also the possibility of contamination of the sample. Is a molecular method that can quantify the amount of the DNA. Comparing data from unknown samples with standard samples, it is possible to determine the meat content of the sample. We analysed sixteen samples of turkey meat products and we found the incidence of chicken at nine samples in the range of the detection range of the reaction 0.1 to 100%. It is appropriate to further verification and testing detection kits used to work for possible use in practice since it has been found to be sufficient sensitivity and specificity to 30 cycle reaction.

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EVALUATION AND COMPARISON OF THE CONTENT OF TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN ONION, GARLIC AND LEEK

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ABSTRACT

Onion, leek and garlic as onion family are a great source of freely available health-promoting and chemoprotective compounds (polyphenols, flavonoids, fructooligosaccharides, thiosulfinates and other sulfur compounds, vitamins). Chemoprotective compounds belong between natural components. Onion, garlic and leek have high nutritional value. They are an important component of our human diet and we used them as a preventive factor for many diseases of civilization (cancer, coronary heart diseases, and atherosclerosis). In this work we watched and evaluated content of total polyphenols and antioxidant activity in onion, garlic and leek. Samples of plant material (onion, garlic and leek) we collected at the stage of full maturity in the area of Pružina. Pružina is area without negative influences and emission sources. Samples of fresh onion, garlic and leek were homogenized and were prepared an extract: 25 g cut onion, garlic and leek extracted by 50 ml 80% ethanol accourding sixteen hours. These extracts were used for analyses. The content of the total polyphenols was determined by using the Folin-Ciocalteu reagent (FCR). Antioxidant activity was measured by using a compound DPPH˙ (2.2-diphenyl-1-picrylhydrazyl). In the present experiment it was detected, that total polyphenols content in samples ranges from 210.67 mg/kg (leek) - 429.58 mg/kg (onion). Statistically significant highest value of total polyphenols was recorded in onion (389.64 to 429.58 mg/kg). Statistically significant the lowest content of total polyphenols was recorded in leek (210.67 - 254.80 mg/kg). Another indicator that has been evaluated and compared was the antioxidant activity of onion, garlic and leek. Statistically significant highest value of antioxidant activity was recorded in onion (20.22 - 25.76%). Statistically significant the lowest value of antioxidant activity was recorded in garlic (4.05 - 5.07%). Based on the measured values of AOA in onion, garlic and leek samples can be classified as follows: onion (20.22 - 25.76%) > leek (8.55 - 12.92%) > garlic (4.05 - 5.07%).

Keywords: 5 **Keywords**: onion; garlic; leek; total polyphenols; antioxidant activity

INTRODUCTION

Vegetables are important components in the human diet and protect against many diseases of civilization especially cardiovascular diseases, high blood pressure, stroke, neurodegeneration, cancer, obesity and diabetes. Family belongs *Liliaceae* among the most important vegetable observation and demonstrated effects on human organism

Onions, garlic, leeks, shallots and other members of the onion family are commonly grown in fresh market gardens. All plants in the *Allium* are herbaceous, biennial vegetables that are known as annuals. Plants in the family *Alliaceae* are derived their characteristic flavor from the enzyme alliinase that acts on sulfur compounds. *Allium*s with their shallow roots system, grown the best in well- prepared soil **(Delamaunt, 2003)**.

Onion (*Allium cepa* var. *cepa*) is member of the lily family or *Amaryllidaceae* and it is classified by the scientific name *Allium cepa*. Onions (*Allium cepa* var. *cepa*) are an important source of bioactive compounds including phenolic compounds, flavonoids, fructooligosaccharides (FOS), thiosulfinates and other sulfur compounds, and many of these compounds have potential beneficial properties for human health **Soinen et al., (2012)**. Phenolic compounds in onions, garlics and leeks are an essential part of the human diet and they are of considerable interest due to their antioxidant properties. These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer **Balasundram et al., (2005)**. Polyphenols are reported to in vitro inhibit cancer cell proliferation, reduce vascularization, protect neurons, stimulate vasodilation and improve insulin secretion **Rio-Del et al., (2010)**.

Onion (*Allium cepa* var. *cepa*) represents a source of cysteine derivatives, which makes it a good antioxidant additive for food **Ostrowska et al., (2004)**. Antioxidants are important components, which they protect against free radicals. Free radicals are known to be the major contributors to degenerative diseases of aging and they are recognised as major factors causing cancer, cardiovascular disorders and diabetes **Calucci et al., (2003)**.

Garlic (*Allium sativum* var. *sativum*) belongs among the oldest of all cultivated plants. It has been used as a medicinal agent for thousands of years. It is a remarkable plant, which has multiple beneficial effects

such as antimicrobial, antithrombotic, hypolipidemic, antiarthritic, hypoglycemic and antitumor activity.

Recently, it has been observed that aged garlic extract exhibited radical scavenging activity. The two major compounds in garlic, S-allylcysteine and S-allylmercapto-L-cysteine, have the highest radical scavenging activity therefor the consumption of garlic may provide some kind of protection from cancer development **Thomson et al., (2003)**.

Leeks (*Allium ampeloprasum* var. *porrum*) are robust winter hardy biennials that do not form a hard bulb like onions or garlic. Leeks are sweeter than onions and have a creamy texture when cooked **(Delamaunt, 2003)**.

Leeks (*Allium ampeloprasum* var. *porrum*) contain vitamin C, vitamin B, vitamin E, copper, potassium, iron, carotenoids, chlorophyll (green tops) and flavonoids. The major flavonoid in leek is kaempferol, with only a small amount of quercetin like spring onions, leeks contain carotenoids and chlorophyll mainly in the green tops.

MATERIAL AND METHODOLOGY

Samples of plant material were collected at full maturity stages from area of Pružina. The samples of soil and plant material were analyzed individually by selected methodologies, and were used fresh material on analysis. Pružina is located under the Strážovské hills - Strážov.

The attitude of the village is in the middle of 381 m.a.s.l. Pružina belongs to the mild cold climate zone, average annual air temperature is 7 °C, annual rainfall is 800 - 1000 mm. Pružina is area without negative influences, emission sources (carbon), relatively pure from content of risk permissible forms point of view (Table 1 and Table 2). Samples of fresh onion, garlic and leek were homogenized and were prepared an extract: 25 g cut onion, garlic and leek extracted by 50 ml 80% ethanol according sixteen hours. These extracts were use for analyze.

Determination of total polyphenols

Total polyphenols were determined by the method of **Lachman, et al., (2003)** and expressed as mg of gallic acid equivalent per kg fresh mater. Gallic acid is usually used as a standard unit for phenolics content determination because a wide spectrum of phenolic compounds. The total polyphenol content was estimated using Folin-Ciocalteau assay. The Folin-Ciocalteau phenol reagent was added to a volumetric flask containing 100 μL of extract. The content was mixed and 5 mL of a sodium carbonate solution (20%) was added after 3 min. The volume was adjusted to 50 mL by adding of distilled water. After 2 hours, the samples were centrifuged for 10 min. and the absorbance was measured at 765 nm of wavelength against blank. The concentration of polyphenols was calculated from a standard curve plotted with known concentration of gallic acid.

Determination of antioxidant activity

Antioxidant activity was measured by the **Brand** and **Williams et al., (1995)** method-using a compound DPPH$^{\cdot}$ (2.2-diphenyl-1-pikrylhydrazyl)). 2.2-diphenyl-1-pikrylhydrazyl (DPPH$^{\cdot}$) was pipetted to cuvette (3.9 m^3) then the value of absorbance, which corresponded to the initial concentration of DPPH$^{\cdot}$ solution in time Ao was written. Then 0.1 cm^3 of the followed solution was added and then the dependence A = f(t) was immediately started to measure. The absorbance of 1, 5 and 10 minutes at 515.6 nm in the spectrophotometer Shimadzu UV/VIS-1240 was mixed and measured. The percentage of inhibition reflects how antioxidant compound are able to remove DPPH$^{\cdot}$ radical at the given time.

Inhibition (%) = (Ao - At / Ao) x 100

RESULTS AND DISCUSSION

Onion, garlic and leek belong among the most valuable kinds of vegetables. They are rich on polyphenols, flavonoids, vitamins and protein. It also contains of phosphorus, calcium and carbohydrates. Plants in the onion family have antihelmintic, antioxidant, antinflammatory and antiseptic effects. The total content of polyphenolic compounds contained in onion, garlic and leek are quite variable, may be affected by post-harvest and climatic conditions. In this work the content of total polyphenols in onion, garlic and leek was watched and evaluated.

In the present experiment it was detected, that total polyphenols content in samples ranges from 210.67 ±16.63 (in variety of leek) to 429.58 ±17.44 (in variety of onion). Statistically significant highest value of total polyphenols was recorded in onion (variety of Lusy, Štutgarská and Všetana). Statistically significant the lowest content of total polyphenols was recorded in

Table 1 Agrochemical characteristic of soil substrate in mg/kg

pH (H_2O)	pH (KCl)	Cox (%)	Humus (%)
7.70	5.60	1.38	2.39

Table 2 Agrochemical characteristic of soil substrate in mg/kg (heavy metals)

Heavy metals	Zn	Cu	Mn	Fe	Cr	Cd	Pb	Co
aqua regia	85.00	22.4	615.0	2642	34.6	1.28	24.40	14.0
limit value	**100.00**	**60.00**	**-**	**-**	**70.00**	**0.40**	**70.00**	**15.00**
HNO_3(c=2 mol.dm^{-3})	6.50	10.50	454	1500	0.50	0.14	3.10	

leek (variety of Arkansas, Nobel, Hannibal). **Andrejiová et al., (2011)** said that the content of total polyphenols in onion was in the interval from 105 to 134 mg/kg**. Armand et al., (2012)** reported that the content of polyphenols in onion was 620 mg/kg. Our values of total polyphenols in onion were in the range from 389.64 ±20.31 mg/kg to 429.58 ±17.44 mg/kg. Polyphenolic compounds have antioxidant properties and play an important role in preventing many of lifestyle diseases. In the work the content of total polyphenols in leek and garlic was also watched. Our values of total polyphenols in leek were 210.67 ±16.63 mg/kg to 254.80 ±10.09 mg/kg.

In the case of garlic were measured lower levels of content of polyphenols (260.62 ±25.56 mg/kg to 279.74 ±16.18 mg/kg) than in onion. **Priecina et al., (2013)** reported that the polyphenols in garlic was in the amounts from 272.28 to 1818.81 mg/kg.

From the results there is obvious that the highest content of total polyphenols is characteristic for onion (389.64 ±20.31 mg/kg to 429.58 ±17.44 mg/kg), followed by garlic with the measured value of 260.62 ±25.56 mg/kg to 279.74 ±16.18 mg/kg. While the lowest content of total polyphenols was recorded in leek (210.67 ±16.63 mg/kg to 254.80 ±10.09 mg/kg. Polyphenolic compounds contained in onions, garlic and leeks are one of the largest categories of phytonutrients, it is essentially the most represented group of secondary plant metabolites. Another indicator that has been evaluated and compared was the antioxidant activity of onion, garlic and leek. The antioxidant activity of vegetables is often associated with a ß-carotene, L-ascorbic acid, vitamin E and present polyphenolic compound such as quercetin, rutin.

Table 3 Average content of total polyphenols (mg/kg) in onion, garlic, leek.

vegetable	variety	TPC (mg/kg)
onion	Lusy	389.64 ±20.3 d
	Všetana	396.71 ±18.10 de
	Štutgarská	429.58 ±17.44 e
garlic	Makoi	260.62 ±25.56 bc
	Mirka	268.09 ±16.61 c
	Matin	279.74 ±16.18 c
leek	Arkansas	225.51 ±20.04 ab
	Nobel	210.67 ±16.63 a
	Hannibal	254.80 ±10.09 bc
	$HD_{0,05}$	26.6589
	$HD_{0,01}$	35.9988

Table 4 Average values of antioxidant activity (% inibition) in onion, garlic, leek.

vegetable	variety	AOA (%)
onion	Lusy	25.76 ±0.53 h
	Všetana	23.09 ±0.79 g
	Štutgarská	20.22 ±0.53 f
garlic	Makoi	4.45 ±0.28 ab
	Mirka	5.07 ±0.47 b
	Matin	4.05 ±0.20 a
leek	Arkansas	8.55 ±0.54 c
	Nobel	10.82 ±0.53d
	Hannibal	12.92 ±0.66 e
	$HD_{0,05}$	0.768657
	$HD_{0,01}$	1.037950

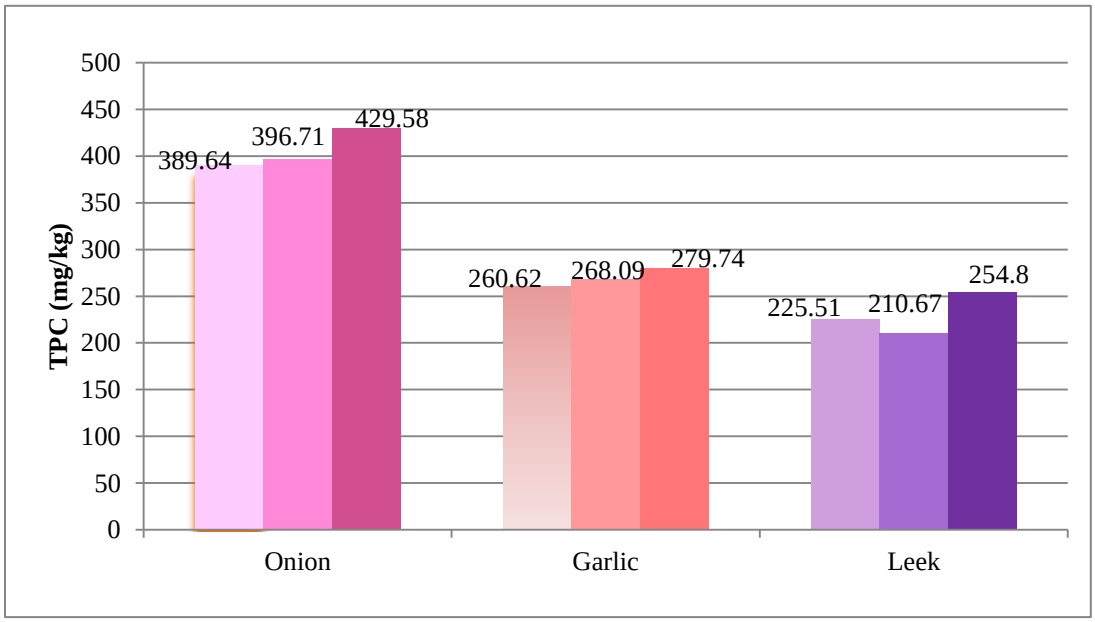

Figure 1 The content of total polyphenols (mg/kg) in onion, garlic and leek.

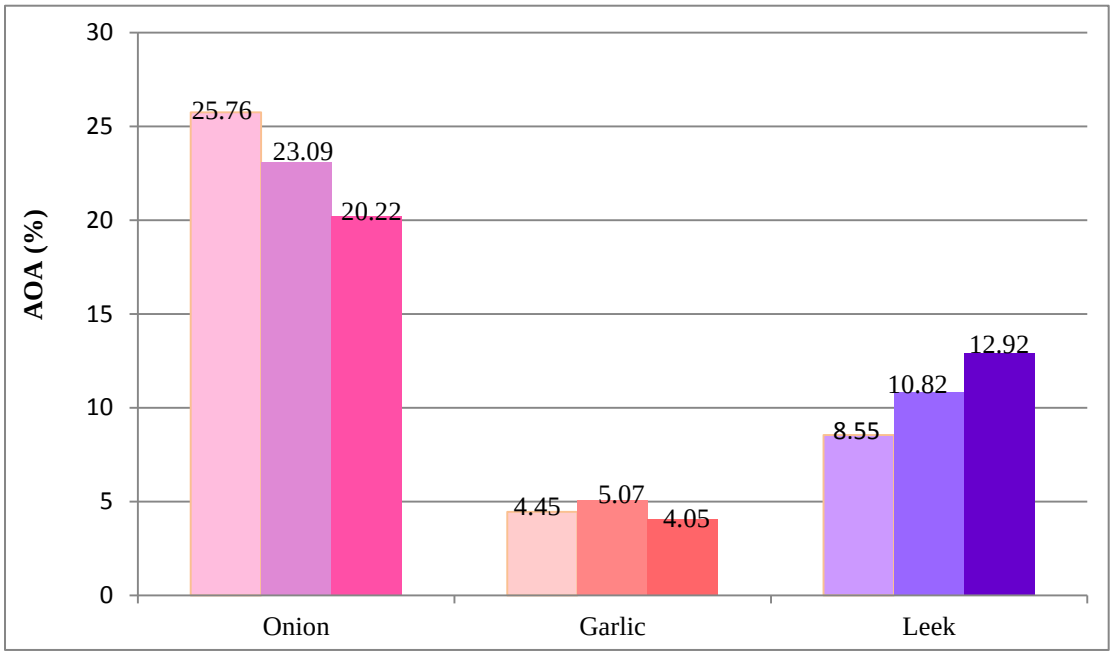

Figure 2 Values of antioxidant activity AOA (% inibition) in onion, garlic, leek.

In the present work it was detected, that antioxidant activity in samples ranges from 4.05% ±0.20% (garlic) to 25.76% ±0.53% (onion). Statistically significant highest value of antioxidant activity was recorded in onion (20.22% ±0.53% to 25.76% ±0.53%). Statistically significant the lowest value of antioxidant activity was recorded in garlic (4.05% ±0.20% to 5.07% ±0.47%). **Prakash et al., (2007**) published that the value of antioxidant activity in onion was in interval from 3.6% to 84.1%. Our results of antioxidant activity in onion was higher than the results **Karadeniz et al., (2005)**, which found that the value of antioxidant activity was 12.5%. In the case of garlic was measured the lowest level of antioxidant activity (in interval from 4.05% ±0.20% to 5.07% ±0.47%) than in onion. **Priecina et al., (2013)** reported that value of antioxidant actvity in garlic was 11.98%. Based on the measured values of AOA in onion, garlic and leek can be samples classified as follows: onion > leek > garlic. Antioxidants in onion, garlic and leek catch reactive form of oxygen, inhibition of enzymes responsible for superoxide anion production, chelation of transition metals involved in process forming radicals and prevention of the peroxidation process by reducing alcoxyl and peroxyl radicals **(Biesaga, 2011)**.

CONCLUSION

The contribution focuses on the total of polyphenol content and antioxidant activity in onion, garlic and leek. The results suggest that the highest value of total polyphenols and antioxidant activity was in onion. In generally these vegetables are also characterized by a high content of chemoprotective compounds (polyphenols, flavonoids, anthocyanins, quercetin, and many more) and minerals (zinc, calcium, iron, selenium, and phosphorus), which positively affecting the human body. The content of total polyphenols and antioxidant activity may be affected by many factors for example postharvest (storage) and climatic conditions (altitude, rainfall, mean annual temperature) and the agrochemical composition of the soil (humus of content, nutrients) and type varieties.

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LOW ADMINISTRATION OF BEE POLLEN IN THE DIET AFFECTS BONE MICROSTRUCTURE IN MALE RATS

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ABSTRACT

Bee pollen is often used as a dietary additive because it contains proteins and is rich in vitamins, minerals and phytochemicals. However, its impact on growth characteristics and bone microstructure is still poorly understood. Therefore, the objective of this study was to investigate the effect of low administration of bee pollen on selected growth characteristics and histological structure of femoral bones in rats. For this purpose, 1-month-old male Wistar rats were randomly divided into two groups of 5 animals each. In the control group (CG), rats were fed a commercial diet throughout the experiment (90 days). Rats of experimental group (EG) received standard diets with a 0.2% addition of bee pollen for the same time period of treatment. At the end of the experiment, macroscopical and microscopical structures of femoral bones from all rats were analysed using analytical scales, sliding instrument, polarized light microscopy and atomic absorption spectrophotometry. The statistical analysis of obtained data did not reveal significant differences for body weight, femoral weight, femoral length, and cortical bone thickness between both investigated groups of rats. However, a higher number of primary and secondary osteons was observed in the central area of *substantia compacta* and near periosteal surfaces in rats from the EG group. Histomorphometrical data of primary osteons' vascular canals, Haversian canals and secondary osteons did not differ between rats from both groups. Also, concentrations of Ca, Mg, Fe and Zn in the bones of rats from the EG group were similar to those from CG group. Our results indicate that 0.2% concentration of bee pollen in the diet significantly affects qualitative histological characteristics of femoral bones in rats. On the other hand, it has no impact on the size of primary and secondary osteons and on the content of Ca, Mg, Fe and Zn in the bones of male rats.

Keywords: bee pollen; femoral bone; rat; qualitative parameters; quantitative parameters.

INTRODUCTION

Bee pollen is often used as food for all developmental stages in the hive **(Almeida-Muradian et al., 2005)**. It contains many essential nutritional elements important for growth and development of animals and humans **(Orzaez Villanueva et al., 2002**; **Haščík et al., 2011**; **Petruška et al., 2012)**. Bees use pollen as their nutritional source of proteins (25 - 30%), carbohydrates (30 - 55%), lipids, including fatty acids and sterols (1 - 20%), vitamins and minerals. Furthermore, bee pollen is rich in carotenoids, flavonoids, phytosterols, polyphenols and other beneficial compounds **(Baltrušaityté et al., 2007**; **Estevinho et al., 2008)**.

This natural product is recognized to be a valuable product with potential for medical and nutritional applications **(Almeida-Muradian et al., 2005)**. It has been reported to trigger beneficial effects in the prevention of prostate problems, arteriosclerosis, gastroenteritis, respiratory diseases, allergy desensitization, improving the cardiovascular and digestive systems, body immunity and delaying aging **(Estevinho et al., 2012)**.

The bee pollen also improves bone mineralization due to high vitamin D content, which increases calcium absorption **(Wang et al., 2007)**. According to **Yamaguchi et al. (2004)** and **Hamamoto et al. (2006)**, it has stimulatory effects on bone formation and inhibitory effects on bone resorption. It also stimulates bone calcification.

In general, growth and bone microstructure of animals are affected by numerous factors, e.g. nutritional regime, genetic factors, sex, age, management conditions, production system. Recent years have witnessed an increasing interest in the use of various feed additives and dietary supplements believed to improve growth characteristics of animals. Therefore, the aim of this study was to determine the effect of low administration of bee pollen on selected growth characteristics (body weight, femoral weight, femoral length and cortical bone thickness) and bone microstructure in male rats.

MATERIAL AND METHODOLOGY

Our study was carried out on ten 1-month-old male Wistar rats. The animals were housed individually in plastic containers (Techniplast, Italy) under the same laboratory conditions of temperature (20 - 24 °C) and relative humidity (55 ±10%) with access to food (feed mixture M3, Bonagro, Czech Republic) and drinking water *ad libitum*. All experiments were provided in accordance

with accepted standards of animal care in accredited laboratory (SK PC 50004) of the Slovak University of Agriculture in Nitra.

At the age of four weeks, the young rats were divided into two groups, of 5 animals each. The control group (CG) was fed with the feed mixture without bee pollen additive. Experimental group (EG) was fed with the bee pollen addition (*Brassica napus*) in concentration of 0.2% for a total of 90 days. All procedures were approved by the Animal Experimental Committee of the Slovak Republic.

At the end of the experiment, all animals were killed, weighed and their femora were used for macroscopical and microscopical analyses. Femora were weighed by analytical scales and their length was measured by a sliding instrument.

For histological analysis, the femora were sectioned at the midshaft of the diaphysis and the segments were fixed in HistoChoice fixative (Amresco, USA). The segments were then dehydrated in increasing grades (40 to 100%) of ethanol and embedded in Biodur epoxy resin (Günter von Hagens, Heidelberg, Germany) according to the method described by **Martiniaková et al. (2008)**. Transverse thin sections (70 - 80 μm) were prepared with a sawing microtome (Leitz 1600, Leica, Wetzlar, Germany) and fixed onto glass slides by Eukitt (Merck, Darmstadt, Germany) as previously described **(Martiniaková et al., 2010)**. The qualitative histological characteristics of the compact bone were determined according to the internationally accepted classification systems of **Enlow and Brown (1956)** and **Ricqlés et al. (1991)**. The quantitative (histomorphometrical) variables were assessed using the software Motic Images Plus 2.0 ML (Motic China Group Co., Ltd.). We measured area, perimeter and the minimum and maximum diameters of 264 primary osteons' vascular canals, 216 Haversian canals and 216 secondary osteons in all views (i.e., anterior, posterior, medial and lateral) of the thin sections in order to minimize inter-animal differences. Diaphyseal cortical bone thickness was also measured by Motic Images Plus 2.0 ML software. Twenty random areas were selected, and average thickness was calculated for each femur. The concentrations of Ca, Mg, Fe and Zn were determined by atomic absorption spectrophotometry (Perkin Elmer 4100 ZL) in a graphite furnace **(Stawarz et al., 2003)**. The bone samples were dried at 105 °C until dry mass was obtained. Then all samples were weighed (minimum 2 g) and digested in concentrated nitric acid at 90 °C for 10 h. The samples were diluted to 25 ml with distilled water before analysis **(Martiniaková et al., 2011)**. All metal concentrations were expressed on a dry weight basis in $\mu g.g^{-1}$.

Statistical analysis was performed using SPSS 8.0 software. All data were expressed as mean ± standard deviation (SD). The unpaired Student's t-test was used for establishing statistical significance ($p < 0.05$) between rats from the CG and EG groups.

RESULTS

Our results demonstrate no significant effect of 0.2% administration of bee pollen on body weight, femoral weight, femoral length, and cortical bone thickness in male rats (Table 1).

Endosteal borders of femoral bones in rats from the CG group were formed by non-vascular bone tissue in all views of thin sections. This tissue contained cellular lamellae and osteocytes. Areas of primary vascular radial bone tissue (formed by branching or non-branching vascular canals radiating from the marrow cavity) were also identified in anterior and posterior views. In the middle part of the compact bone, primary and secondary osteons were observed. The periosteal border was again composed of non-vascular bone tissue, mainly in the anterior and posterior views (Figure 1).

The rats treated by 0.2% concentration of bee pollen displayed a similar microarchitecture to that of the control rats, except for the middle part of compact bone in posterior view. In this view, vascular canals expanded into central area of the bone what indicates that primary vascular radial bone tissue was also identified in the central area of the femur. Also, a higher number of primary and secondary osteons was observed in the middle part of *substantia compacta* and near periosteal surface in these rats (Figure 2).

For the quantitative histological analysis, 264 vascular canals of primary osteons, 216 Haversian canals and 216 secondary osteons were measured. The results are summarized in Tables 2, 3 and 4. We found that all measured variables (area, perimeter, maximum and minimum diameters) of the primary osteons' vascular canals, Haversian canals and secondary osteons did not differ between rats from both investigated groups.

Using atomic absorption spectrophotometry, the concentrations of Ca, Mg, Fe and Zn in femoral bones of all rats were determined. The findings are shown in Table 5. We have found that application of 0.2% concentration of bee pollen had not significant impact on the content of selected mineral elements in the bones of male rats.

DISCUSSION

Bee pollen contains a wide spectrum of amino acids, vitamins, hormones, and minerals, as well as enzymes and co-enzymes necessary for good digestion and growth. **Haro et al. (2000)** reported that male rats fed with multifloral bee pollen (10 g/kg of the diet for 10 days) had increased body weight. However, we revealed a non-significant effect of 0.2% administration of bee pollen on the body weight of rats. Moreover, the data observed by **Gálik** (**2012**) indicate a decrease of feed intake in rats fed with bee pollen (at the same level as it was used in our study). The decrease of feed intake in previous study could be explained by the increase in nutrient supply. In fact, nutrients such as minerals and water soluble vitamins could accelerate nutrients metabolism and increase energy digestibility, what can negatively affect feed intake **(Attia et al., 2009)** and subsequently don't influence growth of rats.

Prolonged intake of 0.2% concentration of bee pollen in our study induced changes in microscopic structure of femoral bones in male rats. In general, bone is a metabolically active organ and changes in bone angiogenesis are closely associated with bone remodeling **(Brandi and Collin-Osdoby, 2006)**.

Table 1 Average body weight, femoral weight, femoral length and cortical bone thickness in control (CG) and experimental (EG) groups of rats.

Rat's group	N	Body weight (g)	Femoral weight (g)	Femoral length (cm)	Cortical bone thickness (mm)
CG	5	374.00 ±9.62	1.05 ±0.19	3.82 ±0.09	0.554 ±0.064
EG	5	357.00 ±24.90	1.07 ±0.06	3.76 ±0.05	0.545 ±0.052
T-test		NS	NS	NS	NS

N: number of rats, NS: non-significant changes

Table 2 Data on primary osteons' vascular canals in rats from CG and EG groups.

Rat´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
CG	146	390.19 ±94.39	71.23 ±9.23	12.51 ±2.31	9.95 ±1.13
EG	118	381.96 ±93.88	70.65 ±9.59	12.42 ±2.49	9.83 ±1.11
T-test		NS	NS	NS	NS

N: number of rats; NS: non-significant changes

Table 3 Data on Haversian canals in rats from CG and EG groups.

Rat´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
CG	109	394.65 ±80.78	73.37 ±20.69	12.89 ±4.26	10.25 ±2.41
EG	107	394.34 ±65.02	71.37 ±5.80	12.34 ±1.42	10.23 ±1.27
T-test		NS	NS	NS	NS

N: number of measured structures; NS: non-significant changes

Table 4 Data on secondary osteons in rats from CG and EG groups.

Rat´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
CG	109	6819.98 ±1720.62	295.30 ±37.76	51.27 ±8.17	41.97 ±5.70
EG	107	6758.20 ±1772.16	294.87 ±39.99	51.52 ±8.87	41.40 ±6.25
T-test		NS	NS	NS	NS

N: number of measured structures; NS: non-significant changes

Table 5 Concentrations of selected mineral elements in femoral bones of rats from CG and EG groups.

Rat's group	N	Ca	Mg	Fe	Zn
		$\mu g.g^{-1}$			
CG	5	29784.61 ±8427.94	1328.67 ±228.59	95.74 ±21.15	109.63 ±30.42
EG	5	32631.37 ±8914.33	1406.91 ±247.34	97.16 ±20.19	112.45 ±30.28
T-test		NS	NS	NS	NS

N: number of rats; NS: non-significant changes

The formation of blood vessels serves as a way of transporting circulating osteoblast **(Eghbali-Fatourechi et al., 2005)** and osteoclast precursors **(Kassem et al., 1991)** to the sites undergoing active remodeling. **Yamaguchi et al. (2006)** reported that rats fed by bee pollen (50 or 100 mg/kg body weight) had higher DNA content (an indication of higher cell numbers) and higher alkaline phosphatase activity (marker of bone formation) in the femur in comparison with those from the control group. These results point to increased bone remodeling in rats administered by bee pollen. Therefore, a higher number of primary and secondary osteons could be observed in our rats from the EG group. It is generally known that aged rats lack true Haversian cortical bone remodeling but not cancellous bone remodeling **(Erben, 1996**; **Reim et al., 2008)**, which can be influenced by various factors, e.g. fatigue loading of long bones **(Bentolila et al., 1998)**, treatment with bone anabolic substances such as PTH, IGF-I, prostaglandins, vitamin D analogies **(Ibbotson et al., 1992**; **Lauritzen et al., 1993**; **Uzawa et al., 1995; Weber et al., 2004)**. Bee pollen extract has been found to have anabolic effects on bone components in the femora of rats **(Yamaguchi et al., 2006)**. There is also evidence that bee pollen stimulates bone remodeling due to high vitamin D content **(Zuo and Xu, 2003)**. These facts indicate that endocortical bone remodeling could also be stimulated by bee pollen administration in rats from the EG group. Therefore, some newly formed remodeling units (primary and secondary osteons) located near endosteal borders extended deep into the middle part of compact bone.

Morphometrical measurements of basic structural units of the compact bone (primary and secondary osteons) didn't show significant differences in their size between rats from CG and EG groups. These results suggest that low administration of bee pollen in the diet did not influence histomorphometry of the osteons in rats.

