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RAPD ANALYSIS OF THE GENETIC POLYMORPHISM IN EUROPEAN WHEAT GENOTYPES

Tímea Kuťka Hlozáková, Zdenka Gálová, Edita Gregová, Martin Vivodík, Želmíra Balážová, Dana Miháliková

ABSTRACT

Wheat (*Triticum aestivum* L.) is one of the main crops for human nutrition. The genetic variability of grown wheat has been reduced by modern agronomic practices, which inturn prompted the importance of search for species that could be useful as a genepool for the improving of flour quality for human consumption or for other industrial uses. Therefore, the aim of this study was to analyze the genetic diversity among 24 European wheat genotypes based on Random Amplified Polymorphism (RAPD) markers. A total of 29 DNA fragments were amplified with an average 4.83 polymorphic fragments per primer. The primer producing the most polymorphic fragments was SIGMA-D-P, where 7 polymorphic amplification products were detected. The lowest number of amplified fragments (3) was detected by using the primer OPB-08. The size of amplified products varied between 300 bp (OPE-07) to 3000 bp (SIGMA-D-P). The diversity index (DI) of the applied RAPD markers ranged from 0.528 (OPB-07) to 0.809 (SIGMA-D-P) with an average of 0.721. The polymorphism information content (PIC) of the markers varied from 0.469 (OPB-07) to 0.798 (SIGMA-D-P) with an average 0.692. Probability of identity (PI) was low ranged from 0.009 (SIGMA-D-P) to 0.165 (OPB-07) with an avarage 0.043. The dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared. Within the dendrogram was separated the unique genotype Insegrain (FRA) from the rest of 23 genotypes which were further subdivided into two subclusters. In the first subclaster were grouped 13 genotypes and the second subcluster involved 10 genotypes. The first subcluster also included the genotype Bagou from France, in which were detected novel high – molecular – weight glutenin subunits using SDS-PAGE. Using 6 RAPD markers only two wheat genotypes have not been distinguished. Through that the information about genetic similarity and differences will be helpful to avoid any possibility of elite germplasm becoming genetically uniform.

Keywords: *Triticum aestivum* L.; PCR; RAPD marker; genetic diversity

INTRODUCTION

Wheat (*Triticum* spp.) is a self-pollinating annual plant, belonging to the family *Poaceae* (grasses), tribe *Triticeae*, genus *Triticum*. According to different classifications, number of species in the genus varies from 5 to 27. The two main groups of commercial wheats are the durums (*Triticum durum* L.) and bread wheats (*Triticum aestivum* L.) with 28 and 42 chromosomes respectively **(Šramková et al., 2009)**.

Bread wheat (*Triticum aestivum* L.) is one of the most important and widely cultivated crops used mainly for human consumption and support in the world.

The importance of wheat is mainly due to the fact that its seed can be ground into flour, which form the basic ingredients of bread and other bakery products, as well as pastas, and thus