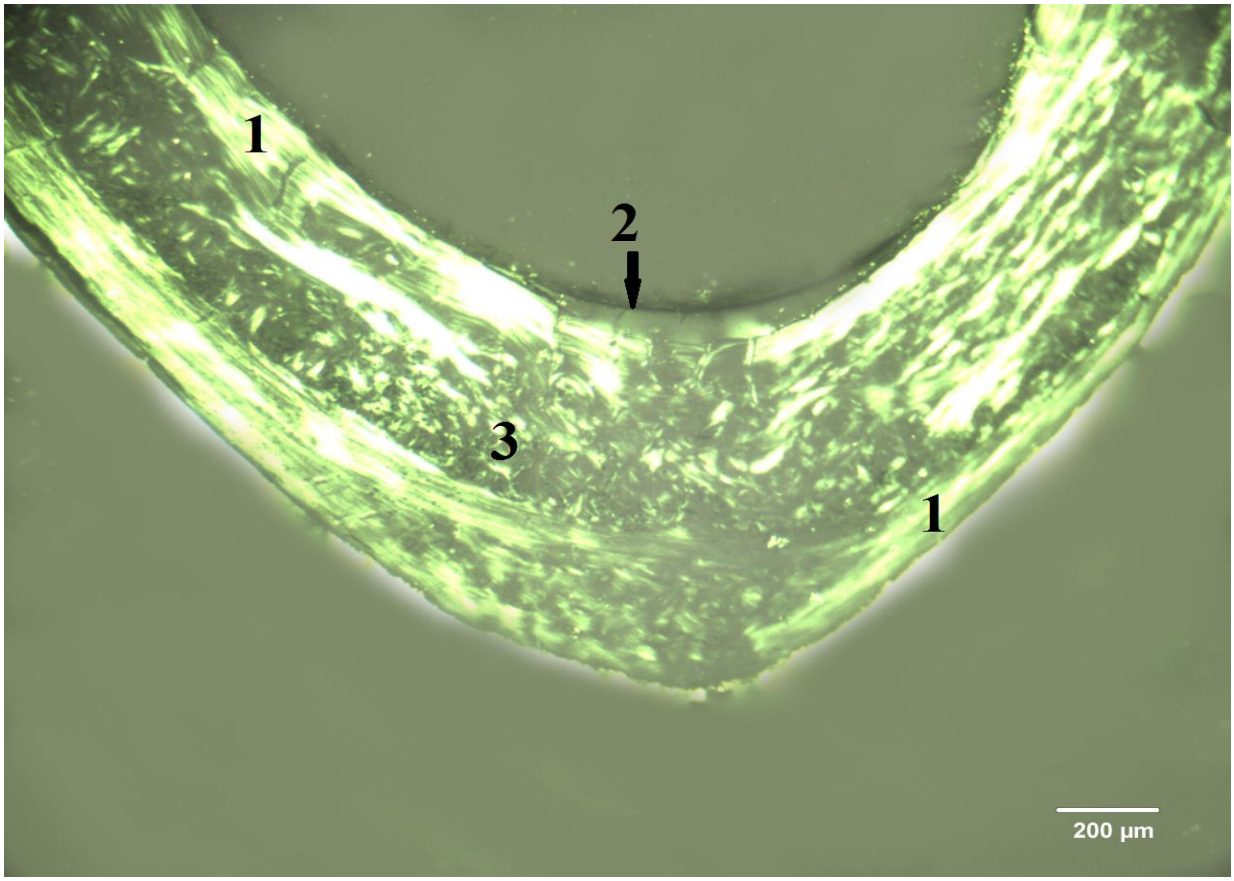

Figure 1 Microstructure of femoral bone in rat from the CG group: 1- non-vascular bone tissue; 2- primary vascular radial bone tissue; 3- primary and secondary osteons.

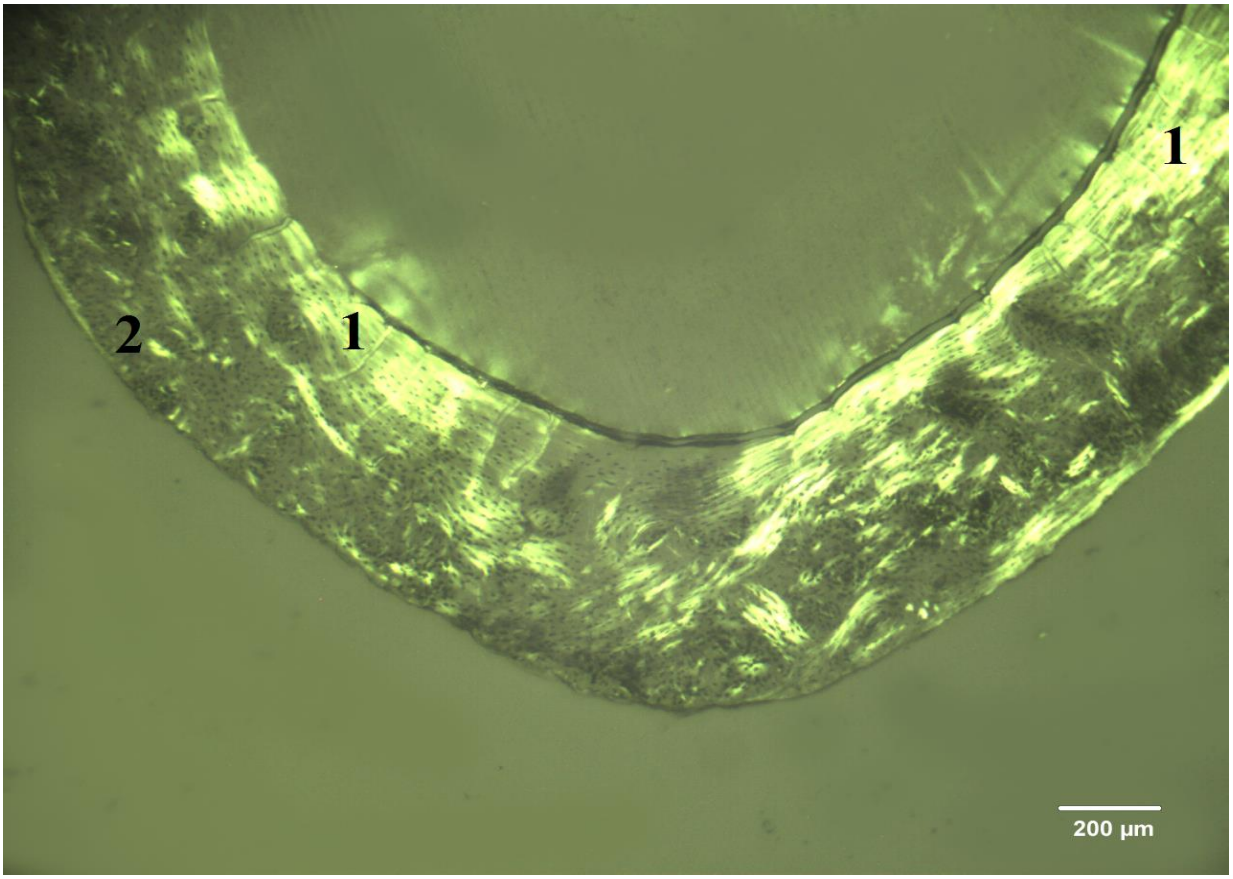

Figure 2 Microstructure of femoral bone in rat from the EG group: 1- primary vascular radial bone tissue; 2- higher number of primary and secondary osteons.

Additionally, our results revealed no demonstrable effect of 0.2% administration of bee pollen on the content of Ca, Mg, Fe and Zn in femoral bones of rats. Bone is generally an important storage organ for essential elements (e.g. Ca, Mg, Fe and Zn). However, their concentrations can be affected by various food additives. Many experimental studies **(Yamaguchi et al., 2006; Wang et al., 2007)** demonstrated positive effects of bee pollen on Ca absorption following its higher deposition in the bone. The better absorption and digestion of Ca in small intestine is attributed to increased contents of vitamin D **(Zuo and Xu, 2003)** and amino acids (e.g. lysine, aspartate, glutamate, ornithine; **Gozálbez, 1984)** in bee pollen. Higher concentration of Ca in femoral bones of rats after peroral administration to 50 and 100 mg/kg bw was observed in the study by **Yamaguchi et al. (2006)**. On the other hand, supplementation with bee pollen in concentrations of 0.5, 1 and 1.5% had no significant impact on Ca content in the bones of broilers **(Oliveira et al., 2013)**, what is consistent with our results. Besides Ca, other minor trace elements (such as Mg, Zn and Fe) can also play important roles in bone metabolism **(O'Neil and Evans, 2004)**. In general, Mg, Zn and Fe are considered to be essential elements for bone formation and bone resorption **(Yamaguchi et al., 1986; D'Haese et al., 1999; Katsumata et al., 2009)**, and they are also primary minerals detected in bee pollen **(Roulston and Cane 2000; Pernal and Currie 2002; Gergen et al., 2006)**. However, we did not observe significant differences related to Mg, Fe and Zn contents in the bones of rats from CG and EG groups. Therefore, it can be concluded that low supplementation with bee pollen (at the concentration used in our study) did not affect Ca, Mg, Fe and Zn levels in femoral bones of male rats.

Additional research dealing with the impact of higher concentrations of bee pollen on bone characteristics is required to gain more information and to verify the results of this study.

CONCLUSION

Our study demonstrates that 0.2% administration of bee pollen in the diet affects the qualitative histological characteristics of femoral bones in male rats. However, it has no significant effect on the size of primary osteons, secondary osteons and on the content of Ca, Mg, Fe and Zn in femoral bones. These results can be applied in experimental studies focusing on the effect of various bee products on bone structure.

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THE ROLE OF SULPHUR ON THE CONTENT OF TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN ONION (*ALLIUM CEPA L.*)

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ABSTRACT

Sulphur is one of the most important elements in plants. Sulphur also positive influences on growth functions (nitrogen metabolism, enzyme activity and protein and oil synthesis), yield and quality bulbs of onion. In this experiment was watched the progress of making the total polyphenols content in different levels of fertilizer sulphur in onion during vegetation. In the work we watched also the influence of sulphur fertilization on the antioxidant activity. Sulphur was added in amounts 7.3 mg.kg^{-1} S, 11 mg.kg^{-1} S and 14,6 mg.kg^{-1} S. Samples of fresh onion we collected at the beginning, in the middle and in the end of vegetation period and we prepared an extract: 50 g cut onion extracted by 100 ml 80% ethanol according sixteen hours. These extracts were used for experiment. The content of total polyphenols was estimated by using Folin-Ciocalteau reagent. The absorbance was measured at 765 nm of wave length against blank. Antioxidant activity was measured using a compound DPPH$^{\cdot}$ (2.2-diphenyl-1-picrylhydrazyl) at 515.6 nm in the spectrophotometer. Our values of total polyphenolic content during vegetation period were in range from 508.16 ±27.59 mg.kg^{-1} to 638.2 ±12.84 mg.kg^{-1}. The highest content of total polyphenolics was recorded at the end of the vegetation period (638.32 ±12.84 mg.kg^{-1}) in III. sampling (incorporation of sulphur in quantity of 14.6 mg S.kg^{-1}soil). This increase was statistically significant (*P-value* = 2.10^{-3}). The lowest content of total polyphenols was measured in the middle of vegetation period (415.41 ±13.32 mg.kg^{-1}) in II. sampling (incorporation of sulphur in quantity of 11 mg S.kg^{-1}soil). Another indicator that has been evaluated and compared was the influence of sulphur fertilization on the antioxidant activity. The highest values of antioxidant activity were recorded at the end of vegetation period in all variants. The lowest value of antioxidant activity (25.41 ±7.67%) was measured in three sampling in II.variant.

Keywords: onion (*Allium cepa* L.); sulphur, soil; total polyphenols; antioxidant activity

INTRODUCTION

Onions (*Allium cepa* L.) is widely used as most important crop among the vegetables and spices in the Slovak Republic. Onions are consumed in different ways, they are consumed uncooked, but is also often cooked before eating. Onions have bioactive compounds including polyphenols and sulphur compounds that are antioxidants and positively affect human health. The consumers preference is for the higher pungent (high sulphur content) onion bulbs **Gambo et al. (2009)**.

Many studies have suggested **(Pellegrini et al., 2009; Pérez-Gregorio et al., 2010; Geetha et al., 2011)** that biologically active phytosubstances such as phenolic acids, flavonoids, quercetin exhibit anti-inflamatory, antiviral, and vasodilating effect and i tis effective in prevention of cancer and heart diseases.

Onion composition is variable and its derived from environmental and genetic factors. Nutrients play a significant role in improving productivity and quality of crops **(Al-Fraihat, 2009)**.

The type and value of fertilizer and the level of application directly influence plant physiology and the biosynthesis of secondary compounds in plants **Naguib et al. (2012)**.

Sulphur is recognized as the fourth major plant nutrient after nitrogen, phosphorus and potassium in crop productivity. It is a consistent of sulphur containing amino acids, which are building blocks for protein in the plant **Shahen et al. (2013)**.

Sulphur is incorporated into onion flavour precursors S-alk(en)yl-L-cysteine sulphoxides (ACSOs) among other compounds **Forney et al. (2010)**.

Sulphur compounds play an important role in carbohydrate metabolism, where sulphur is an indirect component of insulin **(Stipanuk, 2004)**.

Sulphur fertilization affects on the onion quality, and is essential for a good vegetative growth and bulb development in onion **Anwar et al. (2001)**.

Sulphur deficient in plants also had poor utilization of macro and micronutrients **(Kumar and Singh, 1994)**.

The main purpose of this study was to determine influence of sulphure addition on the content of the total polyphenols as well as antioxidant activity of onion.

MATERIAL AND METHODOLOGY

The soil that was used for growing bowl - shaped pots was taken from area called Babindol. This area is without negative influences, emission sources (carbon), relatively

pure from point of view of content permissible forms of risk elements (Table 1).

Six kilograms of soil was weighted into plastic bowl-shaped pots with average of 20 cm and height of 25 cm with foraminate bottom. Basic nutrients were added in the form of aqueous solution. 8 yellow onion variety of Mundo were planted into each container. The experiment was based on four replications. Variants of pot experiments are given in Table 2.

Determination of** total **polyphenols (TCP)

Total polyphenols were determined by the method of **Lachman *et al.* (2003)** and expressed in mg eq. gallic acid per kg fresh mater. Gallic acid is usually used as a standard unit for phenolics content determination because a wide spectrum of phenolic compounds. The total polyphenol content was estimated by using Folin-Ciocalteau reagent. The Folin-Ciocalteau phenol reagent was added to a volumetric flask containing an aliquot of extract. The content was mixed and a sodium carbonate solution (20%) was added after 3 min. The volume was adjusted to 50 mL by adding of distilled water. After 2 hours, the samples were centrifuged for 10 min. and the absorbance was measured at 765 nm of wave length against blank. The concentration of polyphenols was calculated from a standard curve plotted with known concentration of gallic acid.

Determination of antioxidant activity (AOA)

Antioxidant activity was measured by the method **Brand-Williams *et al.* (1995)**, using a compound DPPH (2.2-diphenyl-1-picrylhydrazyl) (Merck).

2.2-diphenyl-1-picrylhydrazyl (DPPH) was pipetted into cuvettes (3.9 cm^3), then was written the value of absorbance, which corresponded to the initial concentration of DPPH solution in time Ao. Then 0.1 cm^3 of the followed solution was added and then was immediately started to measure the dependence A = f(t). The solution in the cuvettes was mixed and measured the absorbance of 1, 5 and 10 minutes at 515.6 nm in the spectrophotometer Shimadzu UV/VIS-1240. The percentage of inhibition reflects how the followed compound is able to remove DPPH radical at the given time.

Inhibition (%) = (Ao - At / Ao) x 100

RESULTS AND DISCUSSION

Sulphur fertilization has a marked effect on the quality of the onion bulbs. Several researches **(Ullah et al., 2008; Al-Fraihat, 2009; Mishu et al., 2013)** reported role of sulphur in onion production.

Table 1 Agrochemical characteristic of soil substrate in $mg.kg^{-1}$.

Agrochemical characteristic	pH (H_2O)	pH (KCl)	Cox (%)	Hum. (%)					
	7.75	6.60	1.19	2.05					
Nutrients ($mg.kg^{-1}$)	N	K	Ca	Mg	P				
	1225	285.80	3091.4	265.70	195.80				
Heavy metals	Zn	Cu	Mn	Fe	Cr	Cd	Pb	Co	Ni
Aqua regia	71.0	20.0	640.0	22785	22.60	0.72	21.20	12.40	29.20
Limit value	**100.0**	**60.0**	**-**	**-**	**70.0**	**0.4**	**70.0**	**15.0**	**40.0**
NH_4NO_3 ($c = 1\ mol.dm^{-3}$)	0.05	0.07	0.21	0.20	0.02	0.04	0.22	0.11	0.15
Critical value	**2.0**	**1.0**	**-**	**-**	**-**	**0.1**	**0.1**	**-**	**1.5**

Legend: *Limit value for Aqua raegia – Slovak decree no. 220/2004 Z.z.
**Critical value for NH_4NO_3 ($c= 1\ mol.dm^{-3}$) – Slovak decree no. 220/2004 Z.z.
- not applicable.

Table 2 Variants of pot experiments.

Variety	Added amount of S ($mg.kg^{-1}$)
Control	0
S1	7,3
S2	11
S3	14,6

S - Sulphur

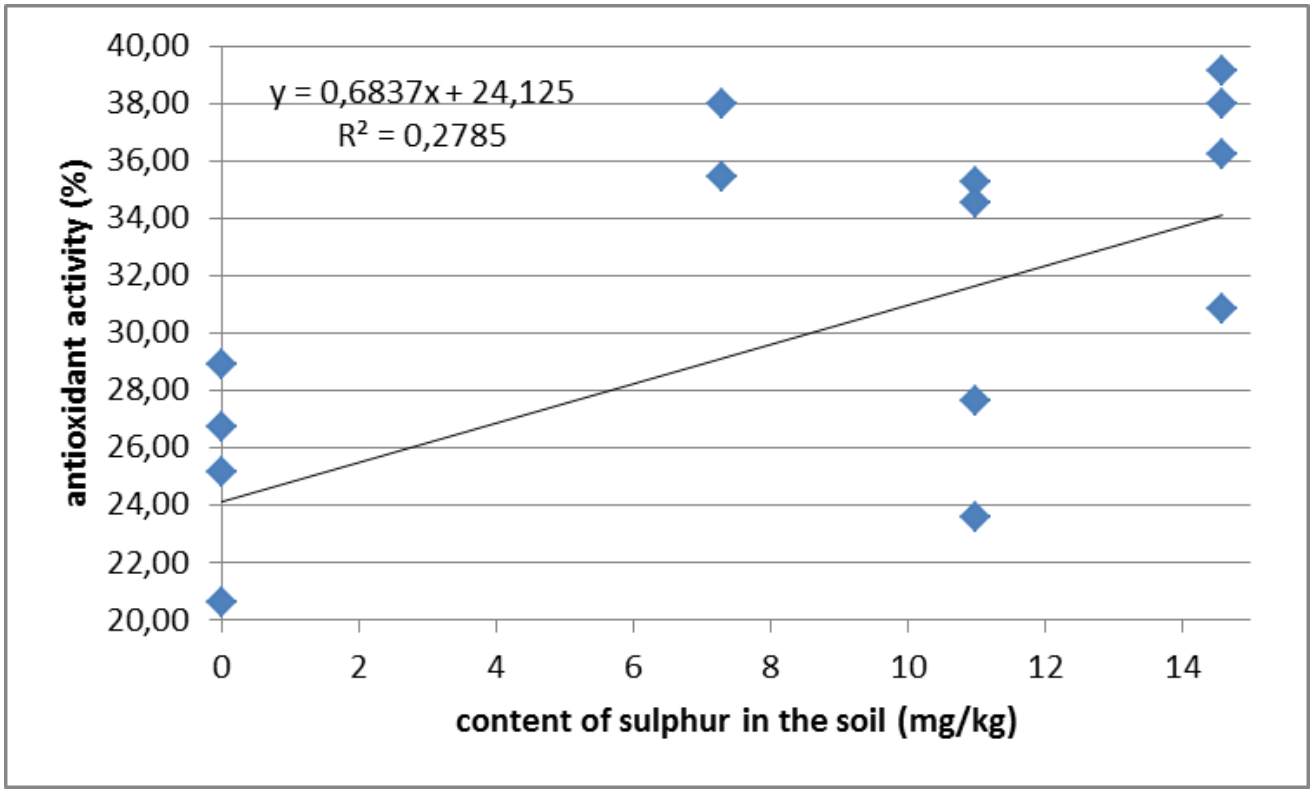

Figure 1 The dependence of the sulphur content in the soil of the AOA (II. sampling).

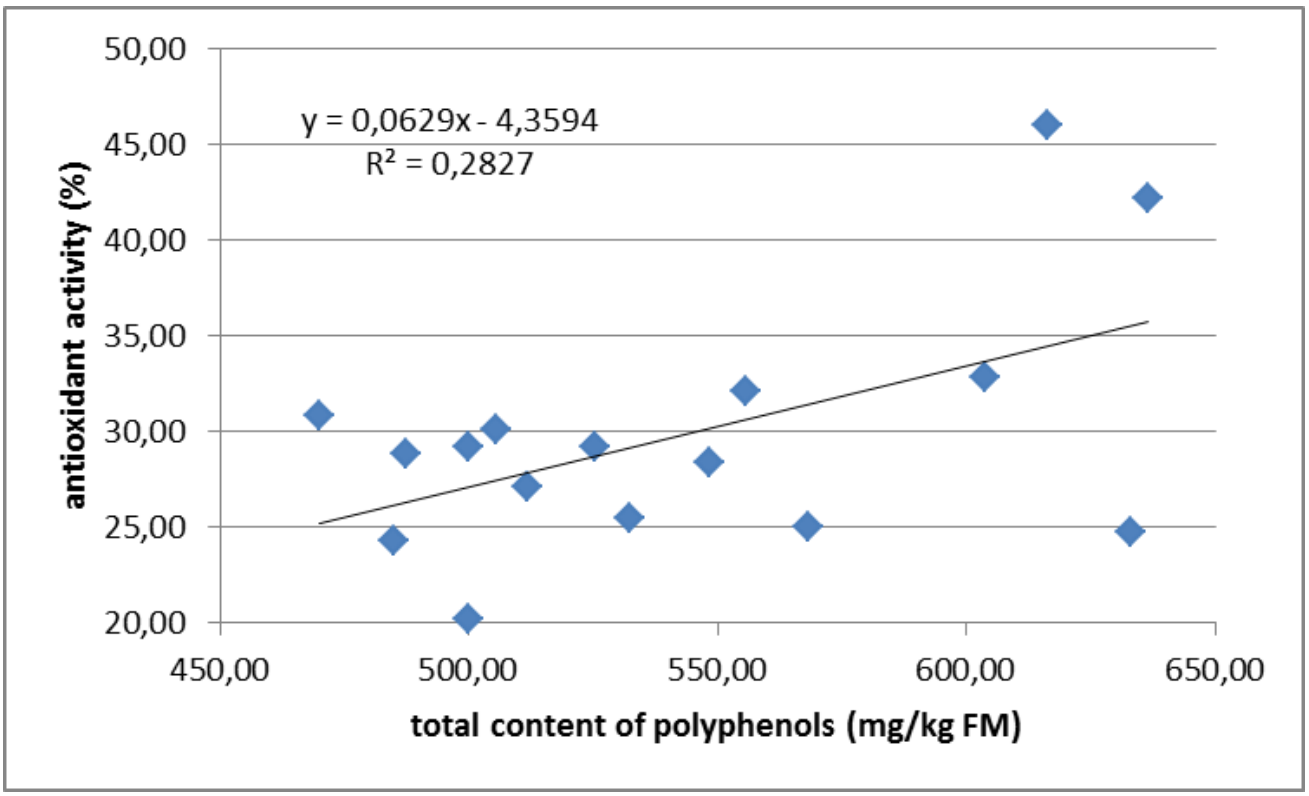

Figure 2 Relationship between TPC and AOA (I. sampling).

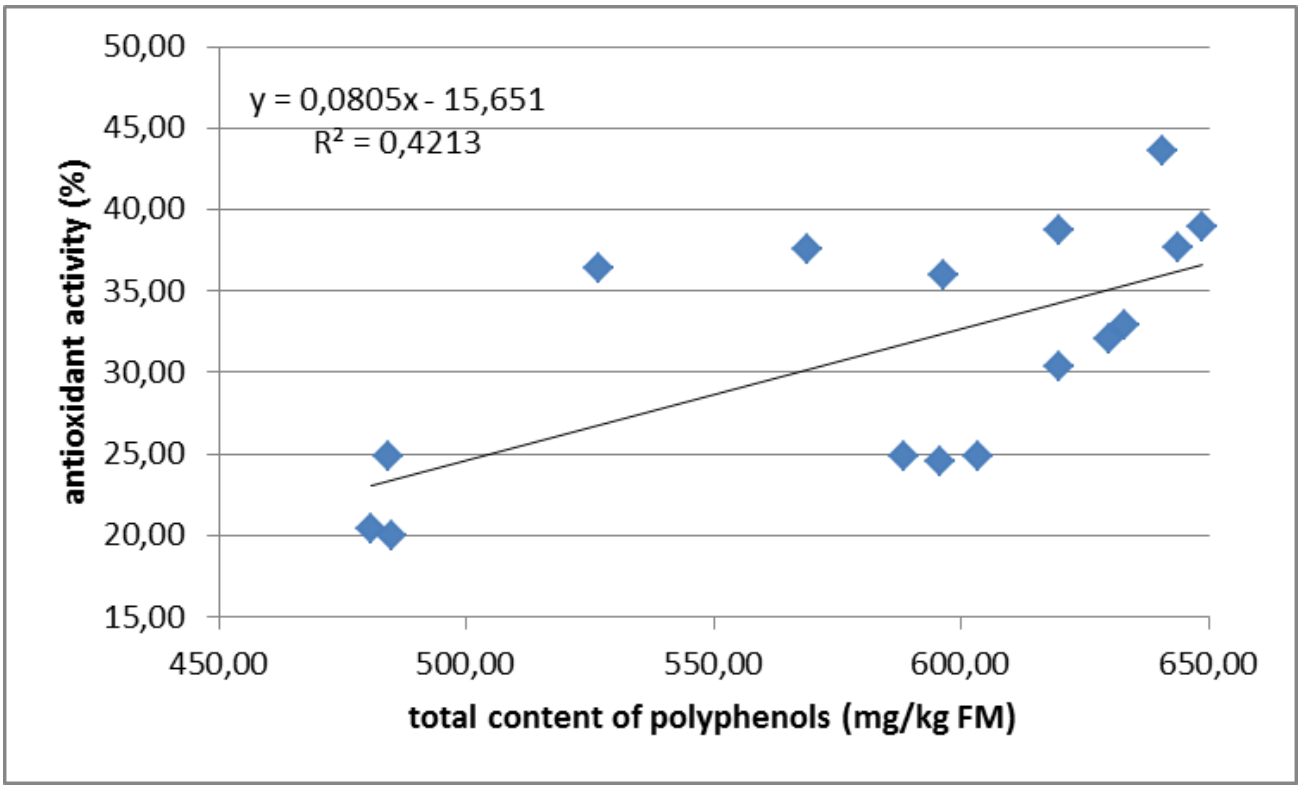

Figure 3 Relationship between TPC and AOA (II.sampling).

Table 3 Dynamics of changes TPC ($mg.kg^{-1}$) in onion after sulphur application.

Variety	I. sampling	II. sampling	III. sampling
Control	545.0 ±19.97	615.73 ±20.15	621.49 ±13.41
Added S1	508.16 ±27.59	573.0 ±12.16	587.0 ±12.77
Added S2	493.57 ±18.86	415.41 ± 13.32	494.05 ±21.80.
Added S3	622.45 ±15.27	580.68 ±19.88	638.32 ±12.84

Table 4 Dynamics of AOA (%) in onion after sulphur application.

Variety	I. sampling	II. sampling	III. sampling
Control	27.92 ±3.36	25.34 ±3.49	30.05 ±3.59
Added S1	25.51 ±4.13	27.35 ±10.85	30.77 ±6.99
Added S2	29.20 ±1,61	30.25 ±5.62	25.41 ±7.67
Added S3	36.43 ±9.56	36.03 ±3.67	39.72 ±2.64

In our experiments the process of total polyphenolic content formation after application of different sulphur levels was observed in onion bulbs during vegetation period. In the work we watched also the influence of sulphur fertilization on the antioxidant activity.

Table 3 shows the progress of making the total polyphenolic content in different levels of sulphur fertilization in onion during vegetation.

There are many scientific works **(Nasreen et al., 2007; Forney et al., 2010, Fatma et al., 2012)** dealing with the influence of sulphur fertilizer on the yield and quality of the onion, but already the influence of sulphur fertilizer on the formation of common total polyphenol content and antioxidant activity are devoted to less work. Our values of total polyphenolic content during vegetation period were in range from 508.16 ±27.59 $mg.kg^{-1}$ to 638.2 ±12.84 $mg.kg^{-1}$. The highest values of total polyphenols were recorded at the end of vegetation period in all variants. The slight decrease in the value of the total polyphenols against first sampling (about 15.8%) was

recorded in second sampling (415.41 ±13.32 mg.kg^{-1}) in variant II (incorporation of sulphur in quantity of 11 mg S.kg^{-1} soil). Increasing tendency to the formation of the polyphenols is not in relation with results, which were obtained in 2013. As is well known the content of polyphenols is affected by factors such as cultivar, growth conditions, use of fertilizers and climate **(Vagen and Slimestad, 2008; Dangour et al., 2009)**. Vegetation period was extremely dry in 2013, but in 2014 the vegetation period was extremely wet with storm character.

Onions require relatively high levels of available sulphur. Sulphur fertilization had a significant effect on the quality of the onion bulbs **(Jaggi and Dixit, 1999; Lancaster et al., 2001)**. **Imen et al. (2013)** reported that the sulphur fertilizers increased the content of total polyphenols.

The highest content of total polyphenolics was measured at the end of the vegetation period (638.32 ±12.84 mg.kg^{-1}) in third collection (incorporation of sulphur in quantity of 14.6 mg S.kg^{-1}soil), this increase was statistically significant (*P-value = 2.10^{-3}*).

In the work we watched the influence of sulphur on the antioxidant activity (Table 4).

Similar progress in the dynamics of antioxidant activity in the value formation was determined. The highest values of antioxidant activity were recorded at the end of vegetation period in all variants. The highest value 39.72 ±2.64% was measured in third collection ((incorporation of sulphur in quantity of 14.6 mg S.kg^{-1}soil), this increase compared to the control variant was statistically significant (*P-value = 2.10^{-3}*). In our results (in I. sampling) we have seen a slight decrease in the value of the antioxidant activity in variant I. (incorporation of sulphur in quantity of 7.3 mg S.kg^{-1} soil), but statistically significant relationship was not recorded (*P-value >0.05*). Between the content of the sulphur in the soil and antioxidant activity (in II. sampling) we have seen a slight positive correlation (*P-value = 3.10^{-2}*) (Figure 1). At work we have found positive correlation between the content of the total polyphenols and antioxidant activity (*P-value = 3.10^{-2}, P-value = 6.10^{-3}*) (Figure 2, 3). These results are in good accordance with **De Pascale et al. (2007)**, who referred to the positive effect of using sulphur-based fertilizers on the total polyphenolics content and antioxidant activity.

CONCLUSION

The current study demonstrated that onion is a rich source of bioactive polyphenolics substances. Different doses of sulphur player important roles on the growth and on the level of the polyphenolics substances in onion. Results of our experiment revealed that doses of sulphur did not have unique effects on the content of the total polyphenols and antioxidant activity. Application of 14.6 mg S kg^{-1} (44 kg S ha^{-1}) resulted in the highest content of the total polyphenols and the highest value of antioxidant activity. In the next research of the influence of sulphur fertilizer on the bioactive components it should be presented complemented results of the influence of other bulbs of applied doses of sulphur and attempts to expand on the small areas cultivation.

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EVALUATION OF INFLUENCE OF THE LOCALITY, THE VINTAGE YEAR, WINE VARIETY AND FERMENTATION PROCESS ON VOLUME OF COOPER AND LEAD IN WINE

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ABSTRACT

We have focused on the influence evaluation of the locality, the vintage year and fermentation process on the volume of copper and lead into grape must and wine. First of all copper and lead volume was assessed into fresh grape musts. Subsequently the musts were fermented. During the wines analyses we found great decrease of copper by the fermentation process. Assessed Cu^{2+} values vary from 0.07 to 0.2 mg.L^{-1} and represent a decrease of the original copper volume from 90 to 97%. On the copper content into grape has probably the significant influence also the precipitation amount, which falling in the second part of the vegetation half a year. Total rainfall in the period before the grape harvesting (the months of August - September**)** was for the first year 153 mm and for second year 137,5 mm. During both observed vintage years it was concerning to the above average values. Copper is not possible to eliminate totally in the protection of the vine against fungal diseases, because against it does not come into existence resistance into a pathogen. For resolution of this problem it is suitable to combine the copper and organic products. Fermentation affect as a biological filter and influence also lead volume. Into analysed wines we found the decrease of the lead volume from 25 to 94%. Maximal assessed Pb^{2+} value into wine was 0.09 mg.L^{-1}. The linear relationship between lead and copper into grape must in relationship to the lead and copper into wine was not statistically demonstrated. We found the statistically significant relationship in lead content into grape must by the influence of the vintage year, which as we supposed, it was connected with the atmospheric precipitation quantity and distribution during the vegetation. On the base of the assessed results of the lead and copper volume into wine, we state that by using of the faultless material and appropriate technological equipment during the wine production, it is possible to eliminate almost completely the problems with these heavy metals content into our wines and it is from the view of technological as well as hygienic.

Keywords: Viticulture; grape must; wine; heavy metals; copper; lead

INTRODUCTION

Intensive soil cultivation is oriented mainly on the economic effect achievement. This can cause to insufficient ecology consideration and subsequently to the environment damage with the toxic elements from the fertilizers, composts and pesticides. In the past we observed such behaviour with the agricultural soil handling. In the present time a view on the ecology and healthy environment is totally different. The grapevine is cultivated mainly by the integrated and environmental friendly way. However the agricultural soil can contain contaminates residues from the past. These contaminants enter after the crop plants intake into the food chain. It belongs here copper and lead.

Mineral substances content into grape must is very unstable value, which is not influenced only by the original amount coming from the vineyard and external environment, but also by the application of the additive matters and residues from the technological machines equipment (Table 1).

Table 1 The highest allowed amounts of the lead and copper into fruit musts, grape musts and wines within the meaning of the valid legislative (*Codex Alimentarius*).

Element	Fruit musts (mg.L^{-1})	Wine (mg.L^{-1})
Lead	0.05 (1)	0.2 (2)
Copper	5.0 (1)	1.0 (3)

(1) Regulation of Ministry of the Agriculture and Rural Development of the Slovak Republic and Ministry of Health of the Slovak Republic from 11 September 2006 No. 18558/2006-SL, by which it is issuing the Chapter of Codex Alimentarius of the Slovak Republic, regulating food contaminants as amended by.

(2) Commission Regulation (EC) No. 1881/2006 from 19 December 2006, by which are setting maximum levels for certain contaminants in foodstuffs.

(3) Commission Regulation (EC) No. 606/2009 from 10 July 2009, from which are laying down certain detailed rules for implementing Council Regulation (EC) No. 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions.

MATERIAL AND METHODOLOGY

After sampling of the biological matter, we processed the grape by the uniform way. Unclarified must (Samotok - juice extract from grapes gained by using only its own weight) was not in touch with any contaminant during whole working process and it could not be enriched with some of observed elements. Into obtained must we measured (1) the sugar content in $kg.hl^{-1}$ on the Slovak Normalized Must-Weight Scale (Slovak: Normalizovaný muštomer), which indicates how many kilograms of sugar are contained in one hundred liters of grape juice, according to Slovak Technical Standard (STN) 25 7621 with modification of the measured values according to the temperature correction, (2) the total acid content by titration (STN 56 0216) and (3) pH potentiometrically.

The observed chemical elements content into must we assessed, after wet way burning according to STN 2676/1990 (Nitric acid, Perchloric acid, Sulphuric acid) by atomic spectrophotometer according to the EU certified method (2676/1990). We worked with analyser SP-9 (PYE-UNICAM). From the reason of the errors elimination by possible heavy metals contamination from the chemical reagents, we made a blank test.

Into fermented must we observed the biodegradation of lead and copper by fermentation process. We performed the analyses after complete musts fermentation, in both observed vintage years 60 days after the grapes pressing. The state of the musts fermentation we measured by assessing of the alcohol content *ebullioscopically.*

The obtained results we evaluated statistically by the analysis of variance and they are listed into enclosed Tables and Graphs. Each measurement we performed four times and we evaluated them by the analysis of variance method.

Experimental sites characteristic

Established

The experimental site is situated at an altitude of from 150 to 250 m.a.s.l. in the central part of the river Nitra. The average annual air temperature is 7.4 °C, during the vegetation period is 13.6 °C. The average total annual rainfall is 593 mm, during the vegetation period is 338 mm. The average annual sunshine duration is 2 200 – 2 400 hours, during the vegetation period is 1 500 – 1 600 hours.

Soil type: typical rendzina, brown rendzina. They are medium-heavy soils and heavy soil, medium textured soils (skeleton content 25 – 50%), medium deep, with slope 12 – 17°.

Spacing: 3x0.9 m. Keeping of grapes: the simple drape grapes. Rootstock**:** Kober 5 BB.

Dolné Krškany locality

The interested area is situated at 150 – 200 m.a.s.l. on the moderately undulating plain to upland. Average annual temperature is 9.7 °C, during the vegetation period 16.6 °C. The average total annual rainfall is 595 mm and during the vegetation period is 333 mm. The average annual sunshine duration is 2 300 – 2 600 hours, during the vegetation period is 1 600 – 1 700 hours.

Soils type includes carbonate chernozem. From the soil granularity view it is concerning to clay – loam soil, moderately deep, with slope 15 – 20°.

Spacing: 3x1.1 m. Keeping of grapes: the simple drape grapes. Rootstock: 5 BB.

Experimental varieties characteristics

Rulandské b i e l e (Pinot blanc)

It is concerning to the variety of French origin, which is cultivating in Alsace from 14 century. It belongs to the Burgundy (Slovak: Burgundské) family and according to the French resources; it was created as burgeon variation from Burgundské sivé (Rulandské šedé). Geo-ecological variety groups: Vitis vinifera L., subspecies sativa D.C., proles occidentalis Negr.

Bunch of grapes is small to middle size (100 – 120 mm long), cylindrical, the stem creates one main spindle, with one to two simple short ailerons near the base. Berries are densely deployed. It belongs to the moderate early variety. The beginning of the blossom accounted for 4^{th} – 16^{th} of June, the beginning of the berries ripening for 6^{th} – 22^{nd} of August and grapes harvesting for 25^{th} September – 10^{th} of October.

Rulandské biele belongs to the groups of variety using for production of the high quality white wines. For achievement of the full wines with a varietal bouquet, which is reflected more in the taste than in the scent, it is demanding relatively high sugar content of the grape must. Its quality acquires fine bouquet by ageing **(Pospíšilová, 2005)**.

S v ä t o v a v r i n e c k é (St. Laurent)

The origin of this variety is not accurately known. Originally it was reproduced as a table variety in Germany. Geo-ecological variety groups: Vitis vinifera L., subspecies sativa D.C., proles occidentalis Negr. Bunch of grapes is moderately large (120 - 150 mm long). Spindle creates aileron near the base. It is cylindrical or cylindrical-conical. The beginning of blossom accounted for 9^{th} – 26^{th} of June., the beginning of the berries ripening for 11^{th} – 28^{th} of August grapes harvesting before 10^{th} of October. With a view to the early ripening it not required top-class positions **(Pospíšilová, 2005)**. However in cooler positions it increases the acid content into berries, what into red wine is not accepted by consumers.

RESULTS AND DISCUSSION

Copper

Copper intake by vitis is estimated for 50 – 100 $g.he^{-1}$ per year. The optimal content into leaves ranges from 10 – 20 ppm. Fertilising is not necessary, while is used 1 – 2 copper fungicide sprayings, what is sufficient to the normal vitis demand **(Vanek, 1996)**. In the past, the Cu^{2+} compounds played a very important task, because they were the most often used for vitis protection, mainly Bordeaux mixture ($CuSO_4$ + $Ca(OH)_2$) against downy mildews (Plasmopara viticola). Traditional vineyard soils, generally contain higher copper resources then soils with the other cultivated plants **(Pfeiffer and Rupp, 1993)**. Therefore it is important that the interval between the copper feeds application and the grapes harvesting is the longest as it is possible. Although it is possible to decrease

copper content into wine by the appropriate technological measures, **Dersch (1993)** recommends spraying by the copper feeds to restrain. If the vineyards are spraying by the fungicides before grapes ripening, it is possible to import the unknown tone and double copper content into wines.

At high copper concentrations in foodstuffs as consumed there may cause the serious changes in the homeostatis body balance. Important is also relationship between copper to iron metabolism and zinc **(Bencko et al., 1984)**. In several samples of unfermented musts from the year 1994 **Bujdoš (1996)** found more than 5 mg.L^{-1} of copper.

Into musts from Oponice and Nitra localities, we determined values Cu^{2+} in the range from 1.06 to 1.74 mg.L^{-1}, in both observed vintage years. Up to double copper amount (3.34 mg.L^{-1}) we determined in one case of Burgundy white wine in Krškany locality. Neither in this case was not exceed the highest allowed amount (5 mg.kg^{-1}), what means the copper content assessed by the Codex Alimentarius (Table 1). By this means highly demonstrated increase was probably caused by the vineyard spraying with sulphate product during bunch closuring. The highest allowed Cu^{2+} amount for the copper content into fruit juice, which is assessed by the Codex Alimentarius, is valid also for the table grapes must, which is consumed in the fresh state. **Ailer (1997)** find out into fresh musts of the variety Chrupka biela, Irsay Oliver and Julski biser Cu^{2+} values in the range from 1.06 to 1.35 mg.L^{-1}.

Application of the copper oxide pesticides significantly influence the copper content into must. **Michlovský and Hanák (1990)** compared the copper content into must of the interspecified vine varieties with limited chemical protection with noble varieties of Vitis vinifera. For a variety of Bianca, the above mentioned authors found out 0.28 mg.L^{-1} Cu^{2+}, what is in comparison with our results lower value and in the noble variety of Rizling vlašský with full chemical protection up to 6.55 mg.L^{-1} Cu^{2+}. **Ailer (1999)** observed the influence of the complementary extra-root nutrition on Cu^{2+} content into must. He used two different leaf fertilizers. Results of his three-year research were ranging in the interval from 0.53 to 1.69 mg.L^{-1}. **Henze and Bauer (2004)** indicate into red Swiss wine the values of Cu^{2+} 0.0528 mg.L^{-1} and into white Spanish wine values of Cu^{2+} 0.0555 mg.L^{-1}.

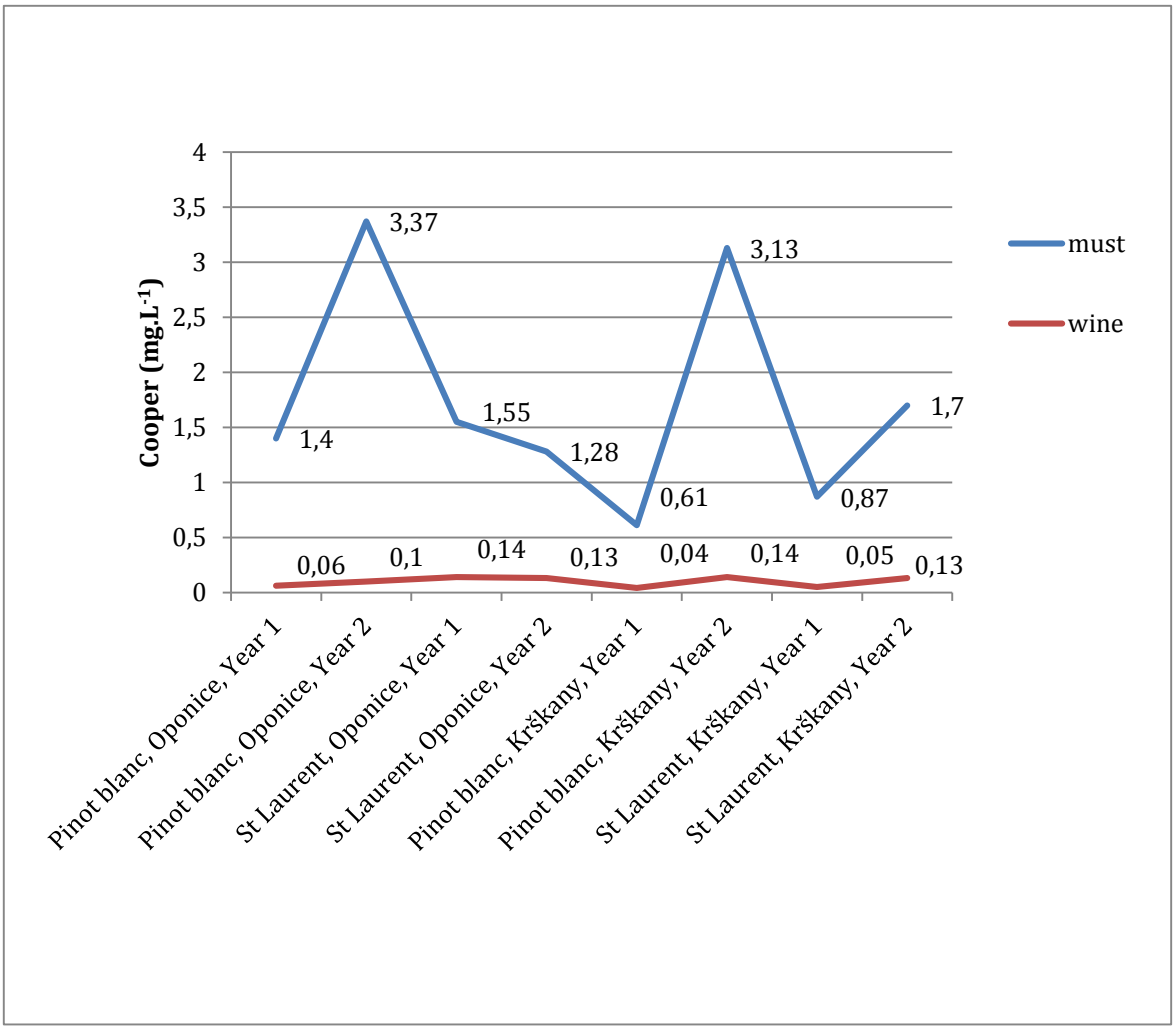

Figure 1 Content of copper in different variants – must and wine (mg.L^{-1}).

Table 2 Results of lead assessment individual variants.

Locality	variety	period	Content of lead (mg.L^{-1})	
			must	wine
Oponice	Pinot blanc	Year 1	0.071	0.004
		Year 2	0.006***	0.003
	St. Laurent	Year 1	0.14	0.04
		Year 2	0.015***	0.002
Krškany	Pinot blanc	Year 1	0.07	0.09
		Year 2	0.021***	0.01
	St. Laurent	Year 1	0.02	0.01
		Year 2	0.016*	0.012

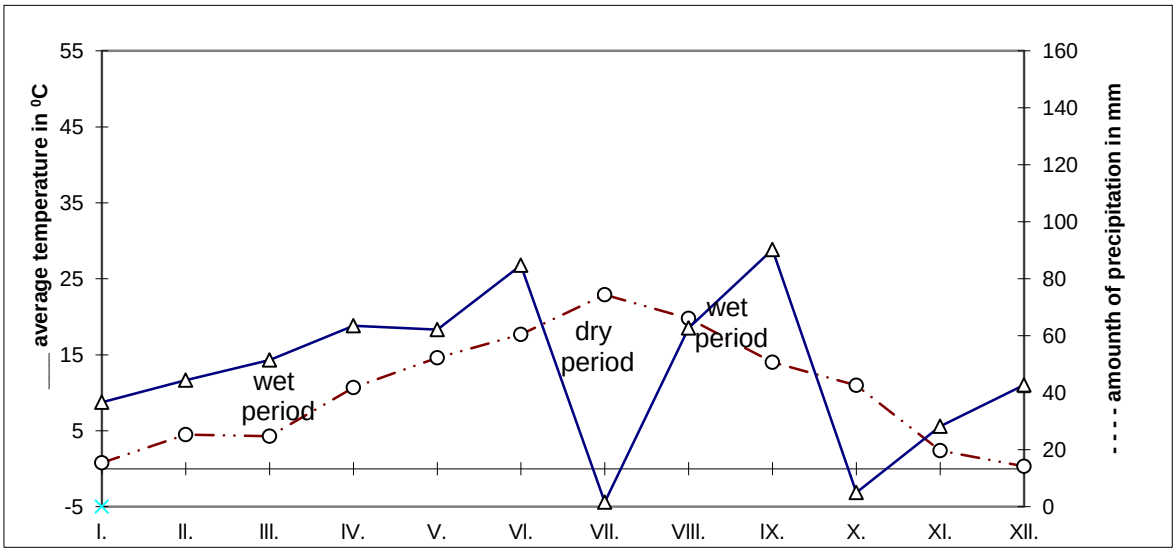

Figure 2 Climatogram of average monthly temperatures and precipitation in the subject area - Year 1.

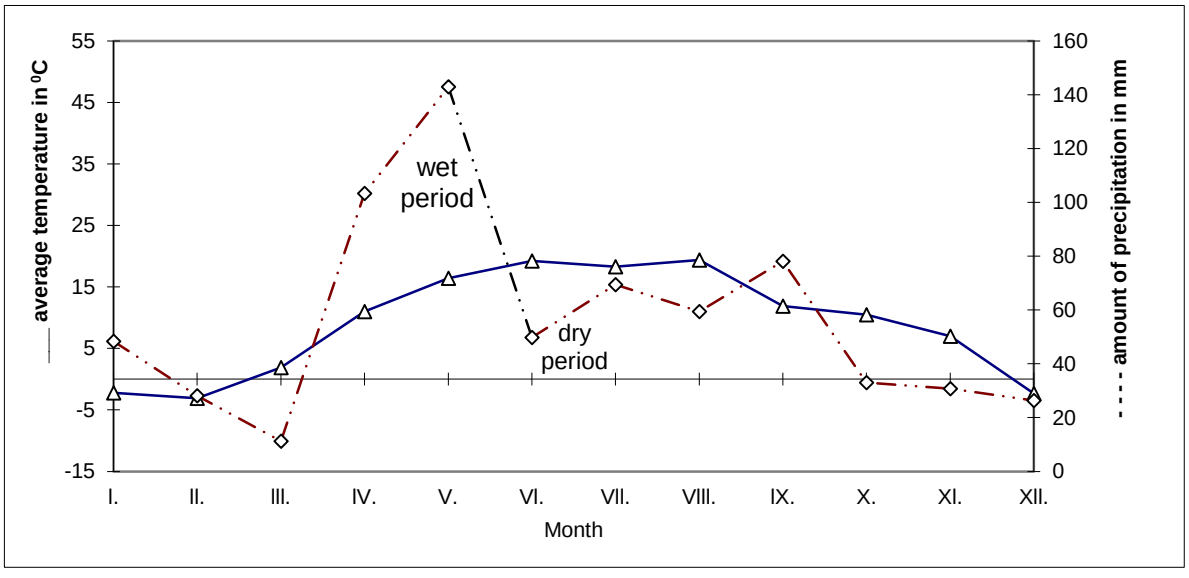

Figure 3 Climatogram of average monthly temperatures and precipitation in the subject area - Year 2.

On the copper content into grape has probably the significant influence also the precipitation amount, which falling in the second part of the vegetation half a year. Total rainfall in the period before the grape harvesting (the months of August - September**)** was for the first year 153 mm and for second year 137.5 mm. During both observed vintage years it was concerning to the above average values. Copper is not possible to eliminate totally in the protection of the vine against fungal diseases, because against it does not come into existence resistance into a pathogen. For resolution of this problem it is suitable to combine the copper and organic products. Into Austrian must from the vintage year 1992, the copper content ranges from 0.4 to 0.7 $mg.L^{-1}$. By the tripe application of Kupfer - Fusilan copper fungicide during the time span 11 – 5 weeks before the grape harvesting, the copper content increased on 2.9 to 5.1 $mg.L^{-1}$ **(Dersch, 1993)**. Therefore this chemical element is not totally trouble-free, mainly in the vintage years with strong infectious pressure of the fungiform diseases, those are demanding an intensive chemical protection by the fungicides. We recommend, mainly during the vintage years with dry autumn, to observe the copper content into grape from vineyards, those were treated by the appliances with Cu^{2+} content. It is important that the interval between applications of the copper oxide appliances is the longest as it is possible. For the integrated cultivation of the vine, it is copper content used for the vineyard protection against fungiform diseases defined by the value 3 $kg.he^{-1}$ per year (Figure 1).

Lead

Lead is an important compound of the energetic, but as also the transportation air pollutants. Lead intake by plants from soil depends on the organic matter in soil, pH value, on the quantity of phosphorus, carbonates, calcium and magnesium. In living organisms, the haematopoietic system, the central nervous system, but as well as the digestive tract are especially sensitive to lead. It causes breakdown of red blood cells and inhibits the iron synthesis with porphyrins. It damages the synthesis of acid delta – aminolevulinic, which is one of the basic haem components. It influences negatively on the variety of enzymes, which is associated with blocking SH groups **(Tölgyessy et al., 1989)**. Lead distribution into grape berries observed **Teissedre (1994)**, while atmospheric lead created only 16,3% from the total amount into berry. The other portion of lead (in average 0.058 $mg.kg^{-1}$) came from soil. Lead content into individual berry parts decreased in the order to grape seed – peel – pulp. **Ailer (1997)** found out into fresh musts of the table grapes varieties Chrupka biela, Irsay Oliver and Julski biser, the average lead value 0.035 $mg.L^{-1}$ during the three-year monitoring.

Lead is a classic example that its tolerated limit was changing with advancing knowledge about its toxicity and with its accurate content assessment into grape cultivating in the unspoilt nature. In the year 1953 the International Organisation of Vine and Wine (O.I.V.) assessed the tolerated threshold limit 0.6 $mg.L^{-1}$. In the year 1975 the threshold limit was decreased on 0.5 $mg.L^{-1}$ and in the year 1987 the limit was set on 0.3 $mg.L^{-1}$ **(Bujdoš and Magdina, 1994)**. At the present time the maximal allowed lead amount into wines is allowed on 0.2 $mg.L^{-1}$ and enologists demand that in the following years is projected a possibility of its further content decrease, as far as the technological advances allow it, because it is undesirable component into wine.

Lead content into undrained musts was ranging from 0.006 to 0.124 $mg.L^{-1}$ and several values exceeded the hygienic limits assessed for fruit juices (0.05 $mg.L^{-1}$) enshrined in the Codex Alimentarius. However it was concerning to the wine grapes, those were dedicated for the further wine processing. Into fermented musts, we mostly found a considerable lead decrease by the fermentation process. Assessed limits, those were ranging from 0.001 to 0.115 $mg.L^{-1}$, do not achieved the threshold hygienic limit for lead content into wine (0.2 $mg.L^{-1}$). From our assessments arise that into wines and musts, those were obtained using appropriate technologies, without possibilities of their secondary contamination, it is a content of this risk element into quantities that do not constitute a danger to the consumer health. Influence of the foliar nutrition on Pb^{2+} content into must of the wine grapes varieties observed **Ailer (1999)**. He set the values in the interval from 0.007 to 0.014 $mg.L^{-1}$. **Eschnauer and Ostapczuk (1992)** set the lead values into the young German wines. Assessed value that average was 0.041 $mg.kg^{-1}$, have been very low and authors do not consider the riskiness of this element. **Henze and Bauer (2004)** identified into red Swiss wine Pb^{2+} values 0.0326 $mg.L^{-1}$ and into white Spanish wine specified Pb^{2+} values 0.089 $mg.L^{-1}$.

In South Africa was the average Pb^{2+} content 0.046 $mg.L^{-1}$ into wines of during the years 1995 – 1996. In Argentina it was found into musts in average 0.138 $mg.L^{-1}$ of this heavy metal, while the lead content into soil and its plant absorption are statistically **(Doboš, 1997)** (Table 2).

Copper and lead biodegradation during the alcoholic fermentation

The mineral substances content decrease into wine is explaining by the yeast utilization during must fermentation, by the solubility decrease of some components into alcoholic solution as well as by the wine stabilization process.

Must contains in average from 3 to 5 $g.L^{-1}$ of the mineral substances, but their content into wine is considerable lower (from 1.5 to 3 $g.L^{-1}$). During the analysis of fermented musts we found considerable high copper content decrease by the fermentation process. Assessed values from 0.07 to 0.2 $mg.L^{-1}$ represent a decrease of the original copper content about 90 to 97%, while between its original quantity amount into must and its final content into wine is not a direct correlation. Whereas the subject of our analysis were fermented, undrained musts in the laboratory conditions, it can be assumed, that by the wine lees extraction the copper content decrease even considerably. In the consequence of this positive fact, it can be almost completely eliminated the possibility of the maximal permissible Cu^{2+} content values exceedance into wines.

It is positive that the fermentation acts as a biological filter also on the lead content. In the analysed fermented wines we found the decrease of the lead content about 25 to 94%, while in most cases it was more than 50%. A large part of these metals is eliminated during the fermentation in the insoluble sulphides form, because of what it is not the limit value exceeded into wines.

CONCLUSION

For intensive agro-technology it is not possible to eliminate completely the negative interventions in the biological environment equilibrium. The individual agro-technological interventions it is therefore possible to realize in the appropriate agro-technologic periods and restrict them on the minimal, however sufficient amount for healthy grapes production without cultivation risks. By using of the faultless material and appropriate technological equipment during the wine production, it is possible to eliminate almost completely problems with copper and lead content into our wines and it is from the view of technologic and hygienic. The analysed wines did not exceed the highest permissible amounts of copper and lead assessed by the valid legislation.

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THE EFFICIENCY, ENERGY INTENSITY AND VISUAL IMPACT OF THE ACCENT LIGHTING IN THE RETAIL GROCERY STORES

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ABSTRACT

Over the last few years, topics of displaying, presentation, lighting, energy saving and issues related to the environment while selling the fresh food (fruits, vegetable, bakery products, meat) are becoming an important matter among traders. However, just bigger companies with transnational capital have devoted their attention to this issue yet. Generally, the energy costs make up 70% of operating costs in retail stores where the cooling system and lighting are the most energy consuming. Accent lighting in modern retails is largely involved in the overall design and atmosphere in shops and plays a crucial role in presenting the goods as well. Using of accent lighting can draw the customer's attention to a specific part of the sales area and achieve the overall harmonization in the store. With the rational using of combination of energy saving and effective accent lighting retailers can achieve not only attractive presentation of displayed products but also appreciable savings in the operation of their stores. It is the only factor that can be exactly measured and controlled. Using a Colour and Lux Meters we found out the intensity and color temperature of accent lighting used in domestic and foreign retail chains for the different kinds of fresh food products. Based on the obtained values we have compiled graphs, which are showing visual comfort. We also identified different types of accent lighting, which we assigned to their impact on emotional involvement of consumers. The starting points were the tests we conducted in simulated laboratory conditions. While searching of a compromise between effective and energy efficient accent lighting we take into consideration consumers' emotional response as well as the annual electricity consumption of different types of light sources. At the end we recommend options for energy-efficient, effective and spectacular lighting while using the optimal number of light sources and their logical organization, automatic control as well as using energy-saving LED lights or practical skylights. Improvements in energy efficiency of technical equipments could reduce the operating costs for retailers as well as enhance the attractiveness of displayed goods and customer satisfaction and thereby increase their sales.

Keywords: retailing; efficiency; visual impact; innovation; accent lighting

INTRODUCTION

Retailing consists of the final activities and steps needed to place merchandise made elsewhere into the hands of the consumer or to provide services to the consumer. In fact, retailing is actually the last step in a supply chain that may stretch from Europe or Asia to your hometown **(Dunne et al., 2013)**. Retailers play a major role in creating and adding value to final goods and services. This ensures that customers needs and wants are satisfied **(Cant, 2005)**. In today era retailing combines the function of retail and wholesale with the function of internal and external trade within one business entity **(Cimler et al., 2007)**. Every retail business, regardless of its financial background is associated with the application of various marketing tools. All the marketing tools that are applied in terms of retail chains are called retail marketing. Retail marketing can be considered as marketing, which can be applied in retailing. It represents all the activities related to the "4P" marketing (product policy, pricing, communication policy, and distribution policy) performed in retailing **(Horská et al., 2010)**.

There are economic tendencies, which fundamentally change the conditions in the global market **(Nagy et al., 2012)**. **Bárta et al. (2009)** emphasize that it is necessary to mind out the human behavior while the marketing application in business. New revolutionary technologies offer incredible potential for the application of marketing and marketing techniques. The retail market is changing with an unbelievable rate and a lot of businessmen are therefore trying to use innovative ideas, which could help them to distinguish themselves from their competitors **(Hallbauer, 2008). Das (2009)** states that while the consumer can pretend his behaviour, his brain activity tells about the true intentions of his purchase decision. One of the new approaches, which connect knowledge from neuropsychology, cognitive psychology and neuroscience with the environment of marketing decision-making, is called neuromarketing **(Vysekalová et al., 2011)**. Neuromarketing presents the relation of medical knowledge; technology and marketing, which through brain screens can help, understand incentives in the brain and how they are processed **(Lindstrom, 2009)**. Neuroeconomics, neuromarketing, neuroaesthetics and neurotheology are just a few of novel disciplines that have been inspired by a combination of ancient knowledge together with recent discoveries about how the human

brain works **(Legrenzi and Umiltá, 2011)**. However, having the best technology or the highest quality solution does not guarantee that prospects will always buy from you. But exciting new findings in brain research suggest that speaking to the true decision maker, the old brain will raise your effectiveness in communicating an idea or selling a product **(Renvoisé and Morin, 2007)**.

Neuromarketing uses a variety of tools and techniques to measure consumer responses and behavior. These include everything from relatively simple and inexpensive approaches, such as eye tracking (measuring eye gaze patterns), analyzing facial expressions, and behavioral experiments (for example, seeing how changes in product displays affect a consumer's choices), to more complex, sensor-based approaches, including biometrics (body signal measures) that measure perspiration, respiration, heart rate, and facial muscle movement (electromyography [EMG]), as well as neurometrics (brain signal measures) that measure electrical activity (electroencephalography [EEG]), and blood flow (functional magnetic resonance imaging [fMRI]) in the brain (Neuromarketing uses a variety of tools and techniques to measure consumer responses and behavior. These include everything from relatively simple and inexpensive approaches, such as eye tracking (measuring eye gaze patterns), analyzing facial expressions, and behavioral experiments (for example, seeing how changes in product displays affect a consumer's choices), to more complex, sensor-based approaches, including biometrics (body signal measures) that measure perspiration, respiration, heart rate, and facial muscle movement (electromyography [EMG]), as well as neurometrics (brain signal measures) that measure electrical activity (electroencephalography [EEG]), and blood flow (functional magnetic resonance imaging [fMRI]) in the brain **(Genco et al., 2013)**.

The structure and circulation of the space has been determined, the atmosphere and thematics of the space must be created through lighting, sound, materials and visual branding. These design elements have the greatest impact on the consumer behaviour in the store. One of the key requirements of operating equipment of modern retail stores is good quality lighting that enhances the image of these stores, attracts potential customers as well as focuses their attention on the displayed products what results in increasing of sales. The importance of good lighting is often still underestimated. However, lighting is incredibly important to any retail environment. General lighting, lighting to emphasise and coloured lighting can all give the store and the merchandise something extra and make them look more appealing. Lighting can bring drama into the store **(Floor, 2006)**.

Poor lighting can cause eye strain and an uncomfortable experience for the consumer. To minimize the possibility of eye strain, the ratio of luminance should decrease between merchandise selling areas. The next layer will complement and bring focus onto the merchandise; this lighting should be flattering for the merchandise and consumer. The final layer is to install functional lighting such as clear exit signs **(Israel, 1994; Lopez, 2003)**.

A case study of **Areni and Kim (1994)** found out that clearer interior store lighting act more positively on consumer perception in the form of time spent by browsing the goods in the store.

Characteristics of the lighting used in the retail food stores can be designed in various ways. The primary purpose of lighting is to improve the display of merchandise. Lighting systems can significantly increase the positive reaction of the consumer to products on display **(Piotrowski and Rogers, 2007)**. They contribute to make an impression about the visual quality of the environment in stores, but can also be used to disguise poor quality of products offered. Lighting is used not only for food accents lighting products, but also to create a photometric reactions in products to be sold in the stores **(Borusiak, 2009)**.

Store design is a fascinating multifaceted field **(Ebster and Garaus, 2011)**. Retail store design factors into window displays, furnishings, lighting, flooring, music and store layout to create a brand or specific appeal.

All the stores are using basic lighting, which is in many stores combined with the accents lighting. This one can play a decisive role, especially when selling the fresh products. Selecting the appropriate combination of different types of lighting in grocery stores undoubtedly contributes to building the image. One could say that the image is considered as the generalized and simplified symbol, which is based on the interplay of ideas, attitudes, views and experiences of man in relation to a particular object **(Kleinová and Kretter, 2011)**.

The image means that the thing is able to break out of the stereotypical average and become original, unique and different **(Banyar, 2006)**.

Basic lighting does not essentially very among the stores operating on the Slovak food market **(Nagyová and Machajová, 2008)**. Even though, the accent lighting is significant and the only one strong marketing tool for some types of unpackaged fresh food (fruits, vegetable, bakery products and meat products) there are still food store chains which do not attach to this element as much importance as they should do.

To choose strategy of presenting new launched as well as already existing products in retail grocery stores correctly by using the optimal mix of marketing tools, it can significantly contribute to the growth of retail turnover **(Kubicová and Kádeková, 2011)**. The matter of effective presentation of goods in retail stores is closely related to the issue of efficiency and energy consumption. Especially while using the lightning as a significant marketing tool it is necessary to take into account the energy consumption since lighting is one of the major cost items in retail stores. On the food market retailers should focus not only on the impressive presentation of the displayed goods but it is important to seek a compromise between the energy consumption of light sources and their effect on consumer perceptions.

Energy consumption of stores may vary greatly depending on their size and segment offered. By far the most energy power in food retailing is used for keeping food cold as well as for presentation of fresh products **(Horská and Berčík, 2013)**.

Global warming is increasingly changing climatic conditions as well as the natural environment. Combating the climate change has become one of the challenges of

today era. More and more retail stores mainly with the multinational capital ties operate on the market keeping in mind the previous fact. Foreign but also domestic retailers are increasingly becoming aware of the need to protect the environment and climate, which impact their business activities **(Jongen and Meulenberg, 2005)**. That is the reason why they are trying to build projects of so-called green stores using the energy-saving initiatives. Some of them are equipped with new-efficient technologies such as solar panels, wind turbines and geothermal wells of course according to the specifics of each individual store.

MATERIAL AND METHODOLOGY

"The Use of Neuromarketing in Visual Food Merchandising" conducted at the Department of Marketing at the Slovak University of Agriculture in Nitra.

The main objective of this paper is to highlight the efficiency, energy intensity and visual impact of the accent lighting in retail food stores in Slovakia and also to point out the retailing solutions that will contribute to the elimination of negative impacts on the environment. The aim of this paper is also to find a compromise between visual impact and energy consumption of different types of accent lighting in selected food store chains operating on the Slovak market and make proposals for the optimal operating and competitive energy lighting in the stores. While preparing this paper we used available book sources such as professional publications of domestic and foreign authors, print journals as well as information available on the different websites and library publications.

To obtain the underlying data we used:
- digital lux meter - Brand: Mastech, type: MS 6612
- digital color meter - Brand: Minolta II
- mobile device EEG (electroencephalogram) - brand EPOC
- 5 different types of lighting (halogen, metal halide-cool, warm-halide, LED, fluorescent)

The first part of the research was carried out in the field in order to measure values of accent lighting in the retail stores. These were used to compare and identify the different types of accent lighting. The measurements of basic and accent lighting for the different kinds of fresh products were made with the intention to find visual comfort in the most significant food store chains operating in Slovakia. To process the above mentioned we used Luxmeter (brand Master - tech) and Color meter (Minolta II). The obtained data were synthesized for each individual type of lighting and after we created a graph showing visual comfort when buying food in different retail stores. The second part of the research was carried out in simulated field conditions. The laboratory was set up to uncover preferences and emotional involvement of respondents by using different types of accent lighting. During the first phase of the laboratory research there was always couple of respondents who visually evaluated the different types of lighting in simulated conditions by means of a questionnaire survey. In simulated conditions we used five different types of lighting, which were designed to provide about the same luminous flux 600lux (except stand no.3) despite of some differences in power. The second phase of the research consisted of consumers´ neurological tests. The wireless EEG device was set on the respondent´s head to read its brain activity in real time. This device was sending data wirelessly with a frequency of 2.4 GHz to the control center where they were recorded into three software solutions. Individual light sources were used to illuminate the fruit (apples and oranges).

To process the obtained data we used basic methods namely comparison, selection and graphic presentation of data. Calculation of energy indicators is based on the following relation:

$$S = \frac{P * \quad t * \quad d * \quad m}{1000} \text{ in } (€)$$

P - Electric power of device (W)
T - Device´s time of use during the day (hours)
D - The number of days
m - Price per 1kW

Table 1 Technical parameters of different types of accent lighting in simulated conditions.

Station	Light Source	Power	Luminous flux	Colour temperature	Colour rendering index
1	Halogen lamp	160W	650lux	2700K	70CRI
2	Metal-halide lamp	70W	580lux	5000K	60CRI
2	Metal-halide lamp	150W	850lux	3000K	85CRI
4	LED lamp	60W	620lux	5600K	75CRI
5	Fluorescent lamp (tube)	72W	540lux	4100K	64CRI

RESULTS AND DISCUSSION

Based on measurements made within selected retail chain stores we made comparisons of the intensity and color temperature of accent lighting used for the fresh food (fruit and vegetable, bread and bakery products). After this we synthesized the conclusions of efficiency and energy intensity in these grocery stores. As the Figure 1 shows, the highest light intensity for fruits and vegetable is used by the Austrian retail chain Billa. On the other hand the lowest intensity of accent lighting was measured in the supermarket chain CBA as this retail tends to use basic lighting simultaneously as accent lighting for fruit and vegetables. Low values of accent lighting intensity for bakery products were measured at several local chains such as COOP, CBA, Nitra Zdroj but also in some foreign retail chains such as Lidl and Hypernova.

By measuring the color temperature of accent lighting in different retail chains we identified light source used and also the color of light emitted from accent lighting. In Figure 2 you can see that accent lighting used for fruits and vegetable in retail chains Tesco, Kaufland and Hypernova produces the warmest color. The light color

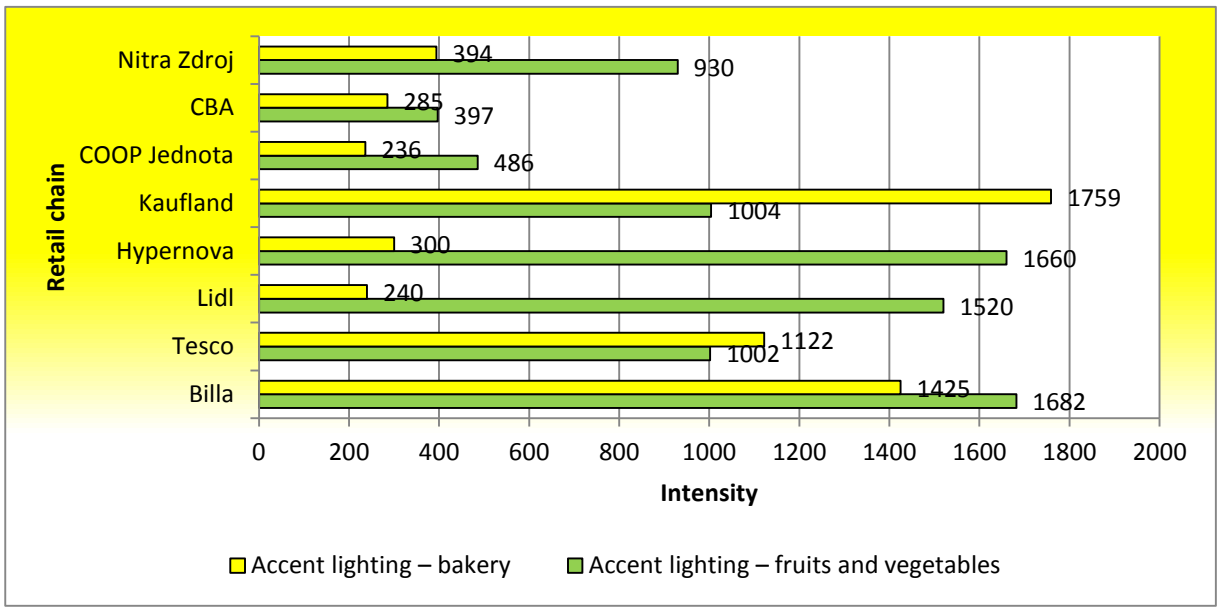

Figure 1 Comparison of accent lighting intensity for fruits, vegetable and bakery products.

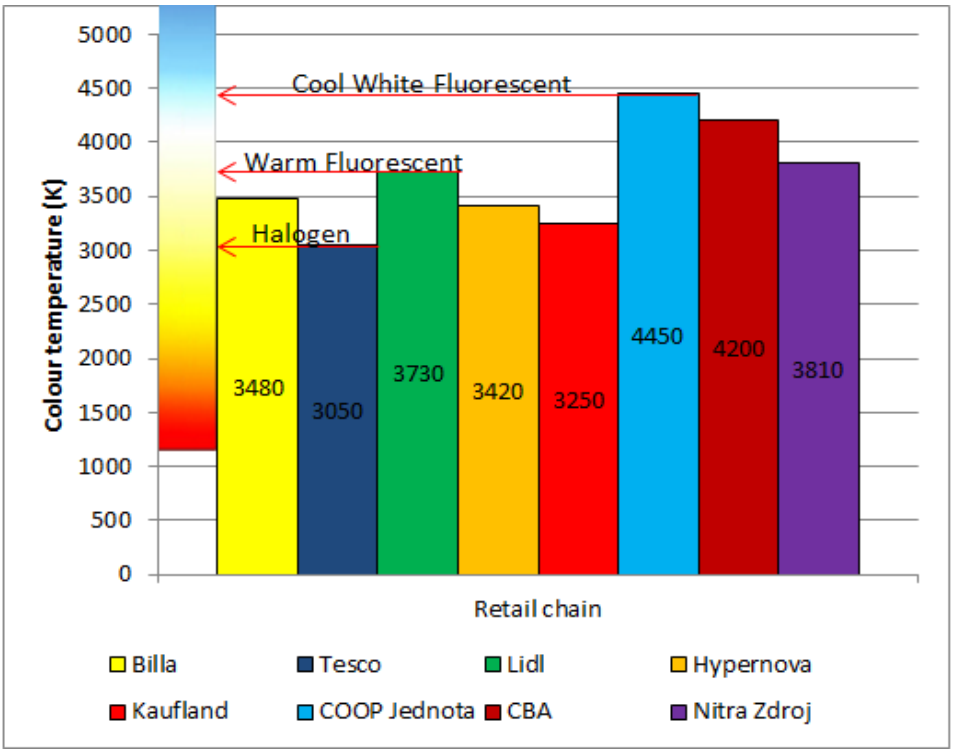

Figure 2 Comparison of accent lighting color temperature for fruit and vegetables.

emitted from the light source is a warm yellow which is typical for halogen light sources or fluorescent lights producing a warm yellow light as in the case of retails Lidl and Nitra Zdroj. Due to the fact that almost no chains operating in the Slovak food market do use LED light source, the coldest accent lighting used for fruits and vegetable has been observed in the COOP. Here the fluorescent light source producing white cold color is used.

As the Figure 3 is showing, for accent lighting used to light up the bread and bakery products retails chains Tesco, Kaufland and Hypernova are using light sources emitting the hottest color light in. Light sources that produce cool colors were used to light up bread and bakery products only in the stores of CBA where the basic lighting is also used as accent lighting for bread and bakery products.

Comparisons of the intensity and color temperature of lighting in five foreign and three domestic retail chains were used to compile graphs of accent lighting visual comfort in these retail food chains.

Based on the observed data and the created graphs of visual comfort we can state that in the case of accent lighting used to illuminate the fruits and vegetable the pleasant environment is typical for the retail chains as it can be seen from Figure 4. On the other hand for accent lighting used for bakery products several deficiencies were found. In Tesco and Kaufland chains there is very bright and unnatural environment causing colors distortion of displayed goods. CBA, COOP and Lidl mostly use accent lighting with a dark color environment for bakery

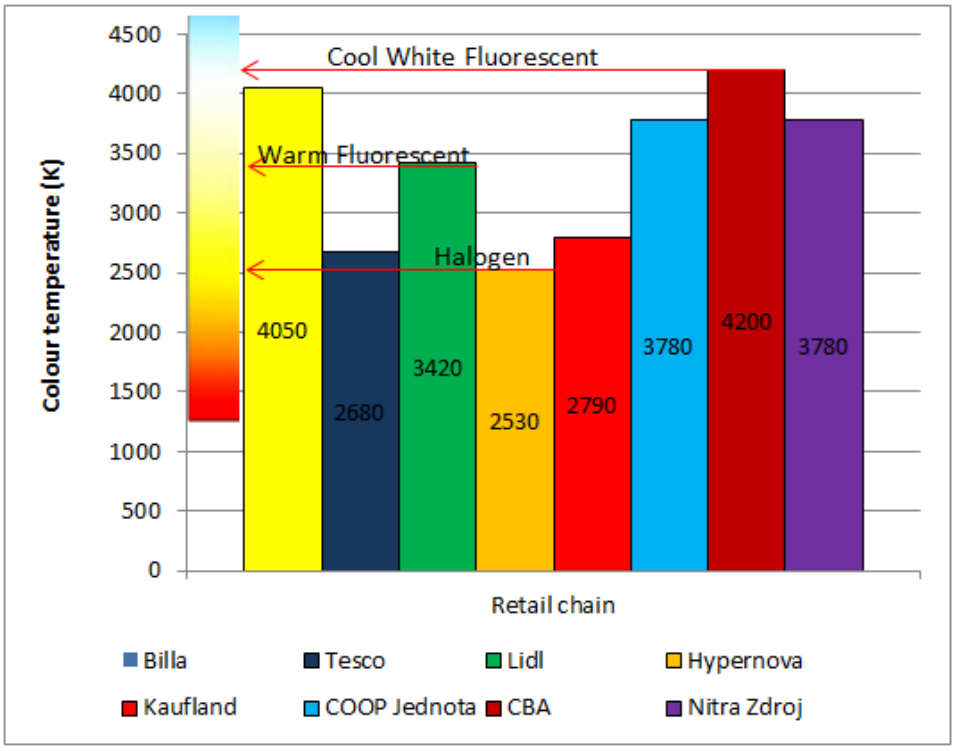

Figure 3 Comparison of accent lighting color temperature for bakery products.

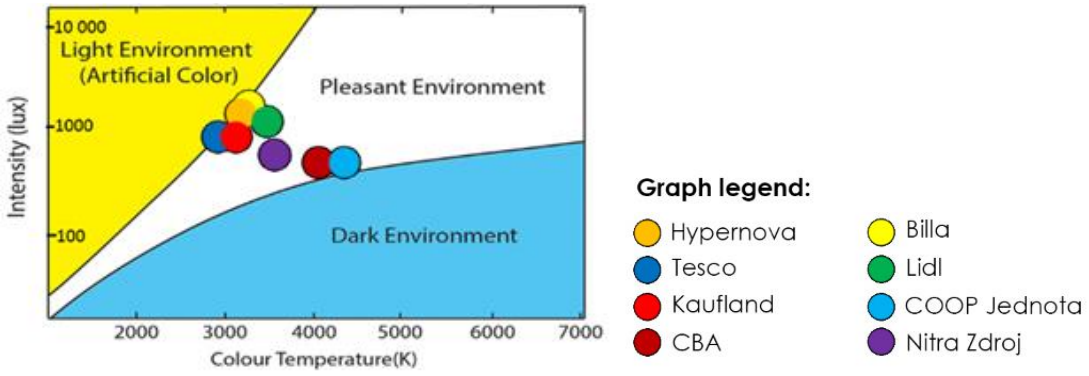

Figure 4 Visual comfort of accent lighting used for fruits and vegetables in retail chains operating in Slovakia.

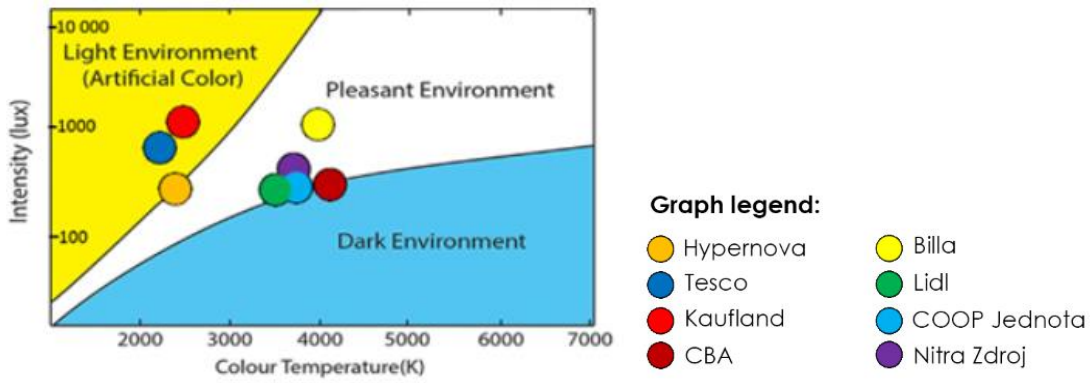

Figure 5 Visual comfort of accent lighting used for fruits and vegetables in retail chains operating in Slovakia.

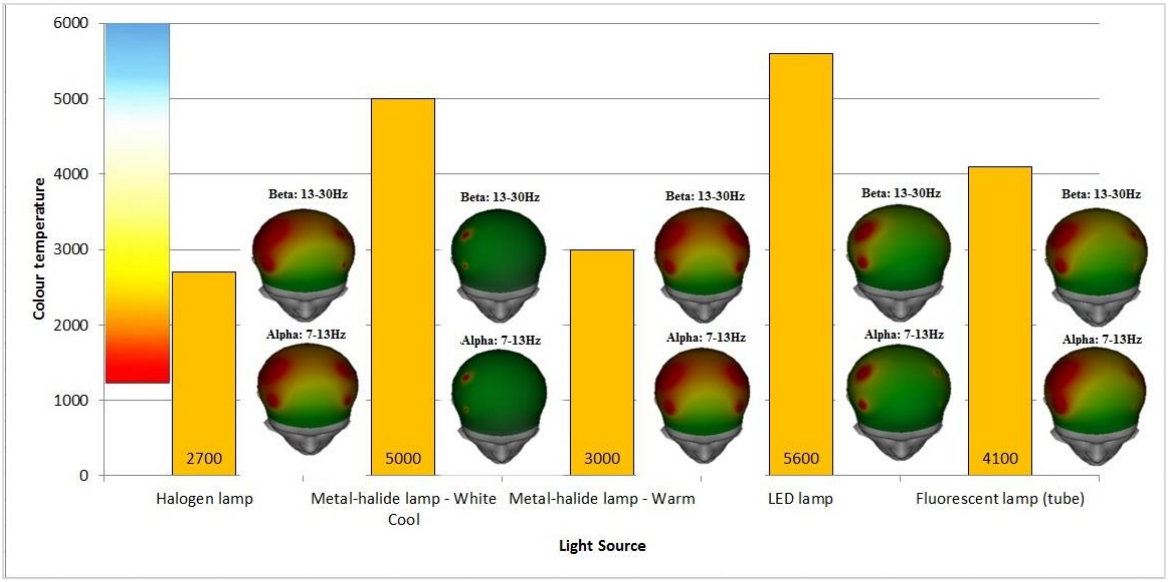

Figure 6 Comparison of respondent's emotional involvement within each type of accent lighting in simulated conditions.

products, which also causes some kind of deficiencies while presenting this type of good (see Figure 5). Lighting in retail chain Billa possibly Hypernova, Lidl or Nitra Zdroj can be considered as the optimal solution. Based on the underlying data and the fact that the stores are not using energy-saving LED light sources we can state that accent lighting creating too bright and unnatural environment contributes to higher energy consumption and lower operating efficiency of these technological resources.

In order to find a compromise among efficiency, energy intensity and visual impact of different types of accent lighting the place with simulated conditions for fresh food sales (fruits and vegetable). Figure 6 shows the respondent's emotional involvement in different types of lighting conditions. The greatest emotional response was observed with using the halogen and metal halide light sources producing a color temperature in the range 2700-3000 K. On the contrary the least impact on the respondent's emotions the cold light sources emitting light: LED and metal halide - cool white cause.

According to the figure 7 the most respondents consider halogen lighting as the most attractive one based on visual evaluation. This type of light source produces warm vibrant colors with a value of 2700K and intensity of 650lx and was used to illuminate the goods under the first stand. However, the disadvantage of this light source is higher energy consumption and its lower lifetime. According to the 40% of respondents the second most attractive type of accent lighting is metal halide lamp with a power of 150W.

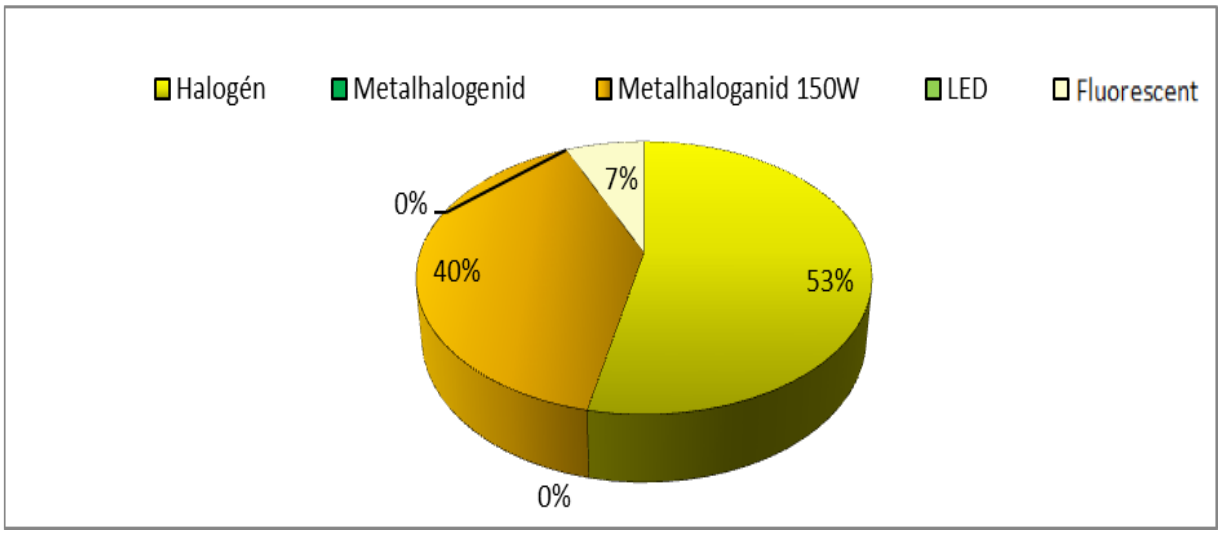

Figure 7 The most attractive accent lighting in simulated conditions - visual evaluation.

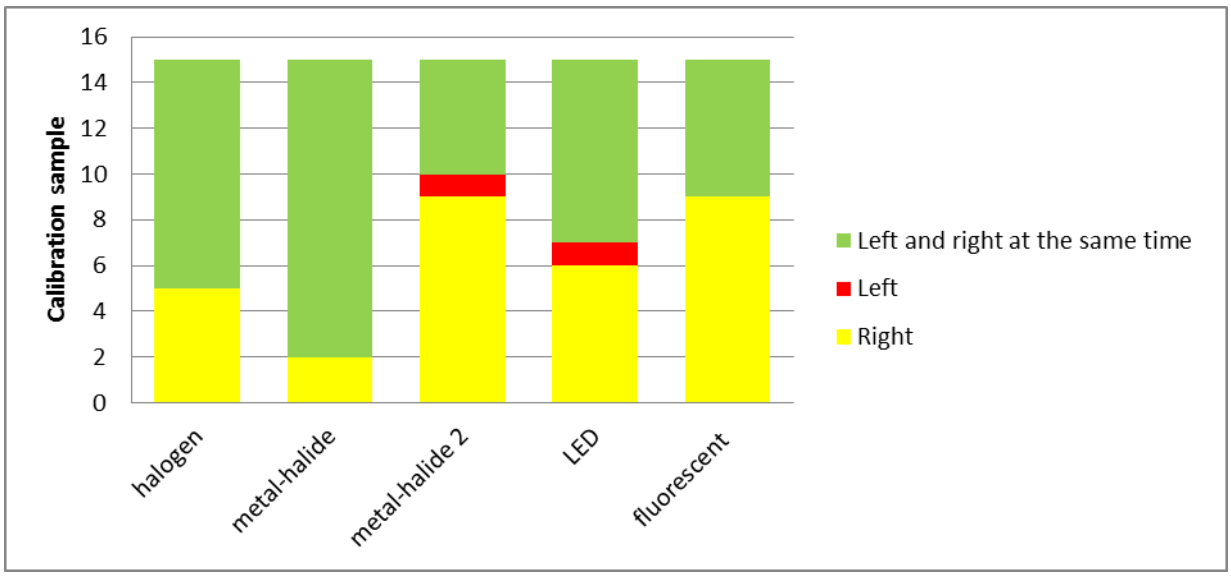

Figure 8 Monitoring the response in the different hemispheres while using different types of lighting - simulated conditions.

Reactions in different parts of the respondents' brain within each type of accent lighting vary what means that the light source does really affect consciously but also unconsciously on consumer response. Watching the origin of the responses in the right and left hemisphere enable us to identify which type of lighting evoked more emotional reactions. The response in the right hemisphere is considered as an emotional response so far the response in the right hemisphere reflects a rational reaction of the respondent. Figure 8 presents the emotional involvement of the respondents for each type of accent lighting.

The most emotional responses arose while using metal halide reflector with 150W. In questionnaire survey consumers rated this one as the second most attractive type of lighting also with fluorescent lighting source. On the other hand the least respondents' emotional reactions arose while using metal halide reflector with power 70W as well as halogen lamps. Paradoxically, the halogen light source was visually rated as the most attractive. Mostly the respondents' brain activity appears on the right side of the human brain, which is emotional hemisphere (less rational hemisphere). Just after this the activity extends also to the other parts of the brain. In general women are more emotional than men and also younger people react more emotional compared to older people. To illustrate in better way the brain activity in its different parts we compared the reactions of five different respondents while watching the fruits (apples and oranges) illuminated with metal halide reflector 150W (Figure 9).

To make this comparison we chose two men and three women in age between 24 and 44 years. Looking at the Figure 9 you can notice that greater emotional response rose in female brain as well as the largest brain activity was measured for the youngest respondent while the same incentive activity.

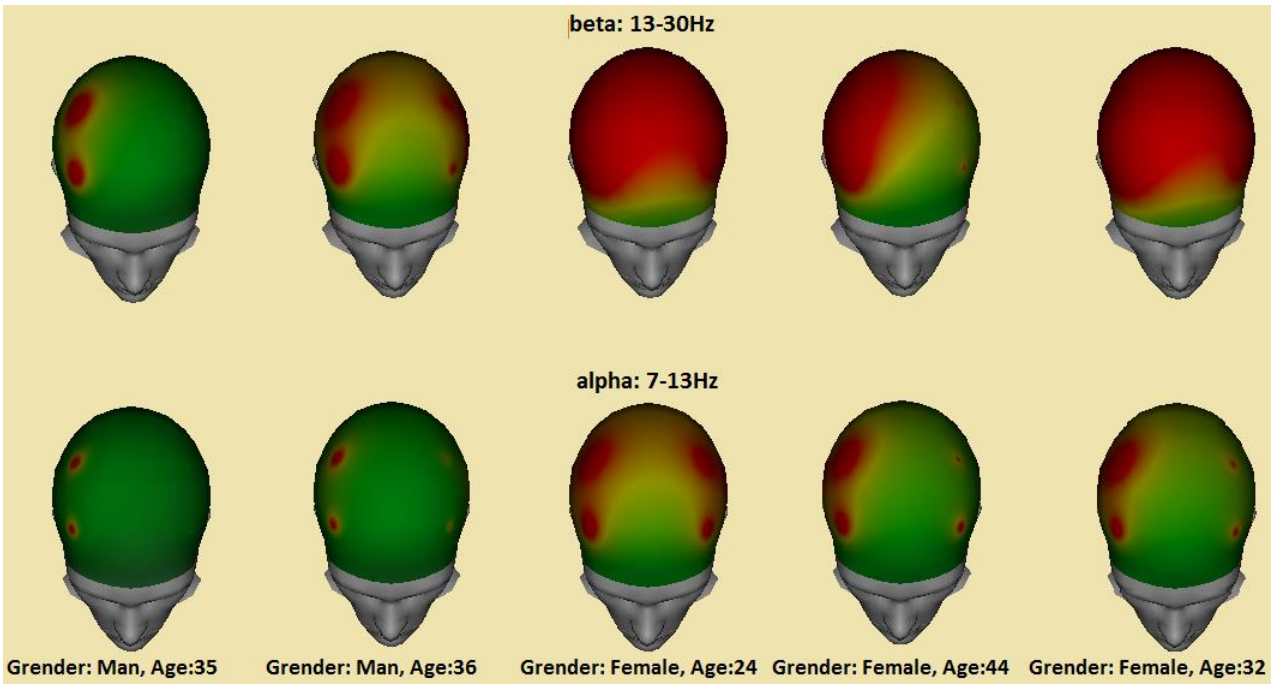

Figure 9 Comparison of respondents' brain activity - simulated conditions.

Table 2 Energy consumption for the different types of light sources.

Type of Light Source:	Halogen	Metalhalid	Fluorescent light source x 2	LED
Power (W)	160	70	72	60
Luminous Flux (lux - lx)	650	580	540	620
Initial Costs (€)	8	53	35	100
Cost of light body replacement in (€)	4.9	13.5	5.3	0
Cost of 30.000 hours operation	73.5	67.5	42.4	0
Lifespan (hours)	2000	6000	8000	35000
Ecology	High energy consumption	Difficult accessories	Mercury, heavy metals	ok
Energy consumption per 1 hour (€)	0.032	0.014	0.0144	0.012
Energy consumption per year (€)	187	82	84	70
Total costs per four year period (€)	826	408	379	280

Continual finding a compromise between reducing the energy consumption while using accent lighting and positive impact on consumer perception of the displayed goods can help trader to achieve savings in the operation of their stores. Lighting of modern retail is largely involved in the overall design and the resulting store atmosphere. The amount as well as type of lighting used in the stores is therefore always different. Anyway, traders are able to regulate their own expenses using the optimal number of light sources, their logical arrangement and automatic control by setting a time sensors either by using of energy-saving LED lights.

Table 2 shows the most common used sources of accent lighting in grocery stores on the Slovak market. Clearly the most widely used type of lighting is fluorescent lighting in Slovakia. The main reason is that it is characterized with relatively low costs and domestic retail chains still do not attach enough importance to the accent lighting. When calculating the energy consumption per four years period it shows the second largest cost savings compared with other types of light source. The negative aspect of this type is the fact that while manufacturing mercury and heavy metals are used what means that it does not contribute to environmental protection. The most energy-efficient is LED lighting according to our test results but at the same time it is characterized with higher initial costs which will return within over 4 years using. The other two types of lighting are used mostly in the international chains operating on the Slovak market food. There is more importance attached to their marketing effect than their energy efficiency.

CONCLUSION

Accent lighting plays an important role in each retail food store while presenting fresh products (fruits, vegetable, bakery products, meat) since it substitutes the packaging promotional function and significantly contributes to the increasing of the attractiveness of the displayed goods. Lighting is not only an important marketing tool that ensures the correct color rendering (color rendering index) of displayed products as well as the right store atmosphere. It is also the only factor that can be exactly measured and controlled. Make improvements by deciding for appropriate light source and energy intensity accent lighting retailers may reduce their costs, increase the attractiveness of the presented goods, contribute to the customer satisfaction and thus realize higher sales. Based on measurements and consumer neurotests we found out that there is the way how to find a compromise between effective and energy-saving type of accent lighting which can at the same time contribute to the cost effective store running. Go in hand with the environmental protection and energy saving we propose to replace traditional fluorescent tubes with the LEDs in retail stores or even in parking lots in front of the stores. The investment is indeed higher but good-implemented project of lighting renovation will return it. The main advantage of LED light sources is the availability of several variations of their color temperatures (cool white - warm white). We propose to choose the appropriate one based on the results of neuromarketing tests for different types of lighting. For the chain stores using accent lighting for the fresh food the better solution is to change halogen headlights to metal halide (warm white) lighting. Metal halide lamps have similar characteristics as halogen sources but they are less energy-intensive. For the fresh fruits and vegetable, the lightning has its specific role and not each type of lighting meets the before mentioned conditions such as color temperature, color rendering index and so on. In this case it is also possible to place automatic sensors and dimmers in underutilized spaces or during the time with less traffic in the store. The most expensive solution but also the most efficient one is to build practical skylights on the roofs of the retail stores. Such kind of design solution would contribute to significant energy savings as well as to create the most natural and comfortable shopping environment. The costs associated with the building of practical skylights could be returned in the form of reduced energy consumption and also satisfied customers when making purchases, which will contribute to increasing sales of the retail chain.

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MORPHOLOGICAL CHARACTERISTICS OF FRUITS, DRUPES AND SEEDS IN GENOTYPES OF *ZIZIPHUS JUJUBA* MILL.

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ABSTRACT

The aim of this work is to discriminate 28 *Ziziphus jujuba* Mill. genotypes (ZJ-01 – ZJ-28) based on weight and morphological characteristics of fruit, stones and seeds. These genotypes maintained in a clone repository (Nova Kachovka, Ukraine) were chosen for investigation as potential species for cultivation and use in Slovakia. The fruits were collected at the period of their full maturity (October); their morphometric parameters were following: weight from 2.90 g (ZJ-13) to 28.99 g (ZJ-22), length from 20.73 mm (ZJ-08) to 44.84 mm (ZJ-04), and width from 16.64 mm (ZJ-13) to 38.87 mm (ZJ-22). Isolated stones were also analysed, and their weight was from 0.173 g (ZJ-07) to 0.731 g (ZJ-20), length from 12.84 mm (ZJ-08) to 28.67 mm (ZJ-04), and width from 5.06 mm (ZJ-07) to 9.74 mm (ZJ-01). The weight of the seeds was ranging from 20.00 mg (ZJ-25) to 69.11 mg (ZJ-12), length from 7.24 mm (ZJ-15) to 11.85 mm (ZJ-04), and width was from 3.86 mm (ZJ-05) to 6.84 mm (ZJ-12). Significant differences in the size, shape and color of fruits, stones and seeds were also identified. The pulp, seedless stones and seeds constitute 92.81% (ZJ-26) - 98.32% (ZJ-04), 1.36% (ZJ-04) - 6.27% (ZJ-20), and 0.25% (QA-24, QA-25) - 1.21% (ZJ-16) of the total fruits weight, respectively The shape index of the fruits was found in the range of 1.04 (ZJ-08) to 2.00 (ZJ-12 and ZJ-25). The shape indexes of stones and seeds were found ranging from 1.74 (ZJ-08) to 4.53 (ZJ-05) and from 1.32 (ZJ-23) to 2.74 (ZJ-04), respectively. The relative weight of pulp was determined in the range from 92.76% (ZJ-13) to 98.32% (ZJ-04) and the relative weight of seed was determined in the range of 0.25% (ZJ-24 and ZJ-25) to 1.16% (ZJ-26). The Chinese jujube genotypes of this study were grouped based on hierarchical clustering of the fruit, stone and seed characteristics.

Keywords: Chinese jujube variability; morphological characteristics; fruit; stones; seed

INTRODUCTION

The genus *Ziziphus* has approximately 40 species **(Li et al., 2007)** that has been cultivated for about 4,000 years in China and grown in this country for over 150 years **(Bonner and Rudolf, 1974; Lyrene, 1979; Mowry et al., 1953)**. Among these species, the most known one named *Ziziphus jujuba* Mill. is a thorny rhamnaceous plant **(Li et al., 2007)** and is mainly cultivated in China and grown to some extent in Russia, northern Africa, southern Europe, the Middle East, and the south-western United States **(Shanmugavasan et al., 2011)**. Its common name is Chinese date or Chinese jujube. In older European texts it may be also called the 'common jujube'. It tends to be a more upright tree with bright green, glabrous leaves. The tree is deciduous, losing its leaves in winter; it flowers in the spring, and the fruits mature in autumn. It is resistant to winter cold **(Guijun and Ferguson, 2010)**. It is a hardy tree of arid region, which can be grown successfully in saline soil under hot, arid environment **(Meena et al., 2003)**. Its fruit is an edible oval drupe with single hard stone **(Nowak a Shulzová, 2002; Azam-Ali et al., 2006)**.

Jujube cultivars vary in size and morphology. After 30 years of growth in an average site, trees of most cultivars will be 40-50 feet tall with a crown diameter of 15-20 feet **(Lyrene and Crocker, 1994)**. Trees bear fruit as early as 1 to 4 years after planting **(Lyrene, 1979)**. Often producing some fruit the second year from seed or grafting **(Lyrene and Crocker, 1994)**. Currently, there are 700 to 800 jujube cultivars in China, including fresh eating, drying, multipurpose (good for both drying and fresh eating), candied, and ornamental. Cultivars for drying, including multipurpose cultivars, formerly dominated and accounted for 90% of the jujube production in China **(Yao, 2012)**. Annual fruit production in China is 450 000 long tons produced on 290 000 hectares **(Hache a Xu, 1995)**. *Zizyphus jujuba* Mill. 'Huizao' is the most commonly planted cultivar in China, with the largest orchard area and the highest annual production **(Shanmugavasan et al., 2011)**. Fruits, leaves, seeds and other parts of *Z. jujuba* plant contain many important biologically active substances. Therefore, their physiotherapeutic effects are used to treat many diseases such as liver disease, fever, sore throat, pharyngitis, bronchitis, diabetes, skin infections, loss of appetite, diarrhea. It is also used for digestive disorders, weakness, urinary problems, and insomnia **(Khare, 1995, Huang et al., 2007, Huang et al., 2008, Adzu et al., 2001, Han and Park, 1986, Kirtikar and Basu, 1984, InAs et al., 2008)**. Alleviates brain nerve disorders **(Heo et al., 2003)**, regulates immune function **(Zhao et al., 2008)** and it's used to reduce blood triglycerides **(Kim, 2002)**. It has anti-cancer **(Lee et al., 2003)**, antifungal, antibacterial, anti-inflammatory **(Al - Reza, et al., 2010, Huang et al., 1990)**, hypotonic, antioxidant **(Zhang et al., 2000, Chang et al., 2010)** and immunostimulatory properties **(Al - Reza**

et al., 2010). It is also used in wound healing **(Ansari et al., 2006)**.

The aim of this work is to determine the differences in basic morphological characteristics of fruit, drupes and seeds between Chinese jujube genotypes, as potential species for cultivation and use in Slovakia.

MATERIAL AND METHODOLOGY

In our experiments, we used the stone fruits (drupes) of 28 Chinese jujube genotypes maintained in a clone repository (Nova Kachovka, Ukraine). The fruits were collected at full maturity (October). At average of 15 fruits, stones and seeds of each genotype were chosen for morphological analysis. The weight (g/mg), height (mm) and width (mm) were determined for all of themusing sliding scale and Axio Vision Rel. 4.8 software **(Carl Zeiss MicroImaging GmbH, Germany)**. Variability of all these parameters was evaluated using descriptive statistics Differences between the genotypes in morphological characteristics were evaluated by analysis of variance and Tukey test. The Pearson correlation coefficients between the selected parameters were calculated. Similarities and/or dissimilarities between genotypes were evaluated by Cluster analysis based separately on fruit, stone and seed data respectively. Statistica 10 software **(StatSoft Inc., USA)** was used to perform all mentioned statistical analyses.

RESULTS AND DISCUSSION

General fruit characteristics

The weight of the whole fruit is one of significant production characteristics of plant species. Further important features of the fruit are its shape, size and color. These parameters of the jujube fruit varied significantly. The fruits were round, oval, apple-, egg- or pear-shaped, etc. Fully mature fruits have brownish-yellow, golden-yellow or reddish to dark brown color. There are also brown spots on the fruit. The images of jujube fruits of various genotypes are shown in Figure 1. High variability of the size, shape and color of these fruits are evident. The fruit pulp also demonstrated varying consistency and different colors (Figure 2).

Fruit weight

As reported by many authors **(Kundi et al., 1989; Gao et al., 2003; Liu et al., 2003; Prasad, 2005; Jiang et al., 2006)**, the average fruit weight of Chinese jujube is usually between 10 and 30 g. **Gao et al., (2012)** determined the fruit weight in the range of 6.7 to 26.7 g, **Karnatovska et al., (2007)** identified in the evaluation of 23 varieties of Chinese jujube in extreme agro ecological conditions in Nova Kachovka (Ukraine), the average weight of the fruit in the range of 1.0 to 9.5 g. **Brindza et al., (2011)** determined the average weight of the fruit in the range of 0.66 to 4.68 g. **Sivakov et al.,** (1988) identified the fruit weight from 5.72 to 10.45 g, **Ecevit et al., (2007)** determined the average fruit weight of 4.52 to 6.12 g. On the other hand, **Guijun and Ferguson, (2010)** determined

Figure 1 Fruits of selected Chinese datle (*Ziziphus jujuba* Mill.) genotypes.

Figure 2 Fruit cross sections of selected Chinese jujube (*Ziziphus jujuba* Mill.) genotypes.

the fruit weight of a certain genotypes to be more than 50 g.

The weight of jujube fruits of present study was in the range of 2.90 to 28.99 g (Table 1). These values are in agreement with those in many reports. The variation coefficient CV characterizes average degree of variability within the tested collection; genotypes reaching extreme values are listed in Table 2.

Fruit length

The jujube fruit reaches the length from 4 to 6 cm. The fruits of the original (wild) forms reach the size of only about 2.5 cm. The pulp is irregularly grooved **(Nowak and Shulzová, 2002, Keys, 1976 Polivka, 2010)**. The given shapes were also determined in genotypes in our collection (Figure 1). The shape of the fruit is conditioned by its height and diameter. The fruit length in our analyses was determined in the range of 20.73 mm (ZJ-08) to 44.84 mm (ZJ-04) (Table 1). The value of the coefficient of variation was 6.11%, which documented low degree of variability of the character within the collection. **Brindza et al., (2011)**

Table 1 The variability of weight and linear parameters of fruits, stones and seeds for the whole collection of Chinese jujube genotypes from the clone repository in Nova Kachovka.

Characteristic	Unit	*min*	*max*	*mean*	*SD*	*CV*
Fruit weight	**G**	2.90	28.99	10.83	0.45	17.93
Stone weight	**G**	0.17	0.73	0.38	0.02	20.34
Seed weight	**mg**	20.00	69.11	48.53	2.69	15.12
Fruit length	**mm**	20.73	44.84	33.68	0.42	6.11
Stone length	**mm**	12.84	28.67	20.62	0.53	5.56
Seed length	**mm**	7.24	11.85	9.74	0.12	3.85
Fruit width	**mm**	16.64	38.87	24.27	0.36	7.07
Stone width	**mm**	5.06	9.47	7.18	0.19	5.82
Seed width	**mm**	3.86	6.84	5.30	0.09	3.95

Legend: *min*, *max* – minimal and maximal values measured, *mean* - arithmetic mean, *SD* - standard error of mean, *CV* - coefficient of variation (%)

Table 2 The fruit/stone/seed weight variability for specific Chinese jujube genotypes from the collection of clone repository in Nova Kachovka.

Genotypes	*mean*	*SD*	*CV*	Genotypes	*mean*	*SD*	*CV*
Fruit weight (g)							
Lowest values				**Highest values**			
ZJ-13	**2.90**	0.08	17.27	**ZJ-22**	28.99	1.14	14.21
ZJ-16	**3.31**	0.08	14.13	**ZJ-17**	26.28	1.95	31.48
ZJ-14	**4.30**	0.10	13.77	**ZJ-04**	18.43	0.72	20.68
ZJ-26	**4.31**	0.07	11.81	**ZJ-02**	18.25	1.22	20.01
ZJ-08	**4.46**	0.11	14.02	**ZJ-10**	15.67	0.60	20.46
Stone weight (g)							
Lowest values				**Highest values**			
ZJ-05	**0.17**	0.01	15.21	**ZJ-20**	0.73	0.03	17.21
ZJ-07	**0.17**	0.01	17.92	**ZJ-11**	0.64	0.03	29.15
ZJ-19	**0.19**	0.01	14.55	**ZJ-10**	0.61	0.04	29.17
ZJ-25	**0.20**	0.00	10.37	**ZJ-03**	0.59	0.03	25.61
ZJ-27	**0.20**	0.01	20.24	**ZJ-12**	0.58	0.03	23.57
Seed weight (mg)							
Lowest values				**Highest values**			
ZJ-25	**20.00**	1.08	16.20	**ZJ-12**	69.11	3.49	15.17
ZJ-05	**25.25**	3.30	26.15	**ZJ-28**	61.50	2.35	12.10
ZJ-07	**26.00**	2.38	25.84	**ZJ-04**	61.00	6.00	13.91
ZJ-13	**34.22**	2.44	21.36	**ZJ-21**	61.00	2.00	4.64
ZJ-24	**42.50**	4.50	21.18	**ZJ-18**	59.57	5.38	23.89

Legend: *mean* - arithmetic mean; *SD* - standard error of *the mean*; *CV* - coefficient of variation (%)

Table 3 Correlation between the fruit weight (g) and other morphological characteristics of evaluated fruits, stones and seeds shown with Pearson coefficients of correlation

Characteristic	Unit	*r*	*sr*	Confidence interval		r^2		*p*
				min	*max*			
Fruit length	**mm**	0.77	4.99	0.56	0.89	0.59		0.00
Fruit width	**mm**	0.97	1.32	0.94	0.99	0.94		0.00
Stone weight	**mm**	0.58	0.14	0.26	0.78	0.33		0.00
Stone length	**mm**	0.69	3.44	0.43	0.85	0.48		0.00
Stone width	**mm**	0.34	1.18	-0.04	0.63	0.12		0.07
Stone thickness	**mm**	0.25	1.35	-0.13	0.57	0.06		0.19
Seed weight	**g**	0.41	0.01	0.01	0.69	0.17		0.04
Seed length	**mm**	0.84	0.92	0.67	0.93	0.71		0.00
Seed width	**mm**	0.51	0.82	0.15	0.75	0.26		0.00

Legend: *r* - Pearson correlation coefficient, *min/max* - 95% confidence interval for *r*, r^2 - coefficient of determination, *p* - significance level ofr

determined the average length of the fruit from 16.77 to 21.67 mm. **Karnatovska et al., (2007)** identified in the evaluation of 23 varieties of Chinese jujube an average length of fruit in the range of 13.0 to 32.2 mm. **Zhang et al., (2011)** determined the average length of fruit at 26.1 mm. **Klymenko and Grygorieva, (2008)** in the range of 14.98 to 29.74 mm. The results of our experiments correspond with the results obtained by the given authors.

Fruit width

In our experiments the fruit width was determined in the range of 16.64 mm (ZJ-13) to 38.87 (ZJ-22) (Table 1). The variation coefficient (7.07%) confirmed moderate degree of variability within the collection. **Brindza et al., 2011** determined fruit width in the range of 12.67 mm to 16.97 mm. **Karnatovska et al., (2007)** identified in the evaluation of 23 varieties of Chinese jujube average fruit width in the range of 11.06 to 23.8 mm. **Zhang et al., (2011)** determined an average width of the fruit at 25.80 mm. **Klymenko and Grygorieva, (2008)** identified in the evaluation of 23 varieties of Chinese jujube fruit width in the range of 11.45 to 18.71 mm. The results of our experiments correspond with the results obtained by the given authors.

Shape index of fruits

The ratio between the length and width of the fruit is known as the shape index. In the assessment of genotypes we determined the shape index ranging from 1.04 (ZJ-08, ZJ-26 – roundish shape) to 2 (ZJ-07, ZJ-25) (Figure 5). We also identified significant differences among the genotypes in the color and shape of the fruit, as illustrated in figure 1.

Stone characteristics

Stone weight

The fruits of Chinese jujube (drupes) contain endocarps (stones), which are highly variable in their shape and size (Figure 2 and Figure 3). Each drupe contains only one stone placed in the middle of the fruit. The stone is hard,

spindle-shaped, elliptic, oblong, pointy at both ends, pointed, deeply wrinkled, red or dark brown to dark gray, 2.5 to 5 cm long **(Dinavand, Zarinkamar, 2006 Bonner and Rudolf, 1974 Husak et al., 1996)**. In evaluated genotypes we determined the weight of stones in the range of 0.173 (ZJ-07) to 0.731 g (ZJ-20) (Table 1). The value of

Figure 3 Stones of selected Chinese jujube (*Ziziphus jujuba* Mill.) genotypes.

Figure 4 Seeds of selected Chinese jujube (*Ziziphus jujuba* Mill.) genotypes.

the coefficient of variation was 20.34%, which documents a high degree of variability of the characteristic within the collection. Genotypes reaching extreme values of a given characteristic are shown in Table 2. The coefficients of variation also documented a significant difference in weight of stones within genotypes. **Brindza et al., 2011** determine the average weight of drupes in the range of 0.153 to 0.845 g. **Karnatovska et al., (2007)**, in the evaluation of 23 varieties of Chinese jujube in extreme agro ecological conditions in Nova Kachovka (Ukraine), identified the average weight of stones in the range of 1.0 to 2.1 g. **Ecevit et al., (2007)**, in the study of 52 genetic resources, determined the average weight of stones in the range from 0.34 to 0.41 g, **Sivakov et al., (1988)** identified the stone weight in the range of 0.28 to 0.65 g and **Ghosh and Mathew, (2002)**, in the study of nine genotypes, in the range of 0.6 to 1.9 g.

Stone length and width

In the assessment of individual fruits in genotypes we determined the stone length and width in the ranges of 12.84 (ZJ-08) - 28.67 mm (ZJ-04) and 5.06 mm (ZJ-07) - 9.74 mm (ZJ-01), respectively (Table 1). Variation coefficients (5.56–5.82%) confirmed low degree of variation in both these parameters. **Brindza et al., (2011)** in the evaluation of 19 genotypes of Chinese jujube determined the average stone length in the range of 10.58–14.35 mm.

Seed characteristics

Seed weight

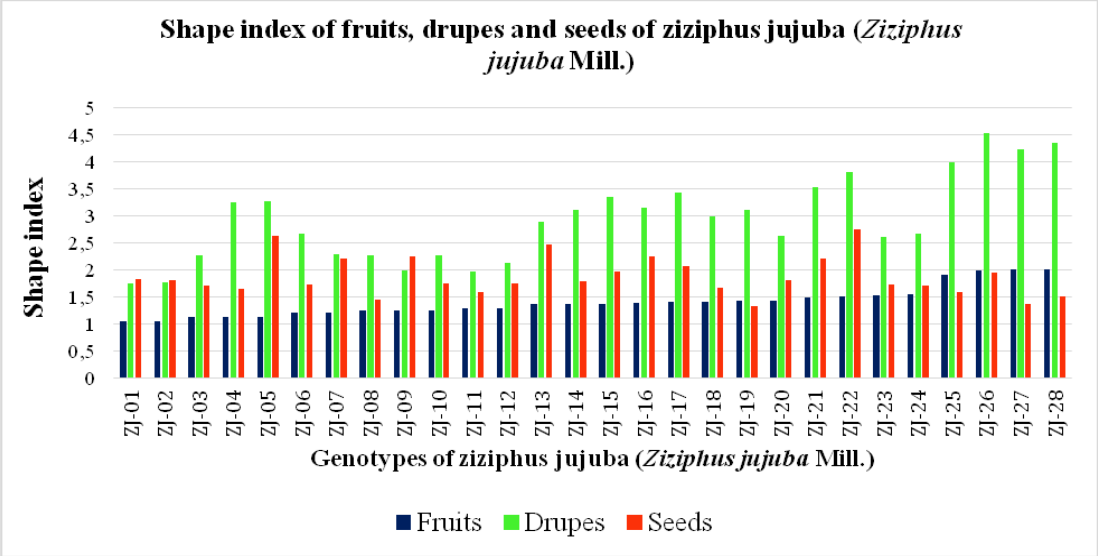

Figure 5 Comparison of the tested Chinese jujube genotypes (*Ziziphus jujuba* Mill.) in the shape index of fruit, drupes and seeds.

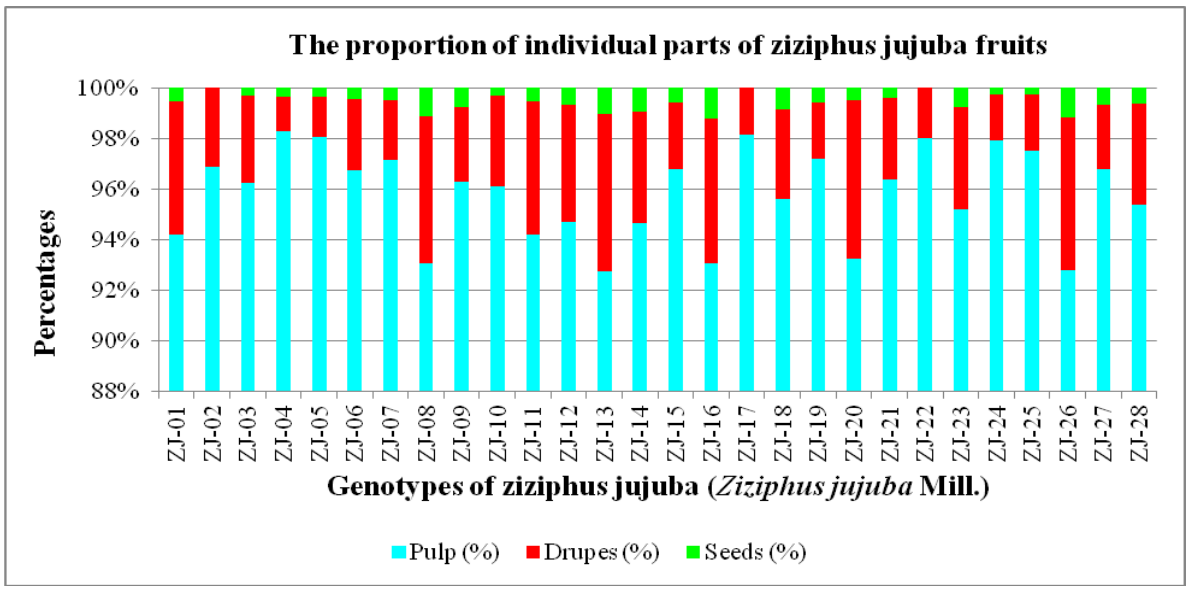

Figure 6 The weight of the pulp, drupes and seeds (%) from the total weight of the fruit (100%) of Chinese jujube (*Ziziphus jujuba* Mill.).

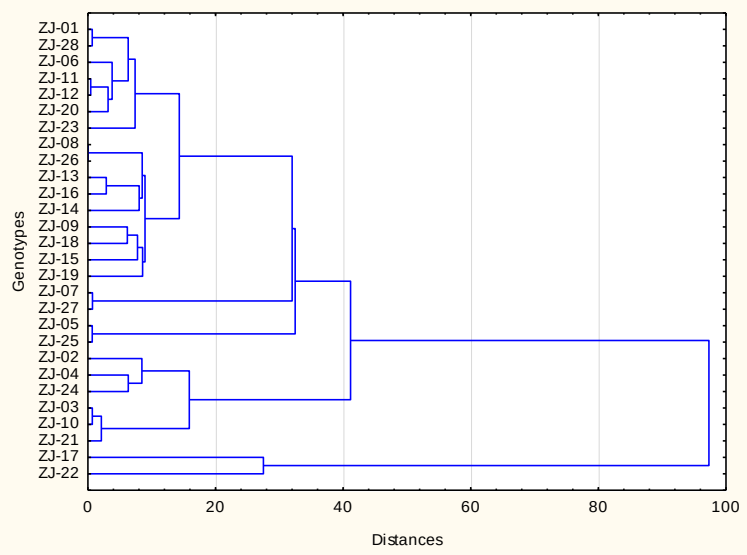

Figure 7 Dendrogram of 28 genotypes of Chinese jujube based on morphometric characteristics of fruits.

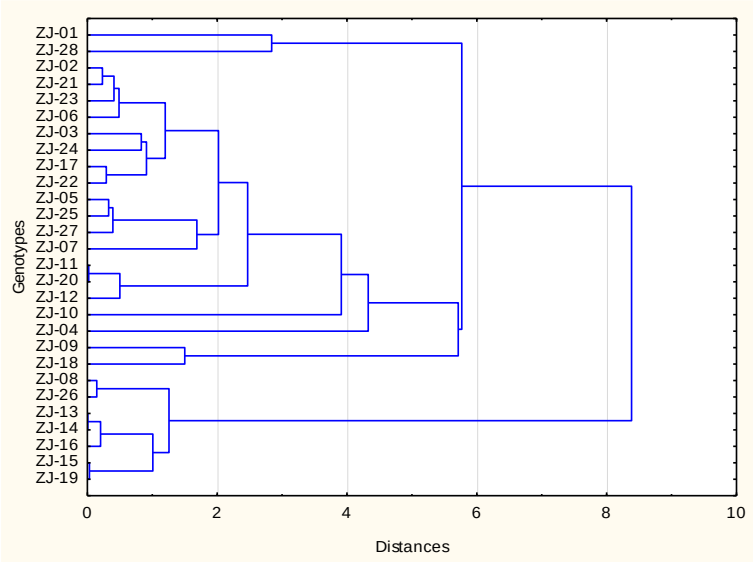

Figure 8 Dendrogram of 28 genotypes of Chinese jujube based on morphometric characteristics of stones.

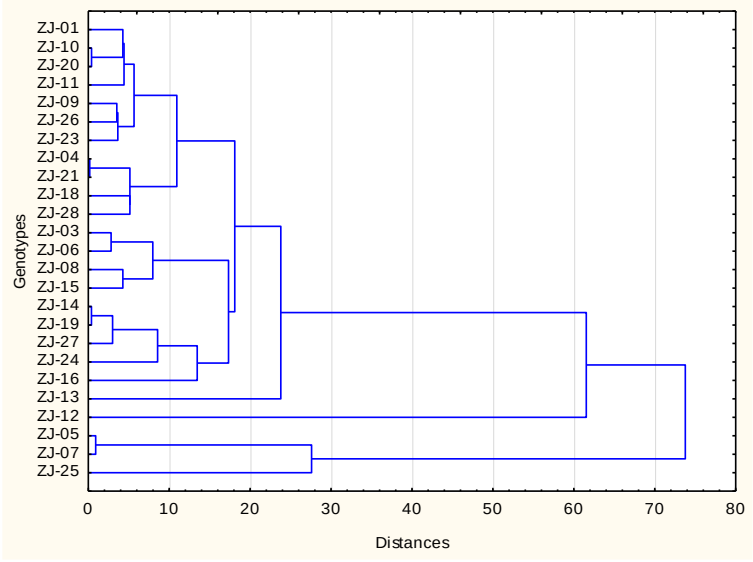

Figure 9 Dendrogram of 28 genotypes of Chinese jujube based on morphometric characteristics of seeds.

Seeds of Chinese datle (up to three) are enclosed in a hard woody inner endocarp – stone. However, some fruits are seedless and formed in a parthenocarpic way **(Pareek, 2001)**. The seeds of Chinese jujube have significant economic value as a raw material for obtaining valuable oil, which has a wide practical use. This is supported by findings of many literary sources. **Al-Reza et al., (2010)** determined 23 components in the oil from Chinese jujube seed. Among the major components included in this oil were eucalyptol, chavicol, eugenol, isoeugenol, ledol, veridiflorol, tumeron and more. Evaluating a collection of genotypes, we determined the seed weight in the range of 20.00 mg (ZJ-25) to 69.11 mg (ZJ-12) (Table 1). The value of variation coefficient (15.22%) documents a medium degree of variability of the characteristic within the collection. Genotypes reaching extreme values of a given characteristic are shown in Table 2. The coefficients of variation also documented a significant difference in the weight of the seeds within genotypes. Some genotypes do not form seeds (ZJ-02, ZJ-17, ZJ-22).

Seed length and width

In the collection of evaluated genotypes we determined the length of seeds in the range of 7.24 (ZJ-15) to 11.85 mm (ZJ-04) (Table 1). Coefficients of variation 3.85% documented low degree of variability of the characteristic within the collection. The width of the seeds was determined in the range of 3.86 mm (ZJ-05) to 6.84 mm (ZJ-12) (Table 1). The value of the coefficient of variation 3.95% documents also a low degree of variability of the characteristic within the collection. Among the genotypes, we also identified significant differences in the color and shape of the seeds, as illustrated in Figure 4. In the evaluated collection of genotypes, we determined the length of the seeds in the range of 3.86 (ZJ-25) to 6.84 mm (ZJ-12) (Table1). Variation coefficient (3.95%) documented low degree of variability of the characteristic within the collection.

Shape indexes of fruits, stones and seeds

The shape of each object can be characterized by the shape index, i.e. the length to width ratio. Figure 5 represents the shape indexes of fruits, stones and seeds. The shape index of the fruits was found in the range of 1.04 (ZJ-08) to 2.00 (ZJ-12 and ZJ-25), so the genotype collection demonstrate significant variability in the shape of the fruit, as seen in Figure 1. The shape indexes of stones and seeds were found ranging from 1.74 (ZJ-08) to 4.53 (ZJ-05) and from 1.32 (ZJ-23) to 2.74 (ZJ-04), respectively. According to Figure 5, it is evident that the shapes of fruits, stones and seeds did not correlate to each other, but were highly specific for jujube genotypes.

Relative weights f pulp, stone and seed

The relative values of pulp (stone, seed) weight in relation to the total fruit weight (relative weight of the pulp, stone or seed) are very important characteristics for practical use of many fruiting plants. A comparison of the evaluated genotypes in a given characteristic is presented in Figure 6. This Figure shows differences between genotypes. The relative weight of pulp was determined in the range from 92.76% (ZJ-13) to 98.32% (ZJ-04). The relative weight of seed was determined in the range of 0.25% (ZJ-24 and ZJ-25) to 1.16% (ZJ-26). These findings have a practical significance for genotype evaluation.

The relationship between specific characteristics

Because of the complexity of addressing the issues, we have identified a relationship between fruit weight and other assessed characteristics on the fruit, with the application of a simple correlation analysis. The results of the analysis are given in Table 3. The results indicated high correlations between the fruit weight and the fruit width (r = 0.971), stone length (r = 0.843), fruit length (r = 0.771) and stone height (r = 0.694). Slight correlation was found between the fruit weight and the stone thickness (r = 0.254) and width (r = 0.341).

Clustering of Chinese jujube e genotypes based on fruit characteristics

The Chinese jujube genotypes of this study were grouped based on hierarchical clustering of the fruit, stone and seed characteristics (Figure 7, Figure 8 and Figure 9). The three dendrograms, obtained from the cluster analysis of the selected morphometric characteristics (fruits, seeds and stones) gave different results. The fruit characters generated dendrogram with three different clusters where in the most dissimilar cluster can be identified genotypes ZJ-17 and ZJ-22 the both with high proportion of pulp in fruits. Dendrogram created from morphometric parameters of stones separated 3 different groups, where genotypes (ZJ-01 and ZJ-28) in the first group and genotypes (ZJ-08, ZJ-26, ZJ-13, ZJ-14, ZJ-15 and ZJ-19) in the second group were different from the rest of the tested genotypes. Performed cluster analysis based on characteristics of seeds created group of distinct genotypes (ZJ-12, ZJ-05, ZJ-07 and ZJ-25).

CONCLUSION

The results of the experiment presented in this work are consistent with the results reported earlier. In evaluating 28 genotypes of Chinese jujube we determined the weight of the fruits in the range from 2.90 g (ZJ-13) to 28.99 g (ZJ-22), length from 20.73 mm (ZJ-08) to 44.84 mm (ZJ-04), and width from 16.64 mm (ZJ-13) to 38.87 mm (ZJ-22). Isolated stones were also analysed, and their weight was from 0.173 g (ZJ-07) to 0.731 g (ZJ-20), length from 12.84 mm (ZJ-08) to 28.67 mm (ZJ-04), and width from 5.06 mm (ZJ-07) to 9.74 mm (ZJ-01). Presented results also showed that significant differences in the evaluated characteristics were found for the studied jujube genotypes. Obtained results are important for breeding new varieties of Chinese jujube as well as their practical use.

The results obtained by these experiments confirmed differences between the evaluation of Chinese jujube genotypes in morphological characteristics of fruit, drupes and seeds. The results also demonstrate that the fruits of Chinese jujube, with their high proportion of pulp, can be used for direct consumption and for preparation of many food products. This species is very suitable for cultivation, dissemination and utilization in Slovakia. It is temperature resistant to -25 °C. Besides fruits, other parts of this plant can be useful, especially for pharmacological and cosmetic use. Seeds are valuable for their oil content. Growing and

utilization of Chinese jujube plant in Slovakia would be mainly used for socio-economic development of family farms.

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INFLUENCE OF GARLIC EXTRACT ON ANTIOXIDANT STATUS OF CHICKEN

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ABSTRACT

In 2006 the European Union banned the feeding of antibiotic growth promoters because of possible risk of drug resistance in human pathogens bacteria. This is the reason for the study of various phytogenic additives and their extracts as a natural source of biologically important compounds. Antimicrobial substances are a commonly included in chicken feed rations. They are used mainly as prevention against various diseases, and also to stimulate growth. The beneficial effects of garlic on animal organism resulting from their antimicrobial, antioxidative and antihypertensive properities. Studies focused on growth, conversion and meat quality of different types of animals indicate its positive effects. In our experiment we studied the influence of garlic extract in a dose of 0, 10 g and 15 g per 1 kg of chicken feed mixture. We focused on weight gains and antioxidant status of an organism. The experiment took 39 days. 54 seven-day-old chickens were included in the experiment. The chickens were weighed once a week, when aged 11, 17, 24, 31 and 38 days, at the same time of the day. The chickens had ad libitum access to feed ration and water. The chickens were taken blood sample at the end of the experiment when 39 days old. Their antioxidant status were measured using ABTS, FRAP and DPPH methods. Our results show that owing to higher concentration of garlic extract in feed ration the antioxidant status of observed chickens was increased. DPPH method showed an increase in antioxidant status of both experimental groups by 38% (a group with a dose of 10 g/kg of mixture) and by 46% (a group with a dose of 15 g/kg of mixture) compared to the control group. When using FRAP method, antioxidant status of both G10 and G15 groups increased by 24%, resp. 16%. No evidential differences in antioxidant activity between the experimental groups and control group were found using ABTS method. The supplement of garlic extract into a feed ration did not have any influence on weight gains of chickens.

Keywords: poultry; garlic extract; antioxidant status

INTRODUCTION

Garlic is one of the earliest plants ever cultivated **(Nevrkla et al., 2013, Prasad et al., 2009)**. It has been known for thousands of years in folk medicine of Greeks and Egyptians **(Horton et al., 1991, Togashi et al., 2008)**. It is known as spice and herbal medicine for treatment as well as prevention against various diseases **(Ashayerizadeh et al., 2009, Khan et al., 2012)**. In body it has various effects such as aggregation of platelets, decrease in arterial blood pressure or prevention against fatty infiltration of liver. Both *in vivo* and *in vitro* studies proved that matured garlic extract stimulates functions of immune system **(Prasad et al., 2009)**.

Garlic contains at least 33 substances containing sulphur, enzymes and amino acids, minerals including selenium. The main active components in garlic are allicin, ajoene, dialkyl polysulfides, s-allylcysteine (SAC), diallylsulfide, S–methyl–cystein sulfoxide and s–allylcysteine sulfoxide which may be responsible for healing effect of garlic **(Togashi et al., 2008)**. Chemistry of garlic is a complex mechanism, which has probably evolved as an individual protective mechanism against microorganisms and other impairment. Whole garlic typically contains 1% of alliin, together with (1)-S-methyl-L-cysteine sulfoxide (methiin) and (1)-S-(trans-1-propenyl)-L-cysteine sulfoxide. S-(2-carboxypropyl) glutathione, g-glutamyl-S-allyl-L-cysteine, g-glutamyl-S-(trans-1-propenyl)-L-cysteine and g-glutamyl-S-allyl-mercapto-L-cysteine are also present in cloves of garlic. Allicin naturally cumulates if garlic is stored in lower temperatures. A garlic bulb contains in average 0.9% of G-glutamylcysteine and 1.8% of alliin. Apart from these main sulphur compounds an unimpaired garlic bulb may also contain a small amount of SAC, but no allicin. SAC is created during catabolism from g-glutamyl cysteine. Typical volatile substances in milled garlic and garlic volatile oils are diallyl sulfid (DAS), diallyl disulfid (DADS), diallyl trisulfide methyl allyl disulfide, methyl allyl trisulfid, 2-vinyl-1,3-dithiin, 3-vinyl-1,2-dithiin a E, Z-ajoene **(Amagase et al., 2001)**. Allicin, a major product of garlic aroma, is known as an antimicrobial substance, which is created from alliin. Enzyme alliinase, and corresponding cystein sulfoxid aliin, can be found in different parts of garlic plant. If a cell is impaired, alliinase lyses alliin into allicin, pyruvate and ammonium.

Considering that garlic contains a high concentration of selenium, an important part of antioxidant system, higher antioxidant potential can be expected **(Horky, 2014a, Horky, 2014b, Horky et al., 2012a, Horky et al., 2013)**. It is not only selenium that takes place in antioxidant

system, but also other substances present in garlic **(Horky et al., 2012b, Jancikova et al., 2012)**. There are several physiological processes in microorganisms which are influenced by allicin, such as biosynthesis of lipids, RNA synthesis or decreasing level of lipids and aggregation of platelets in mammals **(Focke et al., 1990)**. Allicin functions as an antibiotics destroying sulfhydryl group of enzymes, inhibits fermentation and stimulates gastric secretion, which leads to prophylactic precaution against bacterial infections of gastrointestinal tract. In the last decade, garlic has been added into feed doses of poultry due to its influence on gains **(Khan et al., 2012, Togashi et al., 2008)**. Its effect is stronger during first weeks of life of birds **(Togashi et al., 2008)**.

The aim of our study is to find out an influence of garlic extract on weight gains, feed consumption, and weight of carcass bodies and antioxidant status of broiler chickens.

MATERIAL AND METHODOLOGY

Experiment design

54 seven-day-old male chickens of Ross 308 type were included in the experiment. Chickens were divided into 3 groups. One group was a control one, other two groups were fed with a feed ration with added garlic extract of concentration 10 and 15 g per 1 kg of feed mixture.

Animals and their conditions

Chickens were weighed, marked with wing stamps and then divided into groups and put into balancing cages. They were divided into weight-matched groups before the experiment started. Following weightings were done at age of 11, 17, 24, 31 and 38 days, at the same time of the day.

Animals could access water and feed ration *ad libitum*. Feed ration was mixed up from these components: wheat 25%, corn 37%, soya extract grain 28%, sunflower oil 6%, mixture of vitamins and minerals without anticoccidials 3%, monocalciumphosphate 0.8%, grounded calcite 0.2%. Garlic extract of concentration of 10 and 15 g per 1 kg of feed ration was added into feed mixture.

Consumption of feed ration was recorded for each group.

Light regime was set to 6 hours of darkness and 18 hours of light. At the age of seven days temperature was set to 29.9 °C (with relative humidity of 50%). Temperature was being lowered every day by 1 °C to a level of 23 °C.

Sample preparation

The experiment was ended when chickens reached aged of 39 days. Blood was taken from jugular vein into heparin test-tube when chickens were killed by decapitation.

Determination of antioxidant activity and total proteins

Spectrophotometric measurements of antioxidant activity and total proteins were carried out using the BS-400 automated chemical analyser (Mindray, Shenzhencity, China). Transfer of samples and reagents was provided by a robotic arm equipped with a dosing needle (error of dosage not exceeding ±5% of volume). Cuvette contents were mixed immediately after addition of reagents or samples by an automatic mixer including a stirrer.

Determination of total proteins by the Biuret method

The Biuret method is a test used for detecting the presence of peptide bonds. In the presence of peptides, a copper (Hysing & Wiik) ion forms a violet-coloured complex in an alkaline solution.

A 150 µL volume of Biuret reagent (100 mM potassium sodium tartrate, 100 mM sodium hydroxide, 15 mM potassium iodide and 6 mM copper$^{(II)}$ sulfate) is pipetted into a plastic cuvette with subsequent addition of 3 µL of sample. Absorbance is measured at λ = 546 nm after 10 minutes of incubation. Resulting value is calculated from the absorbance value of the pure Biuret reagent and from the absorbance value after 10 minutes of incubation with the sample.

Determination of antioxidant activity by the ABTS test

The procedure for the determination was taken from a publication by Sochor *et al*. **(Sochor et al., 2010a)**. A 150 µL volume of reagent. Seven mM 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet}$) and 4.95 mM potassium peroxodisulphate was mixed with 3 µL of the sample. Absorbance was measured at 660 nm for 10 minutes.

Determination of antioxidant activity by the FRAP method

The procedure for this determination was taken from a paper by Sochor *et al*. (Sochor et al., 2010b). A 150 µL volume of reagent was injected into a plastic cuvette with subsequent addition of a 3 µL sample. Absorbance was measured at 605 nm for 10 minutes.

Determination of antioxidant activity by the DPPH test

This procedure for the determination was taken from publications by Sochor *et al*. **(Sochor et al., 2010a)**. A 150 µL volume of reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl - DPPH$^{\bullet}$) was incubated with 15 µL of the sample. Absorbance was measured at 505 nm for 10 minutes.

RESULTS AND DISCUSSION

When evaluating antioxidant status, antioxidant activity was evaluated using DPPH test, methods FRAP and ABTS. Resulting values of antioxidant activities were converted to 1 gram of protein. According to anticipated hypothesis, in groups with addition of garlic extract we found a direct influence on above-mentioned markers of antioxidant potential of organism.

Evaluation of antioxidant activity

Antioxidant activity is a marker of total amount of antioxidants in a given sample. It is a value, which is used to evaluate the ability of organism to uptake free radicals, protect against their creation or to change them into less reactive forms. Lowered antioxidant activity leads to oxidative stress, which is related to higher rate of impairment of organism (disease, impaired productive and reproductive performance).

When evaluating antioxidant activity using DPPH test (Figure 1) was in both experimental groups observed an increase in antioxidant activity determined by DPPH test by 38% (G10) and 46% (G15) compared to control group.

When evaluating antioxidant activity using FRAP method (Figure 2), increase in antioxidant activity was found in both groups, in G10 by 24% and in G15 by 16%. These values, however, were not statistically significant.

Third method used to evaluate antioxidant activity was ABTS method (Figure 3). No relevant differences between control and experiment groups were found using this antioxidant marker.

Evaluation of weight of chickens

General health, influenced by antioxidant status of an organism, directly affects also efficacy of domestic animals. In our experiment the gains of observed animals were evaluated in time intervals. On days 11, 17 and 24 of the experiment weights of control group animals and G10 and G15 animals did not change considerably. From day 31 weight gains were observed in control group compared to G10 (by 6%) and G15 (by 9%). The same trend was

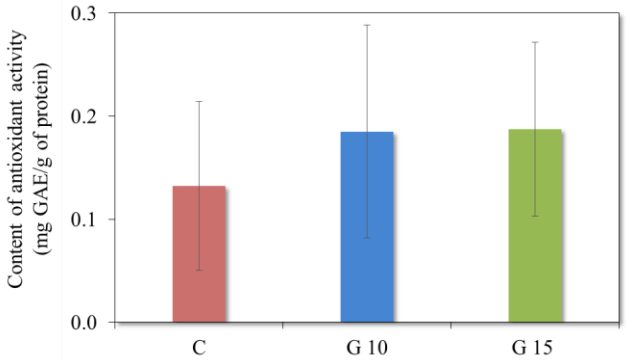

Figure 1 Evaluation of antioxidant activity using DPPH method (C - concentration 0% of garlic extract, G 10 - 10 g/kg of feed ration, G 15 - 15 g/kg of feed ration).

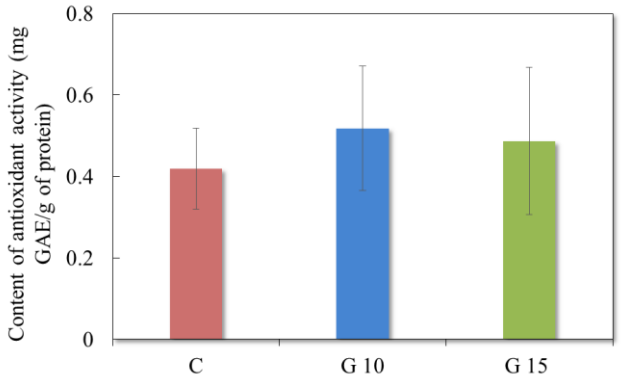

Figure 2 Evaluation of antioxidant activity using FRAP method (C - concentration 0% garlic extract, G 10 - 10 g/kg of feed mixture, G 15 - 15 g/kg of feed mixture).

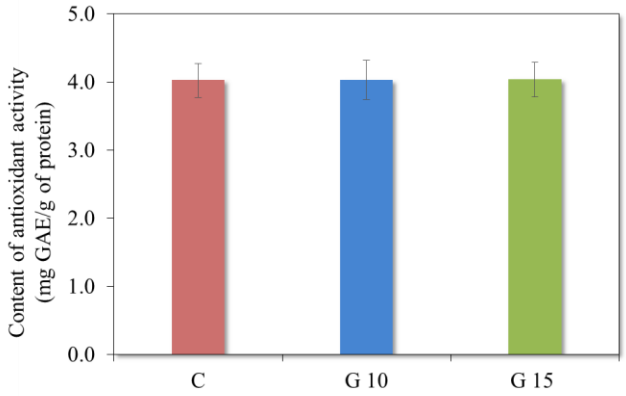

Figure 3 Evaluation of antioxidant activity using ABTS method (C - concentration 0% of garlic extract, G 10 - 10 g/kg of feed mixture, G 15 - 15 g/kg of feed mixture).

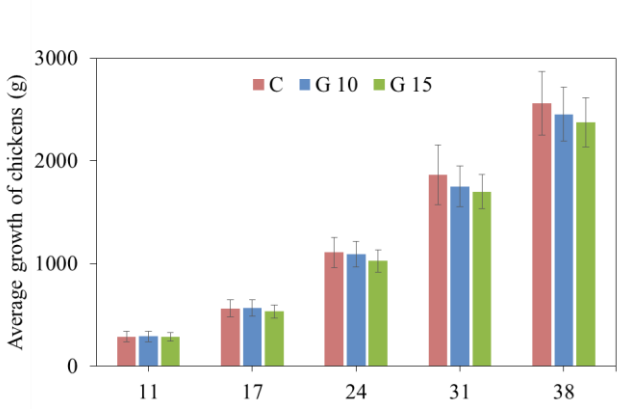

Figure 4 In the figure are shown average weekly weight gains of chickens in grams (C - concentration 0% of garlic extract, G 10 - 10 g/kg of feed mixture, G 15 - 15 g/kg of feed mixture).

obvious also at the end of the experiment (day 38) when weight of animals was higher compared to G10 (by 4%) and G15 (by 7%). Average weights of chickens of individual groups are shown in Figure 4.

Botsoglou et al. (2004) noted that well fed and healthy chickens in clean zoo hygienic conditions of adequate density do not necessarily have to positively react to growth enhancing supplements **(Botsoglou et al., 2004)**.

Differences in results of experiments by different authors may be caused by several reasons: a) difference in a used product - garlic flour, powder or garlic derivatives, b) a kind of used additive substances and concentration of active components which may differ in various experiments, c) complicated chemical composition of garlic **(Amagase et al., 2001)** and d) use of various commercial garlic preparative. Commercial garlic products may be divided according to effective substance into preparatives with raw garlic rich in allicin and preparatives with processed garlic, which contain few allicin **(Khan et al., 2012)**.

Mahmod et al. (2009) states statistically significant influence of addition of 0.5% garlic extract into feed ration on weight gain of broilers and reinforcement of feed conversion. However, influence on yield reinforcement was not proven **(Mahmood et al., 2009)**. **Stanaćev et al. (2010)** in their results state a significantly higher weight in chickens with added 2% of garlic extract into feed ration compared to control group with 0% garlic extract added. Group with added garlic extract also had lower feed conversion compared to control group **(Stanacev et al., 2011)**. According to **Onibi et al. (2009)** supplement of garlic powder in dose of 5 000 mg/kg of feed portion improved live weight, but had no influence on yield of carcasses or quality of inner organs. Adding garlic resulted in decrease in amount of abdominal fat and considerable improvement of oxidative stability of cooled chicken meat of broilers **(Onibi et al., 2009)**. Results of a study by **Raesi et al. (2010)** prove that supplementation of 1 and 3% of garlic powder had a considerable effect on live weight of chickens and improvement of feed conversion. It also had a considerable ($p < 0.001$) influence on yield of broiler carcasses **(Raeesi et al., 2010)**.

Peinado et al. (2012) proved that supplementation of feed ration with garlic derivative PTS-O (propyl propane thiosulphonate) in amount of 45 - 135 mg/kg of feed dose have a beneficial effect on decrease of number of pathogens and potentially pathogenic bacteria in gut, and on improvement of morphological structure of mucous membranes of ileum and production parameters of broiler chickens **(Peinado et al., 2012)**. The results of a study by **Adibmoradi et al. (2006)** point out that supplementation of garlic powder into feed ration is related to morphology of intestines in birds. Considerable differences compared to control group were mainly in dose of 2% of garlic in feed ration. Higher dose of garlic resulted in elongation of villi ($p < 0.05$) in duodenum, jejunum and ileum. Administration of garlic also resulted in considerable decrease in thickness of epithelium in various parts of small intestine. In all parts of small intestine, in groups being administered garlic, there was an increase ($p < 0.05$) in depth of criptae depending on dose of garlic **(Adibmoradi et al., 2006)**.

CONCLUSION

Many studies proved a potential of use of various phytogenic additives, herbs, spices or their essential oils into feed rations of poultry as alternative to antibiotics. More studies on their use are needed, considering the fact that their effect depends on many factors. Efficacy may be influenced by the amount of additive, amount of active substances, digestibility or composition of feed rations. Our expected hypothesis that garlic extract will have a beneficial effect on markers of antioxidant potential of organism and yield of observed animals was not proven. Garlic extract that was in the experiment used in doses of 10 and 15 g per 1 kg of feed ration had no considerable influence on weight gains or antioxidant activity of their organisms. It is possible that selected doses were too low and chicken organism did not react according to our expectations. For this reason in further experiments it would be advisable to increase the dose of garlic extract in chicken diets.

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BIOGENIC AMINES IN SMEAR AND MOULD-RIPENED CHEESES

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ABSTRACT

The aim of the study was the monitoring of six biogenic amines (histamine, tyramine, phenylethylamine, tryptamine, putrescine, and cadaverine) and two polyamines (spermidine and spermine) in 30 samples of dairy products purchased in the Czech Republic, namely in 15 samples of mould-ripened cheeses and in 15 samples of smear-ripened cheeses. A further goal was the microbiological analysis of the individual samples of cheeses (total count of microorganisms, number of enterobacteria, enterococci, lactic acid bacteria, yeasts and moulds). The monitored biogenic amines were analyzed by a high performance liquid chromatography equipped with a UV/VIS DAD detector. The amount of enterobacteria in fresh cheese exceeded 10^5 CFU.g^{-1}. In smear-ripened cheese flavourless (Romadur type), the amount was >10^3 CFU.g^{-1} and 10^4-10^5 CFU.g^{-1} in smear-ripened cheese with flavour. Biogenic amines were observed in two groups of blue cheeses (white veined cheese and blue veined cheese) and smear-ripened cheeses. In both groups, there is a possibility of the presence of biogenic amines because the number of microorganisms and concentration of free amino acids increase during ripening. In ten samples of soft smear-ripening acid cheese and in smear-ripened cheese, the total content of biogenic amines were 22-1000 mg.kg^{-1} and in 5 samples of these cheeses, it was in range 1000-6000 mg.kg^{-1}. The total amount of biogenic amines in the blue cheeses were in range 40-600 mg.kg^{-1}. The presense of the tyramine was observed in the all analysed cheeses. The tyramine producing strains generated more than 900 mg.kg^{-1} of this biogenic amine. The production of tryptamine in the analysed cheeses was not proved by this study. The results of this study show that biogenic amines and polyamines are common in cheese. However, in some cases, they can pose a significant health danger for consumers. Any legislative control authority does not monitor them, as they are secondary metabolites even though they are potential health risks.

Keywords: biogenic amines; high performance liquid chromatography; smear-ripened cheese; mould-ripened cheese

INTRODUCTION

Biogenic amines (cadaverine, histamine, phenylethylamine, putrescine, tyramine, spermidine, spermine; BA) are formed by microorganisms demonstrating decarboxylase activity from corresponding amino acids. Amination and transamination of aldehydes and ketones is also a minor way of creating BA. BAs are present in all foods as products of protein metabolism. They are also present in fermented products, such as smear and mould-ripened cheeses in higher amount **(Loizzo et al., 2012)**. Lack of hygiene or influence of contaminating microflora during cheese ripening can lead to BA formation. Great amount of free amino acids (precursors of BAs) is formed by proteolysis of caseins. Some bacteria species (family *Enterobacteriaceae* or strains of *Lactobacillus*, *Streptococcus*, *Micrococcus*, *Enterococcus* and *Pseudomonas*) are able to produce decarboxylases. Tyramine, histamine, putrescine, cadaverine and tryptamine are usually detected in cheeses **(Silla Santos, 1996; Shalaby, 1996)**. Intensity of decarboxylase reactions depends on the presence of microorganisms (respectively on the presence of appropriate species). Concentration of free amino acids and various environmental factors (for example pH values, oxygen and carbon availability, temperature, the amount of amino acids, water activity can affected the decarboxylase activity **(Halász et al., 1994; Bardocz, 1995)**. BAs are related to alimentary poisoning and they can endanger consumers´ health **(Silla Santos, 1996)**. Tyramine and histamine can affect vascular and central neural system. Putrescine and cadaverine support toxicity of other biogenic amines **(Mayer et al., 2010)**. Amount of BAs in cheeses is influenced by various factors. Main factors during the cheese manufacture are ripening period and storage. Storage of cheese in higher temperatures leads to the increase of BAs. Authors **Mayer et al., (2010)** and **Pachlová et al., (2013)** reported, that the same amounts of BAs were detected in cheese ripened at 5 °C for three weeks and at 20 °C for one week. BA formation can be also influenced by high-pressure treatment during ripening. There can be increased production of peptides and amino acids, which can support of BA production **(Novella-Radríguez et al., 2002)**.

Higher amount of BAs in smear-ripened cheese can be affected by starter or non-starter bacteria (contaminating microflora) during milk fermentation (enterobacteria, enterococci, *Flavobacterium* spp., *Microbacterium* spp., *Bacillus* spp., actinobacteria, etc.). Undesirable pathogens, for example *Listeria monocytogenes*, *Staphylococcus aureus* and pathogenic strains of *Escherichia coli*, can be present in smear-ripened cheese too, due their favourable conditions for growth of this bacteria **(Monnet et al.,**

2010). BA amount is significant in these cheeses because they contain higher amount of decarboxylase-positive bacteria. The strains of *Lactobacillus* produce histamine and putrescine. Some strains, for example *Lactobacillus brevis*, can also create tyramine. Enterococci are important members of microflora in smear-ripened cheese. These bacteria can create tyramine by tyrosine decarboxylation because of its resistance against high temperature and higher NaCl concentration. Significant relation between bacteria species and biogenic amines concentration was observed, for example enterococci and production of phenylethylamine, lactococci and cadaverine and tyramine. In smear-ripened cheeses, there is mainly cadaverine, tyramine, histamine and putrescine detected **(Loizzo et al., 2012)**.

MATERIAL AND METHODOLOGY

Biogenic amines were determined in 30 samples of cheese. There were 9 samples of white veined cheese and 6 samples of blue veined cheese. These products are natural, with no flavour added. The samples of soft smear-ripening acid cheese, soft smear-ripening acid cheese smoked, soft smear-ripening acid cheese flavoured pepper and flavoured garlic, soft cold-ripened cheese unflavoured, flavoured green pepper and flavoured walnuts, smear-ripened cheese flavoured garlic and herbs, flavoured chilli and further 6 samples od smear-ripened cheese were analyzed. All the cheeses were purchased in stores in Czech Republic. The cheeses were stored at temperature 18 °C at least. For each cheese sample, there was identified the total number of aerobic and facultative anaerobic mesophilic microorganisms on Plate Count Agar (PCA), enterobacteria on Endo Agar, enterococci on Slanetz Bartley Agar, lactic cocci on M17 Agar, lactic rods on MRS Agar and yeast and mould on Chloramphenicol Yeast Glucose Agar (CHYGA). Plates with microorganisms were cultivated at temperature 30 °C for 48 hours, plates with Endo Agar at 37 °C for 24 hours and plates with CHYGA at temperature 20 °C for 5 days. Grown colonies were evaluated and converted to $CFU.g^{-1}$ after cultivation.

The production of seven biogenic amines (cadaverine, CAD; histamine, HIS; phenylethylamine, PHE; putrescine, PUT; tyramine, TYR; spermidine, SPD; spermine, SPN) was monitored by an high performance liquid chromatography system equipped with a binary pump; an autosampler (LabAlliance, USA); a column thermostat; a UV/VIS DAD detector (λ = 254 nm); and a degasser (1260 Infinity, Agilent Technologies, USA).

10 ml ($c = 0.6\ mol.L^{-1}$) perchloric acid (Sigma) was added to 1 g of lyophylized cheese sample. The sample was mixed and shaken for 30 minutes, centrifuged (4000 x g) and then an upper part was cast. 7 mL ($c = 0.6\ mol.L^{-1}$) perchloric acid was added to the sediment. This process was repeated 2 times. Then, the mixture was filtered (porosity 0.45 μm) and the filtrate was derivatized with dansyl chloride according to **Dadáková et al., (2009)**. 1.7-heptandiamine was used as internal standard. The derivatized samples were filtered (porosity 0.22 μm) and applied to a column (Cogent HPLC Column HPS C18, 150 x 4.6 mm, 5 μm). Terms of the separation of the observed biogenic amines are described in works of **Buňková et al., (2013)**.

RESULTS AND DISCUSSION

In Czech standard ČSN 56 9606, there is no limit for the total amount of microorganisms in smear-ripened cheese or in blue cheese. The limit is probably not listed because various doses of the culture of microflora is intentionally added to these cheeses depending on the type of the cheese and microorganisms (MO), which affects the aroma significantly, grow there during the ripening **(Buňková et al., 2012)**. Also, the standards often focus on determining microorganisms causing diseases in human, or microorganisms able to devalue the final product. The presence of such microorganisms is usually not expected in cheese (or they are present in very low numbers) because microorganisms in the starter culture are supposed to overgrow them. Identified amounts of MO might include mesophilic starter culture of the genera *Lactococcus* and *Leuconostoc*, bacteria *Brevibacterium linens*, coryneform bacteria, staphylococci and yeast **(Görner et al., 2004)**. On the other hand, many lactic acid bacteria, like coryneform bacteria, are more difficult to cultivate and that is why they are not detected on PCA. This might lead to the conclusion that only microorganisms without specific cultivation requirements can be identified on PCA and thus they can be classified among contaminating microflora (without causing any significant changes in the final product).

Soft smear-ripening acid cheese

In tested samples of soft smear-ripening acid cheese, the total amount of microorganisms (CFU) was defined to be between 10^5-10^9 $CFU.g^{-1}$. Most of microorganisms were determined in samples of soft smear-ripening acid cheese unflavoured, followed by flavoured cheeses and the least of MO was present in samples of cold-ripened cheese. Numbers of enterobacteria were always over 10^5 $CFU.g^{-1}$ in soft smear-ripening acid cheese, $>10^3$ $CFU.g^{-1}$ in samples of smear cold-ripened cheese unflavoured and 10^4-10^5 $CFU.g^{-1}$ in flavoured cheeses. Lactic acid bacteria were present in cheese in amounts ranging from 10^6-10^8 $CFU.g^{-1}$ mostly, in samples of soft smear-ripening acid cheese (10^7-10^9 $CFU.g^{-1}$). According to **Bockelmann, (2002)**, the number of lactic acid bacteria ranged between 10^2-10^7 $CFU.g^{-1}$ depending on the cheese sample. Further they state that the amount of lactic acid bacteria exceeded 10^7 $CFU.g^{-1}$ in some of the cheese samples. Similar results were achieved in this study. Enterococci were detected in 10 cheese samples with their numbers ranging from 10^3-10^6 $CFU.g^{-1}$. Most of the enterococci was found in soft smear-ripening acid cheese flavoured with its amount over $>10^5$ $CFU.g^{-1}$. In samples of cheese type like Limburger and Romadur unflavoured, there was a high amount of enterococci determined $>10^3$ $CFU.g^{-1}$, which corresponds with the studies of **ockelmann et al., (2005)** where presence of enterococci in Limburger and Romadur cheeses was set to be in an amount of $>10^4$ $CFU.g^{-1}$. Lactic acid bacteria form a very large group of microaerophilic to facultative anaerobic gram-positive cocci and rods fermenting saccharides to produce lactic acid. There are included genera *Lactococcus*, *Streptococcus*,

Table 1 Contend of biogenic amines in mg.kg^{-1} in tested smear-ripened cheese.

	Biogenic amines [mg.kg^{-1}]						
Samples of cheese	**Phenylethylamine**	**Putrescine**	**Cadaverine**	**Histamine**	**Tyramine**	**Spermidine**	**Spermine**
Soft smear-ripening acid cheese	11.6 ±0.8	2408.5 ±53.4	1816 ±45.9	373.8 ±11.9	1398 ±76.9	26.1 ±0.2	29.7 ±1.3
Soft smear-ripening acid cheese smoked	ND	701.2 ±14.4	1518 ±18.1	220.9 ±16.8	1118.9 ±78.3	33.3 ±1.7	38.1 ±3.2
Soft smear-ripening acid cheese flavoured pepper	ND	108.1 ±8.2	594.4 ±49.0	396.1 ±28.2	243.4 ±21.6	26.4 ±1.1	33.6 ±2.2
Soft smear-ripening acid cheese flavoured garlic	ND	407.1 ±13.3	773.8 ±35.5	302.22 ±22.1	554.8 ±17.8	31.7 ±1.0	55.7 ±1.9
Soft ripened cold cheese unflavoured	ND	ND	ND	ND	13.9 ±1.0	ND	17.2 ±1.3
Soft ripened cold cheese flavoured green pepper	ND	ND	ND	ND	12.6 ±0.4	ND	16.8 ±1.3
Soft ripened cold cheese flavoured walnuts	ND	ND	ND	ND	7.8 ±0.7	ND	14.9 ±0.4
Smear-ripened cheese flavoured garlic and herbs	ND	352.7 ±11.1	ND	ND	345.8 ±11.1	ND	32.3 ±0.7
Smear-ripened cheese flavoured chilli	ND	148.53 ±11.4	3.9 ±0.1	4.4 ±0.2	167.4 ±10.2	4.4 ±0.2	37.2 ±2.3
Smear-ripened cheese	ND	18.7 ±1.3	ND	34.1 ±0.8	38.0 ±1.0	3.0 ±0.1	57.7 ±0.7
	ND	ND	ND	ND	51.0 ±4.5	3.8 ±0.3	95.4 ±4.8
	ND	ND	4.5 ±0.3	ND	60.4 ±2.8	ND	58.4 ±3.3
	ND	ND	ND	ND	16.3 ±0.9	8.4 ±0.3	42.6 ±1.6
	ND	ND	ND	ND	44.4 ±1.2	6.4 ±0.2	85.1 ±1.9
	254.8 ±14.6	ND	2389.2 ±108.9	96.7 ±4.5	920.7 ±24.8	ND	60.2 ±4.0

* Tryptamin ND.

Enterococcus, *Pediococcus*, *Leuconostoc* and *Lactobacillus*. The amount of lactic acid bacteria exceeded 10^7 CFU.g^{-1} in some cheese samples. Similar results were acquired in this study as well. A great amount of yeast can be found in smear-ripened cheese because it is a kind of MO that gives typical aroma and taste to the cheese. Amount of yeast and mould in samples ranged in 10^4-10^6 CFU.g^{-1}.

Biogenic amines in soft smear-ripening acid cheese and in smear-ripened cheese

In ten samples of smear-ripened cheese, the total content of BA was 22-1000 mg.kg^{-1} and in 5 samples of these cheeses it was in range of 1000-6000 mg.kg^{-1}. **Standarová et al., (2010)** states in their work that values for cheese stored at 20 °C for 6 weeks are >4000 mg.kg^{-1}. Concluding from our research, the raised amount of BA in five submitted samples could be caused by improper storage of the cheese in stores. Tyramine, spermine, spermidine, putrescine, cadaverine, histamine and phenylethylamine were detected in group of smear-ripened cheeses. Putrescine was found in seven samples in the maximal amount >2000 mg.kg^{-1}. According to **Loizzo et al., (2012)**, the amount of putrescine was 532,2 mg.kg^{-1}. **Standarová et al., (2010)** claim that the putrescine amount is 212 mg.kg^{-1} when stored at 5 °C during 4 weeks. Cadaverine was detected in seven cheese samples as well. Its numbers ranged from 3-2400 mg.kg^{-1}. **Loizzo et al., (2012)** presented that the amount of cadaverine is >700 mg.kg^{-1}. According to **Standarová et al., (2010)** the cadaverine amount in this sort of cheese stored at 5 °C for 4 weeks was >400 mg.kg^{-1}. Tyramine, along with spermine, was detected in all analyzed samples of cheeses. It was present in rather wide range 8-1398 mg.kg^{-1}. **Mayer et al., (2010); Loizzo et al., (2012); Standarová et al., (2010)** detected tyramine in the same intervals. The amount of histamine in the studied samples of cheeses was 4-400 mg.kg^{-1}. This BA was found in seven samples. **Loizzo et al., (2012)** present the amount to be 168.3 mg.kg^{-1}, **Standarová et al., (2010)** stated 216 mg.kg^{-1} of histamine in soft smear-ripening acid cheese and the values goes up to 500 mg.kg^{-1} in the study of **Mayer et al., (2010)**. Spermidine was found in 9 out of 15 samples of analyzed cheeses. Compared to the other biogenic amines, its amount in the samples was rather low.

Mould cheese

Groups of determined microorganisms in samples of blue cheese were same like in the samples of smear-ripened cheese. The total number of microorganisms in all of the observed samples was 10^5-10^7 CFU.g^{-1}. Enterobacteria was detected only in sample of white veined cheese (dry matter 28% w/w, fdm-fat in the dry matter 49.5% w/w) with its amount being 5.7 x 10^3 CFU.g^{-1}. The amount of enterococci was determined only in sample of white

Table 2 Content of biogenic amines in mg.kg^{-1} in tested mould-ripened cheeses.

Characterisation of samples of cheese			Concentration of biogenic amines [mg.kg^{-1}]				
	dry matter (% w/w)	fdm (% w/w)	Putrescine	Cadaverine	Tyramine	Spermidine	Spermine
White veined cheese	26.4	48	15.8 ±1.2	ND	20.0 ±0.8	ND	38.3 ±1.1
	51	60	20.1 ±1.7	ND	8.8 ±0.7	ND	25.9 ±2.2
	60	50	16.9 ±1.1	ND	10.2 ±0.8	ND	33.7 ±1.4
	23	46	11.1 ±0.9	ND	9.1 ±0.5	5.5 ±0.3	23.8 ±0.7
	49	43	19.2 ±1.2	ND	11.9 ±0.5	ND	23.4 ±0.8
	52	46	16.1 ±1.1	ND	16.3 ±1.5	ND	38.9 ±3.1
	31	50.5	ND	ND	18.1 ±1.5	5.1 ±0.4	48.2 ±0.9
	22.5	45	117.5 ±7.5	311.1 ±9.8	35.7 ±3.1	5.9 ±0.4	77.8 ±2.4
	28	49.5	10.3 ±0.4	ND	11.1 ±0.7	ND	25.1 ±1.3
Blue veined cheese	52	50	6.4 ±0.1	10.2 ±0.5	72.2 ±4.2	15.4 ±1.2	132.5 ±8.6
	50	52	11.9 ±0.9	ND	127.8 ±4.9	8.3 ±0.6	124.4 ±2.2
	31	53	23.0 ±1.7	ND	87.9 ±2.3	8.4 ±0.2	171.9 ±4.5
	48	48	11.0 ±0.6	ND	40.6 ±2.3	21.1 ±1.9	116.9 ±7.1
	50	52	5.5 ±0.2	ND	220.5 ±11.7	10.5 ±0.8	199.8 ±13.2
	50	53	17.9 ±0.9	ND	66.1 ±5.1	13.8 ±0.5	168.1 ±11.2

* Tryptamine, Phenylethylamine, Histamine ND, fdm – fat in dry matter

veined cheese with fdm 46% w/w. Numbers of lactic acid bacteria were approximately 10^5-10^8 CFU.g^{-1} (lactic acid cocci) and 10^6-10^8 CFU.g^{-1} (*Lactobacillus* spp.). The amount of yeast and mould ranged from 10^5-10^8 CFU.g^{-1}.

The total amount of microorganisms in blue veined cheese was higher than the amount in white veined cheese and it was 10^6-10^8 CFU.g^{-1}. Presence of enterobacteria was detected only in two samples of blue veined cheese (dry matter 52% w/w, fdm 50% w/w and dry matter 31% w/w, fdm 53% w/w). In the blue veined cheese (dry matter 52% w/w, fdm 50% w/w), the number of enterobacteria was 2,9.10^3 CFU.g^{-1} and 2,2.10^5 CFU.g^{-1} in the sample of blue veined cheese (dry matter 48% w/w, fdm 48% w/w). Enterococci were not determined in the blue veined cheese (52% w/w, fdm 50% w/w) while in other cheeses, the amount of enterococci was in order of 10^5-10^7 CFU.g^{-1}. The numbers of lactic acid bacteria (both cocci and lactobacilli) were 10^5-10^8 CFU.g^{-1}. The amount of yeast and mould in the tested cheese samples ranged from 10^6-10^8 CFU.g^{-1} with the exception of blue veined cheese (dry matter 50% w/w, fdm 53% w/w) where growth of yeast and mould was rather low. Generally, the total numbers of microorganisms in samples of white veined cheese were in lower orders than in samples of blue veined cheese.

Biogenic amines in blue cheese

Tyramine, spermine, putrescine, cadaverine and spermidine are biogenic amines determined in group of blue cheeses. The total amount of biogenic amines in the group was in range 40-600 mg.kg^{-1} with higher amount of BA being determined in blue veined cheese (200-500 mg.kg^{-1}). **Komprda et al., (2009)** analyzed blue veined cheese in his study and the same interval for the total amount of BA was presented. In samples of white veined cheese, the total amount of BA reached moderate values <100 mg.kg^{-1}, only two samples of this group exceeded this value. **Mayer et al., (2010)** analyzed five samples of the group and the total volume of BA was determined to be >100 mg.kg^{-1} in all tested samples. In our study putrescine was found in 14 samples while, on the other hand, cadaverine was detected only in two samples in amount 10 mg.kg^{-1} and 300 mg.kg^{-1}. The total amount of BA in white veined cheese (49.5% fdm) reached value 46.4 mg.kg^{-1}. Among all the blue veined cheese, the highest value was reached by the cheese (52% fdm) whose value of BA amount was 436.3 mg.kg^{-1}. The concentration of tryptamine and phenylethylamine was low or not detected at all in these cheeses (Table 2.).

Four biogenic amines (tyramine, spermine, spermidine and putrescine) were detected in all samples of the blue veined cheese group. Putrescine and spermidine reached values <100 mg.kg^{-1}. Putrescine was determined in all samples of white veined cheese with the exception of one cheese (50.5% fdm). In sample of cheese (45% fdm), the value of putrescine concentration was 117.5 ±7.5 mg.kg^{-1}. In sample of white veined cheese (50% fdm), the concentration was 10.17 ±0.5 mg.kg^{-1}. On the other hand, tyramine and spermine exceeded this value. The concentration of tyramine in white veined cheese (52%

fdm) was determined to be 220.5 ±11.7 mg.kg^{-1}. The values of spermine exceeding 100 mg.kg^{-1} were found in all samples of this cheese group.

Three biogenic amines-tryptamine, phenylethylamine and histamine-were not detected in any of 15 tested samples. The cadaverine value was 311.13 ±9.6 mg.kg^{-1} in sample of white veined cheese (45% fdm) which was the highest value. Such amount of biogenic amine can be considered toxic. Moreover, cadaverine can increase unwanted toxic effects of the other biogenic amines (histamine and tyramine) or it can inhibit detoxification system, respectively **(Shalaby, 1996)**.

White veined cheeses and blue veined cheeses each contain very different amount of biogenic amines. This amount is rather variable even within one type of cheese or it can be different in various layers of the cheese **(Fernandes, 2008; Buňková et al., 2010)**. The normal amounts of biogenic amines in food and drinks (approximately <100 mg.kg^{-1}) do not pose any significant threat to a healthy human because they are metabolized by detoxification activity of microorganisms in the human intestinal tract **(Halász et al., 1994; Shalaby, 1996)**. **Shalaby (1996)** suggested that the sum of histamine + tyramine + putrescine + cadaverine amounts in cheese should not go beyond 900 mg.kg^{-1}. If the sum of concentration of tested biogenic amines reaches over 200 mg.kg^{-1}, it can be considered as a toxicologically relevant amount **(Halász et al., 1994)**.

SUMMARY

The amount of enterobacteria in fresh cheese exceeded 10^5 CFU.g^{-1}. In smear-ripened cheese flavourless (Romadur type), the amount was >10^3 CFU.g^{-1} and 10^4-10^5 CFU.g^{-1} in smear-ripened cheese with flavour. Enterococci are usually present in various environments. They are very frequent in cheese and they play important role during ripening when they create typical cheese aroma. Formerly, enterococci presence was considered as a sign of poor hygiene. However, it was found out that many enterococci strains are able to produce various antibacterial proteins against pathogenic microorganisms, for example *Listeria monocytogenes*. As non-starter microorganisms, enterococci can also contribute to the development of taste and aroma of the cheese **(Fox et al., 2004; Buňková et al., 2012)**. Number of enterococci was in range 10^4-10^5 CFU.g^{-1} in white veined cheese and 10^5-10^7 CFU.g^{-1} in blue veined cheese. The values of lactic cocci were 10^6-10^8 CFU.g^{-1} in both analyzed cheese groups. Yeast and mould were detected in both groups in amount 10^5-10^8 CFU.g^{-1}. Enterobacteria in food show lack of hygiene during the food production. The most important bacteria in foods are *Escherichia*, *Shigella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Proteus* and *Yersinia*. The presence of enterobacteria was found in 12 samples out of 30 tested cheese types (enterobacteria was not detected in cold-ripened cheese).

The ability to form biogenic amines was described for certain microorganisms, especially *Enterobacteriaceae*, *Pseudomonas* spp. **(Buňková et al., 2010)** and for lactic acid bacteria **(Buňková et al., 2009; Lorencová et al., 2012)**. However, biogenic amines exist more in cheese due to secondary contamination by microorganisms **(Pleva et al., 2012)**. Biogenic amines were studied in two groups: blue cheeses (white veined and blue veined) and smear-ripened cheeses. These cheeses are part of group of soft ripened cheeses. Both groups are considered to contain biogenic amines because the number of microorganisms, as precursors of biogenic amines and free amino acids, is rising during ripening. It is very difficult to determine biogenic amines and polyamines in food. Their amount depends on various external factors. For example, putrescine and cadaverine increase effects of histamine and tyramine. The toxic dose for human organism is not defined exactly and scientist state wide range of possible toxic dose 200-800 mg.kg^{-1} **(Halász et al., 1994; Silla Santos, 1996)**. Some of the analyzed cheeses could pose health risks for a consumer because this amount was exceeded.

These substances can be natural part of materials, for example spermine and spermidine are growth factors and that is why they are present in animal tissue and they can pass into final products **(Kalač and Křížek, 2005)**. Further, biogenic amines can be present starter and non-starter microorganisms. The origin of detected putrescine could be milk used in production because putrescine is natural component of raw milk **(Santos et al., 2003)** or it can be created by decarboxylation of L-arginine and L-ornithine **(Agostinelli et al., 2010; Igarashi and Kashiwagi, 2010; Fuell et al., 2010; Larqué et al., 2007)**. A lot of authors published that lactic acid bacteria (LAB) is one of biogenic amine producers **(Buňková et al., 2009; Ladero et al., 2012; Linares et al., 2011)**. Pasteurized milk was used in production of the cheeses and there may be thermotolerant microorganisms (for example *Enterococcus* genus) in milk modified that way. This group of microorganisms can produce biogenic amines and that is why non-starter microorganisms can be origin of biogenic amines **(Novella-Rodriguez et al., 2004, 2002; Ladero et al., 2010, 2011)**. The presence of histamine and cadaverine can serve as indicator of hygiene during production **(Stadnik and Dolatowski, 2010; Halász et al., 1994)**. Further, biogenic amines can act as contaminants **(Coton et al., 2012)**.

The decrease of biogenic amine amount can be caused by spices and spice mixes because some of them have inhibitory effects on microorganisms and so they can reduce the synthesis of biogenic amines **(Komprda et al., 2004, 2009; Naila, 2010)**.

CONCLUSION

The results of this study show that biogenic amines and polyamines are common in cheese and they can pose a significant health danger for consumers in some cases. They are not monitored by any legislative control authority as they are secondary metabolites even though they are health risks potentially. Biogenic amines were observed in two groups of blue cheeses (white veined cheese and blue veined cheese) and smear-ripened cheeses. Those cheeses belong to group of soft ripened cheese. In both groups, there is a possibility of presence of biogenic amines because the number of microorganisms increases during ripening with the microorganisms posing as precursors for biogenic amines and free amino acids.

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EFFECT OF FUNGICIDAL TREATMENT ON DIGESTIBILITY OF MYCOTOXINS IN VITRO

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ABSTRACT

In this experiment, the effect of fungicidal treatment on the release of various mycotoxins was tested in rumen fluid in vitro. The three groups of barley crop with different fungicide treatment were included in the experiment. The first group served as the control one without fungicide treatment. The second group of barley (variant A) was treated with Hutton (0.8 L/ha at BBCH 36) + Zantar (1.5 L/ha at BBCH 65). The third group of barley (variant B) was treated with the combination of Hutton (0.8 L/ha at BBCH 36) + Prosaro EC250 (0.75 L/ha at BBCH 65). In the original mass of barely, ten levels of mycotoxins were established. Subsequently, the samples were incubated in the machine Daisy II for 24 hours. The cellulase and pepsin enzymes were used in the incubation. Following mycotoxins were determined in the incubation fluid such as dexynivalenol, zearalenone, deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol. In the variant A, the level of dexynivalenolu was higher by 36%, zearalenone by about 2%, deoxynivalenol-3-glucoside by 12%, and 3-acetyl-deoxynivalenol by 39%. Low levels of the mycotoxins were found out in the variant B. Deoxynivalenol level was lower by 19%, zearalenone by 30%, deoxynivalenol-3-glucoside by 37% ($p < 0.05$). The 3-acetyl-deoxynivalenol level was higher by 12% in a comparison with the control group. The obtained results showed that the fungicidal treatment and digestive enzymes could eliminate the transition of mycotoxins into incubative (rumen) liquid, and thereby to reduce the risk of the load of the organism by the mycotoxins. According to the results, it is obvious that low levels of various mycotoxins presented in the barley grains, as well as the transition of these mycotoxins in the incubation fluid were decreased. Some fungicides can play a significant role in the occurrence of mycotoxins barely grain.

Keywords: mycotoxins; barley; in vitro; digestibility

INTRODUCTION

Barley is classified as one of the most important cereals in the Czech Republic. It is used for livestock feed and food industry - especially malting **(Belakova et al., 2014, Horky et al., 2012a)**. Mycotoxins are fungal secondary metabolites having mutagenic, carcinogenic, and cytotoxic effects. They can often contaminate agricultural commodities in spite of the various protective measures **(Jancikova et al., 2012a)**. Recently, the attention has been focused on the so-called masked mycotoxins. The deoxynivalenol-3-glucoside and 3-acetyldeoxynivalenol metabolized from deoxynivalenol are the most common occurring. The presence of the masked mycotoxins presents the same hazard as the occurrence of classic mycotoxins. In the food productive process, the production of malt, beer, and bread are back metabolized to deoxynivalenol **(Horky et al., 2013**, **Zachariasova et al., 2012)**. Fusarium mycotoxins in foods are the most frequent type of contamination. Deoxynivalenon mycotoxins, zearalenone and T-toxin are responsible for the extensive damage to both feed and food. They directly threaten the health of consumers **(Horky, 2014a, Maul et al., 2014)**. Fungicides are pesticides that are used to eliminate harmful phytopathogenic fungi on crop plants and the substances of organic origin. Fungicides have the ability to eliminate the occurrence of fungal biomass in plants and thereby to reduce the risk of mycotoxin production **(Jancikova et al., 2012b, Schmidt-Heydt et al., 2013)**. After the fungicidal treatment, the development of mold is significantly reduced. Untreated plants can be characterized by higher levels of mold up to 260% **(Pirgozliev et al., 2012**). The similar effect as fungicides may have as well as the antioxidant enzymes **(Horky, 2014a, Horky et al., 2012b)**. The susceptibility of animals to mycotoxins is different. The least susceptible animals are ruminants due to their buffering ability of the rumen **(Horky, 2014b, Nevrkla et al., 2013)**. The aim of the experiment was to test the effect of fungicidal treatment of barley on the release of various mycotoxins in rumen fluid in vitro.

MATERIAL AND METHODOLOGY

Barley samples coming from Libcany area (the Czech Republic) were put in the experiment from the harvest in 2012. The barley was artificially treated with *Fusarium culmorum* (WGSm. Sacc. Strain KM16902; DON chemotype). The inoculation with a conidia suspension of the pathogenic isolate of *F. culmorum* (concentration 0.5 mil. conidia/1 mL of inoculum; spray dose of 200 $L.ha^{-1}$) was performed in the optimal vegetative phase according to the methodology of **Tvarůžek et al.** (**2012**). In the inoculation period, the vegetation was sprayed with clean water before the inoculation in dry and sunny weather. Subsequently, the chemical treatment with

fungicides was applied in the barley. The first group was untreated and served as the control one. The second group of barley (variant A) was treated with Hutton (0.8 L/ha at BBCH 36) + Zantar (1.5 L/ha, BBCH 65). The third group of barley (variant B) was treated with the combination of Hutton (0.8 L/ha, BBCH 36) + Prosaro EC250 (0.75 L/ha, BBCH 65).

Composition of Fungicides:

***Hutton* - active substance:**

Prothiokonazol 100 g/L 2-[2-(1-chlorcyklopropyl)-3-(2-chlorfenyl)-2-hydroxypropyl]-2,4-dihydro-1,2,4-triazol-3-thion.

Spiroxamin 250 g/L [(8-terc-butyl-1,4-dioxaspiro[4.5]dekan-2-yl)methyl]ethyl(propyl)amin.

Tebukonazol 100 g/L 1-p-chlorofenyl-4,4-dimethyl-3-(1H-1,2,4- triazol-1-ylmethyl)-pentan-3-ol

***Prosaro 250 EC* - active substance:**

Prothiokonazol 125 g/L 2-[2-(1-chlorcyklopropyl)-3-(2-chlorfenyl)-2-hydroxypropyl]-2,4- dihydro-1,2,4-triazol-3-thion.

Tebukonazol 125 g/L 1-p-chlorofenyl-4,4- dimethyl-3-(1H-1,2,4- triazol-1-ylmethyl)-pentan- 3-ol.

***Zantara* - active substance:**

Bixafen 50 g/L N-(3,4-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide.

Tebukonazol 166 g/L (3-1-(4-chlorfenyl)-4,4-dimethyl-3-[(1H-1,2,4-triazol-1-ylmethyl]pentan-3-ol.

Before the incubation (in Daisy II), all barley samples were analyzed for the content of individual mycotoxins. From each group, the three samples were collected and analyzed. The results of average concentrations of mycotoxins are listed in Table 1. The barley samples were grounded on the laboratory mill with mesh size 1 mm. The machine Daisy II Incubator - ANKOM Technology, New York was used for the incubation. A 4 g milled sample was taken for the incubation divided into the incubation bags - F57 (Ankom, Macedonia) in the amount of 0.25 g per incubation bag.

Preparation of Solutions

A 1.5 liter of solution was used for one incubation. For preparation of pepsin solution, 3 g of pepsin (*Pepsin from porcine gastric mucosa* 800 - 2500 units/mg protein - (Sigma-Aldrich, Germany)) dissolved in 1.5 liters of 0.1 M HCl then heated to 40 °C. Immediately, it was put in the incubation. Acetate buffer (pH 4.6): 10.2 g sodium acetate (3 H_2O) was dissolved in 1.5 l of distilled water. The pH value was modified using acetic acid or NaOH. In the preparation of cellulase solution, 1.5 g of cellulase was dissolved (*Cellulase Trichoderma viride*, 3-10 units/mg solid - Sigma Aldrich, Germany) in 1.5 liters of acetate buffer heated to 40 °C then the incubation could start. The incubation lasted for 24 hours at 37 °C. The obtained cultured fluids were analyzed on the concentration of mycotoxins.

Determination of Mycotoxins

Solid Samples - Extraction

A 2 g barley sample was weighed to PTFE centrifuge tubes (50 ml) followed by the addition of 10 ml of distilled water acidified (0.2% formic acid). Then the sample was shaked, closed and left for 30 minutes due to the wetting of the matrix. A 10 ml of acetonitrile was added in the sample with water followed by the extraction on the laboratory mixer for 30 minutes (240 RPM). The 4 g of $MgSO_4$ and 1 g of NaCl were put in the cuvette and shaken vigorously for 1 minute. The prepared sample was centrifuged for 5 minutes (10,000 RPM). After centrifuging, the sample was taken (approx. 1.5 ml) for purification using a microfilter with a porosity of 0.2 μm (centrifugation for 2 min, 5000 RPM). The sample was transferred to the vials and prepared for analysis. The samples were stored at -18 °C in glass vials before the analysis. For the identification and quantitative determination of the mycotoxins, Acquity UPLC® System (Waters, Milford, MS, USA) in a connection with tandem mass spectrometer QTRAP® (AB Sciex, Toronto, ON, Kanada) is used for the instrumentation of ultra-efficient liquid chromatograph Acquity UPLC ® System (Waters, Milford, MS, USA).

Table 1 Concentration of detected mycotoxins in control, A and B variant of the fungicidal treatment

Mycotoxins	Variant of fungicidal treatment		
	Control	A	B
Deoxynivalenol	12360.9 ±2045.5	19852.7 ±2173.8	11287.2 ±2718.8
deoxynivalenol-3-glukosid	6774.5 ±502.5	9042.7 ±678.3	5035.3 ±494.9
3-acetyl-deoxynivalenol	1449.4 ±219.4	1969.8 ±257.4	1135.8 ±230.3
Zearalenon	3737.5 ±880.3	4096.3 ±702.9	2947.2 ±704.0
Beta-zearalenol	89.9 ±18.2	107.3 ±16.5	58.8 ±8.1
Alternariol	56.3 ±12.1	13.5 ±5.2	12.1 ±4.2
Alternariol-methylether	2.3 ±0.9	2.6 ±0.9	2.4 ±0.1
Enniatin B	391.5 ±102.7	432.4 ±109.2	460.3 ±104.3
Enniatin A	4.5 ±2.8	4.7 ±3.3	5.5 ±3.0
Enniatin A1	25.8 ±12.4	27.7 ±18.3	32.4 ±13.0

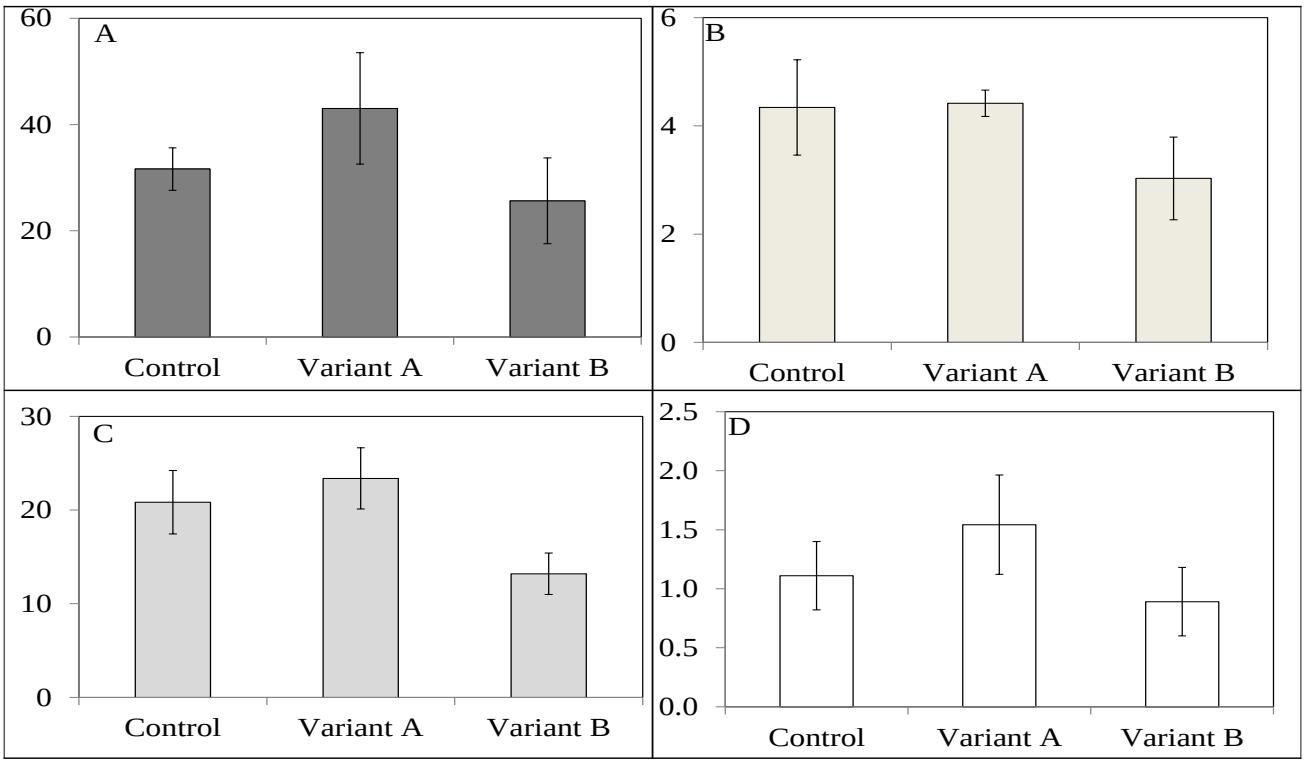

Figure 1 The concentration of mycotoxins detected in the incubation fluid (mg/kg): dexynivalenol (A); zearalenone (B); deoxynivalenol-3-glucoside (C); 3-acetyl-deoxynivalenol (D).

The program Analyst ® (Thermo Fisher Scientific) is used for data processing.

Liquid Samples

Liquid samples were purified using a microfilter with a porosity of 0.2 µm (centrifugation for 2 min, 5000 RPM) before the instrumental analysis. Furthermore, the solid samples were stored at -18 °C and measured using instrumentation consisting of ultra-efficient liquid chromatograph Acquity UPLC ® System (Waters, Milford, MS, USA) in connection with tandem mass spectrometer QTRAP ® (Applied Biosystems, Toronto, ON, Canada).

Determination of Mycotoxins

The total of 57 mycotoxins of microscopic filamentous fungi of the genus *Fusarium*, *Penicillium*, *Aspergillus*, *Alternaria*, *Claviceps* a *Stachybotrys* were set such as Fusarenon X, nivalenol, deoxynivalenol, alfa-zearalenol, beta-zearalenol, zearalenon, 3-acetyl-deoxynivalenol, patulin, alternariol, alternariol-methylether, deoxynivalenol-3-glukoside, enniatin B, enniatin B1, enniatin A, enniatin A1, ergokornin, ergokorninin, ergokristin, ergokristinin, ergokryptin, ergokryptinin, ergosin, ergosinin, ergometrin, ergotamin, ergotaminin, agroklavin, neosolaniol, diacetoxyscirpenol, fumonisin B1, fumonisin B2, fumonisin B3, 15-acetyl-deoxynivalenol, aflatoxin B1, aflatoxin B2, aflatoxin G2, aflatoxin G1, HT-2 toxin, T-2 toxin, sterigmatocystin, ochratoxin A, citrinin, beauvericin, cyklopiazon acid, mycophenolic acid, penicillin acid, rokfortin C, tentoxin, tenuazonic acid, verrucarol, verruculogen, penitrem A, stachybotrylaktam, phomopsin A, gliotoxin, meleagrin, paxillin.

Statistics

The data were processed statistically using STATISTICA.CZ, version 10.0 (the Czech Republic). The results were expressed as mean ± standard deviation (SD). Statistical significance was determined by examining the basic differences between groups ANOVA and Scheffé's test (one-way analysis). The differences with $p < 0.05$ were considered to be significant.

RESULTS

During the analyzing of the incubated fluid, the following mycotoxins such as deoxynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol were detected. The mycotoxins that were analyzed in the original mass of barley such as beta-zearalenol, alternariol, alternariol-methylether, enniatin B, enniatin A, enniatin A1 were below the detection limit indicating the fact that the mycotoxins were largely eliminated by digestive enzymes. The highest concentrations were measured in deoxynivalenol fungicidal treatment of the variant A (by 36%) compared with the control group of barley. Conversely, the variant B had a lower concentration of deoxynivalenol about 19% (Figure 1A). The identical value of the mycotoxin was observed in the control and fungicide variants A during the assessing levels of zearalenone in the incubation fluid. The variant B had lower levels of zearalenone by up 30% compared with the control group in the incubation fluid (Figure 1B). The two masked mycotoxins deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenolwere were also detected in the incubation fluid. In the variant A, Deoxynivalenol-3-glucoside (Figure 1C) was increased by

12% compared with the control group. The variant B had significantly lower levels of mycotoxin by 37% ($p < 0.05$). High concentrations of 3-acetyl-deoxynivalenol was measured for variant A (39%). In the variant B, the quantities of the mycotoxin in the incubation fluid was increased by 12% compared with the control group (Figure 1D).

DISCUSSION

In our experiment, the effects of fungicidal treatment on digestibility of the individual mycotoxins were compared with the usage of Daisy II incubator. The following mycotoxins were detected in the incubation fluid such as dexynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol. In the experiment, in which Fusarium toxins were added to the feed dose of ruminants in the amount of 60 and 30%, the reduction of synthesis of microbial protein was observed. In ruminal environment, the amount of Fusarium mycotoxins was significantly decreased **(Hildebrand et al., 2012)**. In our experiment, the similar effect was observed on the release of mycotoxins in the ruminal environment during the effect of enzymes (cellulase, pepsin). During the incubation of mold feed (for 24 hours), barley base, rapeseed cakes, alfalfa hay and barley straw (fungus 70%) was not affected by the degradation of dry matter of the individual components of the diet. The occurrence of mycotoxins was not monitored in the experiment. The mycotoxins such as aflatoxin 0-30%, deoxynivalenol 0-50%, T-2 toxin 0-70%, zearalenone 0-40%, deoxynivalenol 0-35%, ochratoxin A 50-100% are degraded in their derivatives with varying efficiency in the rumen. The susceptibility of animals to mycotoxins is different. The ruminants are the least susceptible category because of the buffering ability of the rumen. They are able to reduce and tolerate higher levels of mycotoxins **(Undi & Wittenberg, 1996)**. The rumen microorganisms apparently metabolize toxins into non-toxic metabolites. We can also agree with these findings in comparison with the results of our experiment. After the incubation in a mixture of enzymes (pepsin, cellulase), the mycotoxins such as beta-zearalenol, alternariol, alternariol-methylether, enniatin B, enniatin A, enniatin A1 were degraded with high efficiency. Dexynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol were in the original mass of barley at very high concentrations. It was probably the reason why they were analyzed in the incubation fluid. From the findings of other studies, it could be suggested that the rumen environment completely eliminated the mycotoxin called ochratoxin A infected wheat straw **(Abdelhamid et al., 1992)**. Berthiller **(Belakova et al., 2014)** investigated the back hydrolysis of deoxynivalenol-3-glucoside to the original mycotoxin (deoxinivalenol) in the stomach monogastry (in vitro). Deoxynivalenol-3-glucoside was resistant to the acidic environment of the stomach incubated in 0.2 M hydrochloric acid for 24 hours at 37°C. Conversely, some of the lactic acid bacteria were able to hydrolyze deoxynivalenol-3-glucoside back to deoxinivalenol.

CONCLUSION

In the experiment, the effect of fungicidal treatment on the release of various mycotoxins was observed in rumen fluid in vitro. The experiment included the three groups of barley using different fungicide treatment. The first control group was without fungicidal treatment. The second group of barley (variant A) was treated with Hutton (0.8 L/ha at BBCH 36) + Zantar (1.5 L/ha at BBCH 65). The third group of barley (variant B) was treated with the combination of Hutton (0.8 L/ha at BBCH 36) + Prosaro EC250 (0.75 L/ha at BBCH 65). In the mass, the level of ten mycotoxins was measured. Then the samples of barley were incubated in the incubator Daisy II for 24 hours using the cellulase enzyme and pepsin. The dexynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol were determined in the incubation fluid. In the variant A, dexynivalenol level was higher by 36%, zearalenone by about 2%, deoxynivalenol-3-glucoside by 12% and 3-acetyl-deoxynivalenol by 39%. Low levels of the mycotoxins were found out in the variant B, the level of deoxynivalenol was lower by 19%, zearalenone by 30%, deoxynivalenol-3-glucoside by 37% ($p < 0.05$), and 3-acetyl-deoxynivalenol by 12%. According to the results, it is obvious that low levels of various mycotoxins presented in the barley grains, as well as the transition of these mycotoxins in the incubation fluid were decreased. Some fungicides can play a significant role in the occurrence of mycotoxins barely grain.

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MORPHOLOGICAL AND ANTIOXIDANT CHARACTERISTICS OF QUINCE (*CYDONIA OBLONGA* MILL.) AND CHINESE QUINCE FRUIT (*PSEUDOCYDONIA SINENSIS* SCHNEID.)

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ABSTRACT

Quince (*Cydonia oblonga* Mill.) is a fruit species, whose fruits have a high therapeutic value and therefore are used in many countries in traditional medicine. Chinese quince (*Pseudocydonia sinensis* Schneid.) is a lesser used species, although it is a relative of quince. The aim of the study was to evaluate some morphological characters of both kinds of fruit and antioxidant activity of morphological parts of the fruit. For these experiments, two genotypes were used from each species growing in the Arboretum Mlyňany (Slovakia). We determined the antioxidant activity of different parts by the DPPH method. In the genotypes from the evaluated species *C. oblonga*/*P. sinensis* we determined the average weight of the fruit in the fresh condition to be in the range 147.61 – 253.27 g / 197.85 – 466.38 g, the exocarp weight 28.50 – 43.89 g / 24.85 – 45.10 g, the mesocarp weight 116.36 – 204.99 g / 160.30 – 389.80 g, the seed weight 1.05 – 1.54 g / 9.22 – 17.42 g, the height of fruit 74.09 – 80.88 mm / 98.06 – 124.48 mm, average of fruit 60.11 – 81.51 mm / 62.33 – 88.64 mm. In aqueous extracts we determined antioxidant activity of the species *C. oblonga* / *P. sinensis* in dry exocarp in the range 43.52 – 67.73% / 52.76 – 82.20%, in fresh mesocarp 7.36 –14.78% / 15.30 – 23.50%, in dry mesocarp 30.92 – 41.30% / 41.68 – 50.15% and dry endocarp 55.19 – 76.44% / 91.20 – 92.72%. We determined antioxidant activity in methanolic extracts of the species *C. oblonga* / *P. sinensis* in dry exocarp in the range 93.29 – 93.32% / 91.87 – 93.25%, in fresh mesocarp 10.29 – 36.0% / 17.10 – 17.11%, in dry mesocarp 54.55 – 74.11% / 80.39 – 84.11% and in dry endocarp 95.14 – 95.39% / 94.97 – 95.62%. Results document that the fruits of both species can be practically used in the preparation of many dishes, while they can be used as raw material for pharmaceutical and cosmetic use.

Keywords: quince; Chinese quince; fruits, morphology; antioxidant activity

INTRODUCTION

Quince fruit (*Cydonia oblonga* Mill.) in terms of taxonomy belongs to the genus *Cydonia* and the *Rosaceae* family **(Bollinger, 2005)**. This species comes from Asia Minor **(Purves et al., 2004)**. The fruits are big hairy and pear – shaped (var. *pyriformis*) or apple – shaped (var. *maliformis*) pomes yellow color with typical flavor and aroma **(Wagner, 2011)**. In folk medicine quince is used in teas for sore throat, upset stomach and diarrhea. Quince seed infusion is used as a gargle mouth, or mixed with glycerol as emollient for cracked skin twigs. The knowledge indicates that different parts of quince fruits are used as traditional medicines in disorders and diseases of the respiratory system, cough, bronchitis, for fever in digestive disorders, vomiting and diarrhea, for constipation and bloating, inflammation of the kidneys, urinary tract and bladder, cardiovascular and metabolic diseases such as hypertension, hypercholesterolemia, hyperlipidemia, diabetes mellitus, and other **(Khoubnasabjafari and Jouyban, 2011)**. Many previous studies show positive effects of different parts of quinces fruits in various forms on human health. **Hemmati et al., (2010)** studied therapeutic effect of mucilage of quince seed on skin injuries caused by T-2 toxin with positive regenerative effect. Seed mucilage has antiallergic effect and regenerative effect in atopic eczema (**Silva et al., 2002)**. **Khademi, (2009)** describes the hipolipidemic effect of tea and positive effect in lowering cholesterol levels. **Aslan et al., (2010)** reports in his work anti-diabetic effect, **Shinomiya et al., (2009)** and **Jouyban et al., (2010)** describe the hypolipidemic effect of quince leaf decoction on kidney disease caused by hypercholesterolemia. **Magalhães et al., (2009)** deals with antioxidant effect. Many epidemiological studies show the amount of the beneficial effects of quince fruit that need to continue to monitor. Other studies point out that consuming fruits of the family *Rosaceae* is generally beneficial to health effects. However, protectionist effect of various micronutrients and phytochemicals is unclear. Polyphenols as the largest and quantitatively the most important group of phytochemicals may explain part of this effect **(Erlund et al., 2008)**. Chinese quince (*Pseudocydonia sinensis* Schneid.) belongs to the family *Rosaceaea* and for the first time was described by Camilo Karl Schneider as Quince oblong (*Cydonia oblonga* Mill.). It is the only one species from *Pseudocydonia* genus **(USDA, 2013)**. Fruit of the Chinese quince is yellow colored eatable pomes. It has elliptical (var. *ellipsoidea*) or ovoid (var. *ovoidea*) shape. It ripe in October as fruits of Quince oblong those are not eatable directly after harvest

due to bitter pinching taste. The consumption it is possible after appropriate canning heat treatment like compotes, fruit spreads, marmalades, jams, fruit jellies, candied pulp, sweetened syrups and juices combined with ginger, honey and so on **(Facciola, 1990)**. Fruits are very big, 18cm long **(Yan Li et al., 2012)**. Fruits of the Chinese quince are used especially in folk medicine as antitussives that central or peripheral suppress cough. They contain some medically complex of active ingredients including organic acids, flavonoids of rutin and quercetin. Fruits are used for treatment of astma, cold, sore throat, mastitis and tuberculosis in Korea **(NPRI, 1998)**. Another using of these fruits is in household. They are very aromatic so they are often placed into bowl on the table and enrich the room by pleasant spicy fragrance. Hard, dark red wood is used for production of frames **(Khoshbakht and Hammer, 2006)**.

MATERIAL AND METHODOLOGY

The object of our experiment was evaluating the morphological and antioxidant parameters from two genotypes of quince (CO1 – var. *pyryformis*, CO2 – var. *maliformis*) and two genotypes of Chinese quince (PS1 – var. *ellipsoidea*, PS2 – var. *ovoidea*) from the Arboretum Mlynany of Slovak Academy of Sciences (Figure 1). Antioxidant activity of tested quince and Chinese quince genotypes was determined by spectrophotometer Thermo Scientific GENESYS 20. All samples pericarp were homogenized enough for 30 seconds and water and methanolic extract (1 g of native sample of pulp with 25 mL distilled water or methanol) were subsequently prepared from each sample, which was after 8 hours of mixing and filtration subjected to measurement of antioxidant activity by DPPH method. This method lies in reaction of tested substance with stable radical diphenylpicrylhydrazyl – •DPPH (1,1-diphenyl-2-(2,4,6-trinitrophenyl hydrazyl). In the reaction, the radical is reduced and DPPH-H (diphenylpicrylhydrazin) is formed. The reaction is monitored spectrophotometrically. Decrease of absorbance at 515 nm was measured after a certain constant time **(Brand - Williams et al., 1995)**, in our experiment after 10 minutes. Values of antioxidant activity were classified as high (>70% of inhibition), average (40 – 70% of inhibition) and low (<40% of inhibition).

RESULTS AND DISCUSSION

In the first part of the experiment we provide morphological analysis of fruits. In the genotypes from evaluated species *C. oblonga* / *P. sinensis* we determined the average weight of the fruit in fresh condition in the range 147.61 – 253.27 g / 197.85 – 466.38 g, exocarp´s weight 28.50 – 43.89 g / 24.85 – 45.10 g, mesocarp´s weight 116.36 – 204.99 g / 160.30 – 389.80 g, seeds´ weight 1.05 – 1.54 g / 9.22 – 17.42 g, fruit´s height 74.09 – 80.88 mm / 98.06 – 124.48 mm, fruit´s diameter 60.11 – 81.51 mm / 62.33 – 88.64 mm. The obtained experimental data presented in Table 1 and Table 2.

Klimenko, (1993) found in a study of the quinces elongate average weight in the range of 40.00 to 234 grams. In some varieties determine the weight of 300 g (Portugalska) to 2 kg (Berecki). **Salaš, (2001)** determine the weight of the fruit depending on variety from 100 to 1200 g. Comparing our results with those of these authors, we found some consistency. It is common knowledge that the morphological features of the variety are specific and related to the shape of the fruits.

Figure 1 Variability in the shape of fruits quince (*Cydonia oblonga* Mill., CO1 – var. *pyriformis*, CO2 – var. *maliformis*) and Chinese quince (*Pseudocydonia sinensis* Schneid., PS1 – var. *ellipsoidea*, PS2 – var. *ovoidea*); (Photography: A. Monka, 2013).

Table 1 Variability of morphological parameters of fruits quince (*Cydonia oblonga* Mill., CO1 – var. *pyriformis*, CO2 – var. *maliformis*).

Parameters		Sample	Variety	*n*	*Min.*	*Max.*	$\bar{x}$	*CV*
Weight (g)	Whole fruit	CO1	*pyriformis*	10	105.09	205.67	147.61	19.51
		CO2	*maliformis*	10	195.40	301.81	253.27	11.99
	Exocarp	CO1	*pyriformis*	10	21.79	37.49	28.50	19.33
		CO2	*maliformis*	10	33.42	55.03	43.89	16.05
	Mesocarp	CO1	*pyriformis*	10	79.52	163.72	116.36	20.43
		CO2	*maliformis*	10	158.40	246.20	204.99	11.64
	Pubes	CO1	*pyriformis*	10	0.01	0.16	0.07	70.85
		CO2	*maliformis*	10	0.02	0.16	0.08	54.79
	Seeds	CO1	*pyriformis*	10	0.39	1.45	1.05	35.68
		CO2	*maliformis*	10	1.22	1.97	1.54	16.99
Height fruit (mm)		CO1	*pyriformis*	10	71.16	99.60	80.88	10.41
		CO2	*maliformis*	10	65.40	82.34	74.09	7.67
Average fruit (mm)	Middle fruit	CO1	*pyriformis*	10	46.55	68.08	60.11	10.12
		CO2	*maliformis*	10	77.92	89.55	81.51	4.84
	10 mm under apical part of the Stem	CO1	*pyriformis*	10	29.20	38.28	33.14	9.93
		CO2	*maliformis*	10	30.57	41.67	36.10	9.60
	10 mm above basal part of the Fruit	CO1	*pyriformis*	10	32.71	47.06	38.80	12.49
		CO2	*maliformis*	10	32.09	45.60	37.10	9.87
Core (mm)	Height	CO1	*pyriformis*	10	13.58	32.22	27.93	21.08
		CO2	*maliformis*	10	20.35	35.20	25.79	16.98
	Width	CO1	*pyriformis*	10	10.80	27.29	21.43	22.16
		CO2	*maliformis*	10	20.27	33.17	27.73	15.57
Number of seeds in fruits (pc)		CO1	*pyriformis*	10	7.00	23.00	16.50	35.94
		CO2	*maliformis*	10	24.00	42.00	31.80	19.99
Height seeds (mm)		PS1	*ellipsoidea*	10	6.77	8.34	7.53	6.33
		PS2	*ovoidea*	10	8.58	9.65	9.04	4.40
Width seed (mm)		PS1	*ellipsoidea*	10	4.41	5.27	4.88	6.10
		PS2	*ovoidea*	10	4.73	5.52	5.13	5.81

Legend: *n* – number of fruits; *X* – mean – average set; *min*. – minimum value measured in the file; *max*. – maximum value measured in the file; *CV* – coefficient of variation %.

Found in a study of the quinces number of seeds in the range of 32 – 54. Many users do not use the elongate quince seeds. In doing so, the seeds are very valuable resource because they contain up to 22% of slime. Slime is composed of carbohydrates, pentosan, oil, enzymes of cyanogenic glycoside amygdalin, which gives the typical fruit aroma and taste. The seeds swell in water, resulting slime has characteristic effects that are used not only in medicine, but also in cosmetics. Slime is reassuring, anti-irritant, Mitigation and softening the skin and mucous membranes, especially in the treatment of frostbite, bedsores. A fine layer of slime has unique effects. It is used in diseases and inflammations of the mouth and larynx. The cooling effect of slime and is used in inflammatory processes, the defects of the skin (itching, burning, etc.). In cosmetics is used for the preparation of bland protective creams and various dips, but mucilage solutions for the treatment of small, small cracks on the lips, face and nipples. In the pharmaceutical art, and are used for medicinal as mucilaginosum mucilages for the preparation of syrups, drops, and the solution cough, expectoration of sputum respectively to respiratory diseases **(Súkeník, 1997)**. With correlation analysis we determined the degree of tightness of the total weight of the fruits and weight of the individual parts of the fruits when evaluated shaped elongated forms *quinces* and *Chinese quince* (Tab. 3). Among the total weight and the weight of the fruits exocarp we identified statistically significant tightness, which is documented with correlation coefficient for fruits and quince ranging from r = 0.81 (var. *pyrimorfis*) to r = 0.85 (var. *maliformis*) and for fruits Chinese quince in the range from r = 0.50 (var. *ovoidea*) to r = 0.79 (var. *ellipsoidea*). Betweenw eight of fruits and weight of mesocarp of quince and Chinese quince determined high degree of linear dependencies.

Table 2 Variability of morphological parameters of fruits Chinese quince (Pseudocydonia sinensis Schneid., PS1 – var. ellipsoidea, PS2 – var. ovoidea).

Parameters		Sample	Variety	*n*	*min.*	*max.*	$\bar{x}$	*CV*
Weight (g)	Whole fruit	PS1	*ellipsoidea*	10	144.18	273.30	197.85	21.22
		PS2	*ovoidea*	10	346.70	596.80	466.38	19.52
	Exocarp	PS1	*ellipsoidea*	10	19.08	33.46	24.85	21.37
		PS2	*ovoidea*	10	35.05	56.77	45.10	14.09
	Mesocarp	PS1	*ellipsoidea*	10	116.78	233.62	160.30	22.89
		PS2	*ovoidea*	10	261.40	514.50	389.80	22.69
	Seeds	PS1	*ellipsoidea*	10	6.42	13.03	9.22	24.07
		PS2	*ovoidea*	10	13.98	22.79	17.42	17.62
Height fruit (mm)		PS1	*ellipsoidea*	10	86.57	109.61	98.06	8.27
		PS2	*ovoidea*	10	112.18	132.37	124.48	5.10
Average fruit (mm)	Middle fruit	PS1	*ellipsoidea*	10	51.21	70.91	62.33	8.98
		PS2	*ovoidea*	10	77.96	100.25	88.64	9.08
	10 mm under apical part of the Stem	PS1	*ellipsoidea*	10	29.87	47.00	36.31	14.42
		PS2	*ovoidea*	10	38.50	53.98	44.42	12.84
	10 mm above basal part of the Fruit	PS1	*ellipsoidea*	10	24.67	38.58	32.17	14.48
		PS2	*ovoidea*	10	25.56	42.69	33.84	15.72
Core (mm)	Height	PS1	*ellipsoidea*	10	54.50	86.72	66.29	12.98
		PS2	*ovoidea*	10	59.37	84.30	76.44	12.17
	Width	PS1	*ellipsoidea*	10	25.08	32.52	27.85	9.35
		PS2	*ovoidea*	10	27.67	47.19	39.44	14.10
Number of seeds in fruits (pc)		PS1	*ellipsoidea*	10	49.00	203.00	140.50	34.14
		PS2	*ovoidea*	10	161.00	219.00	198.20	7.55
Height seeds (mm)		PS1	*ellipsoidea*	10	6.77	8.34	7.53	6.33
		PS2	*ovoidea*	10	8.58	9.65	9.04	4.40
Width seed (mm)		PS1	*ellipsoidea*	10	4.41	5.27	4.88	6.10
		PS2	*ovoidea*	10	4.73	5.52	5.13	5.81

Legend: n – number of fruits; X – mean – average set; min. – minimum value measured in the file; max. – maximum value measured in the file; CV – coefficient of variation %.

This ours resultes also documented a correlation coefficient determined for both test species ranging from r = 0.98 (CO - var. *pyriformis*) to r = 0.99 (PS - var. *ovoidea*). Between fruit weight and seed weight, we identified for fruits quinces correlation coefficient from r = 0.15 (var. *maliformis*) to r = 0.69 (var. *pyrimorfis*) for fruits and Chinese quince from r = 0.58 (var. *ovoidea*) to r = 0.62 (var. *ellipsoidea*). In aqueous extract we determined antioxidant activity at the species *C. oblonga* / *P. sinensis* in dry exocarp in the range 43.52 – 67.73% / 52.76 – 82.20%, in fresh mesocarp 7.36 – 14.78% / 15.30 – 23.50%, in dry mesocarp 30.92 – 41.30% / 41.68 – 50.15% and dry endocarp 55.19 – 76.44% / 91.20 – 92.72% (Table 4). We determined antioxidant activity in methanolic extracts at the species *C. oblonga* / *P. sinensis* in dry exocarp in the range 93.29 – 93.32% / 91.87 – 93.25%, in fresh mesocarp 10.29 – 36.0% / 17.10 – 17.11%, in dry mesocarp 54.55 – 74.11% / 80.39 – 84.11% and in dry endocarp 95.14 – 95.39% / 94.97 – 95.62% (Table 4). **Tzanakis et al., (2006)** found the average value of antioxidant activity in the dry mesocarp at 65.63% and **Karadenüz et al., (2005)** at 60.40%. **Zavadilová, (2010)** determined the average value of antioxidant activity in fresh mesocarp for genotype var. maliformis values in the range from 21.15 to 36.16% for genotype and var. pyriformis from 16.17 to 36.91%. In comparison to our results we have set significant matches. We have confirmed experimentally comparatively higher antioxidant activity in dry mesocarp compared to fresh mesocarp. This is logical, since the content of the drying and the concentration of all components increase, but can also occur in oxidation of certain biologically active substances. We experimentally confirmed relatively high antioxidant activity of the Quince oblong and the Chinese quince products. Methanol extracts from dry exocarp and endocarp worked effectively against $^{\bullet}$DPPH radical than from dry and fresh mezocarp. We can classify antioxidant activity of dry exocarp and endocarp like high and almost identical. Pericarp includes exocarp, mezocarp and endocarp. Results document that the fruits of both species can be practically used in the preparation of many dishes, while they can be used as raw material for pharmaceutical and cosmetic use.

Table 3 Correlation coefficients of the linear relationship between the weight of the whole fruit weight and fruit basic parts of evaluated varieties of quince (*Cydonia oblonga* Mill.) and Chinese quince *(Pseudocydonia sinensis* Schneid.) by Pearson.

Sample	Variety	*r*	Confidence Interval $r_{95\%}$	r^2	*p*
Weight whole fruit (g) – Weight exocarp (g)					
CO1	*pyriformis*	0.811	0.370<= r >= 0.953	0.657	0.004
CO2	*maliformis*	0.855	0.489<= r >= 0.965	0.731	0.001
PS1	*ellipsoidea*	0.793	0.327<= r >= 0.949	0.629	0.006
PS2	*ovoidea*	0.501	-0.187<= r >= 0.859	0.251	0.140
Weight whole fruit (g) – Weight mesocarp (g)					
CO1	*pyriformis*	0.989	0.955<= r >= 0.997	0.979	0.000
CO2	*maliformis*	0.991	0.961<= r >= 0.998	0.982	0.000
PS1	*ellipsoidea*	0.993	0.973<= r >= 0.998	0.987	0.000
PS2	*ovoidea*	0.995	0.980<= r >= 0.999	0.991	0.000
Weight whole fruit (g) – Weight seeds (g)					
CO1	p*yriformis*	0.685	0.098<= r >= 0.918	0.469	0.028
CO2	*maliformis*	0.147	-0.531<= r >= 0.710	0.021	0.685
PS1	*ellipsoidea*	0.621	-0.013<= r >= 0.899	0.386	0.055
PS2	*ovoidea*	0.576	-0.083<= r >= 0.884	0.332	0.081

Legend: *r* – Pearson correlation coefficient, *min/max* – 95% confidence interval for *r*, r^2 – coefficient of determination, *p* – significance level.

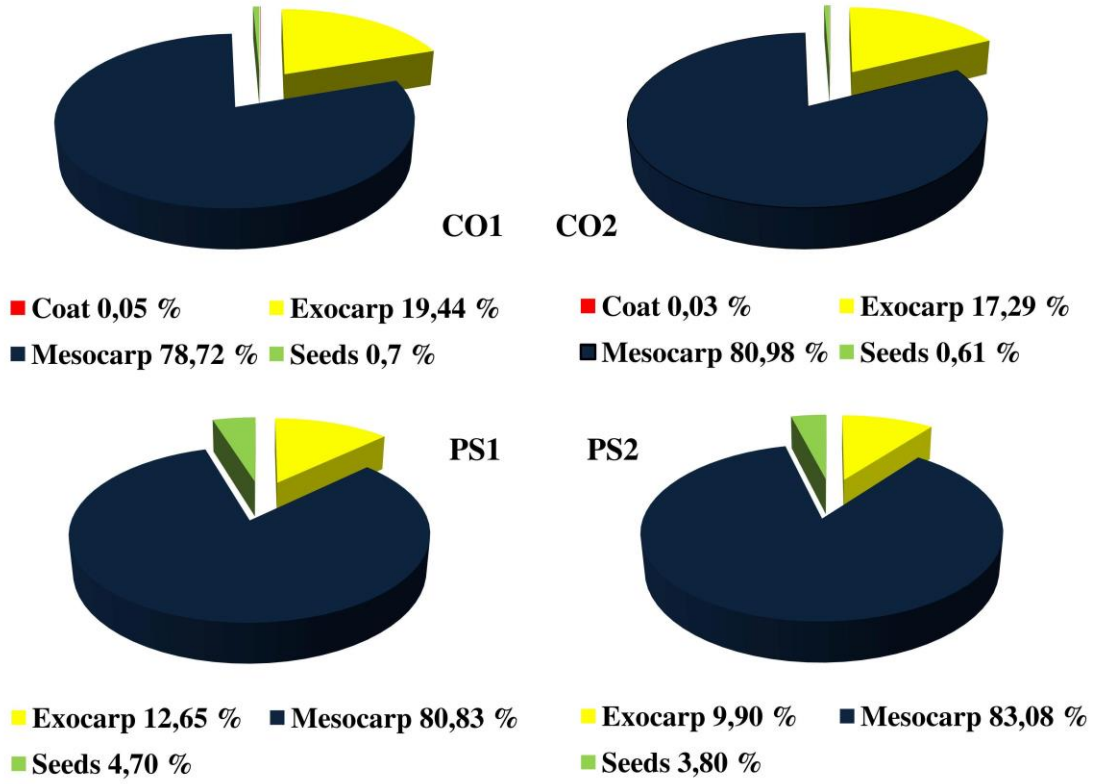

Figure 2 Share pubes, exocarp, mesocarpu and seeds by weight of the total weight of quince fruits (*Cydonia oblonga* Mill., CO1 – *var. pyriformis*, CO2 – *var. maliformis)* and Chinese quince (Pseudocy*donia sinensis Schneid*., PS1 – var. *ellipsoidea*, PS2 – *var. ovoidea*) in fresh condition (whole fruit – 100%).

Table 4 Antioxidant activity of dry endocarp, fresh and dry mesocarp, dry endocarp quince (*Cydonia oblonga* Mill., CO1 – var. *pyriformis*, CO2 – var. *maliformis*) and Chinese quince fruit (*Pseudocydonia sinensis* Schneid., PS1 – var. *ellipsoidea, PS2* – var. *ovoidea*) in methanolic and water extracts to •DPPH in %.

Cydonia oblonga Mill.										
Sample	**Variety**	***n***	***min.***		***max.***		$\bar{x}$		***CV***	
			M	**W**	**M**	**W**	**M**	**W**	**M**	**W**
Dry exocarp										
CO1	*pyriformis*	5	92.66	43.07	93.87	43.79	93.32	43.52	0.47	0.70
CO2	*maliformis*	5	93.16	66.85	93.39	68.26	93.29	67.73	0.11	0.79
Fresh mesocarp										
CO1	*pyriformis*	5	7.12	7.18	17.96	7.63	10.29	7.36	42.34	2.50
CO2	*maliformis*	5	30.40	14.01	38.53	15.35	36.00	14.78	9.57	4.48
Dry mesocarp										
CO1	*pyriformis*	5	48.24	28.28	57.34	33.42	54.55	30.92	6.74	7.88
CO2	*maliformis*	5	73.52	40.57	74.93	42.19	74.11	41.30	0.73	1.69
Dry endocarp										
CO1	*pyriformis*	5	95.03	54.74	95.68	55.82	95.39	55.19	0.26	0.80
CO2	*maliformis*	5	94.92	74.54	95.33	78.03	95.14	76.44	0.21	1.97
***Pseudocydonia sinensis* Schneid.**										
Dry exocarp										
PS1	*ellipsoidea*	5	93.16	80.25	93.40	84.57	93.25	82.20	0.11	2.60
PS2	*ovoidea*	5	91.62	50.07	92.23	55.36	91.87	52.76	0.25	3.72
Fresh mesocarp										
PS1	*ellipsoidea*	5	15.97	22.66	18.83	23.85	17.11	23.50	6.12	2.06
PS2	*ovoidea*	5	15.91	14.09	18.23	17.41	17.10	15.30	5.35	9.41
Dry mesocarp										
PS1	*ellipsoidea*	5	83.63	46.79	84.94	54.58	84.11	50.15	0.59	5.70
PS2	*ovoidea*	5	79.84	40.19	80.84	46.15	80.39	41.68	0.51	6.04
Dry endocarp										
PS1	*ellipsoidea*	5	94.79	92.12	95.05	93.09	94.97	92.72	0.12	0.40
PS2	*ovoidea*	5	95.33	90.98	95.88	91.42	95.62	91.20	0.26	0.20

Legend: *n* – number of fruits; *X* – mean – average set; *min*. – minimum value measured in the file; *max*. – maximum value measured in the file; CV – coefficient of variation %.; M – methanolic extract; W – water extract.

CONCLUSION

Quince is among the forgotten species. In the past, the fruit pulp is mainly used for the preparation of various food products. Its advantages are high in pectin, which is used to thicken fruit juices. The individual parts of the fruit and other plant parts are used and to date used in folk medicine. Therefore, in many regions indicates quince as "pharmacy in the garden" The results confirm the thesis that it is entitled. In all essential parts of the fruit we set a high antioxidant activity, which demonstrates the presence of biologically active components. In aqueous extracts we determined antioxidant activity of the species *C. oblonga* in dry exocarp in the range 43.52 – 67.73%, in fresh mesocarp 7.36 – 14.78%, in dry mesocarp 30.92 – 41.30% and dry endocarp 55.19 – 76.44%. This is confirmed by much literary knowledge. Chinese quince in terms of Europe used very little. It is a relative of quinces elongate. The results presented in the work confirmed that the fruits that have a higher weight than with quince oblong are also an important raw material for the preparation of various food products and a source of biologically active components. This was confirmed by the results of the high antioxidant activity of the essential parts of the fruit. In aqueous extracts we determined antioxidant activity of the species *P. sinensis* in dry exocarp in the range 52.76 – 82.20%, in fresh mesocarp 15.30 – 23.50%, in dry mesocarp 41.68 – 50.15% and dry endocarp 91.20 – 92.72%.

Both fruit can be used to significantly expand their production, processing and practical use in Slovakia. They are very good subject for practical use, especially for family farms and preparation of highly valuable food

products with high biological value and therapeutic effects. Therefore, a good subject for the socio-economic development of micro, bio-economy and landscaping.

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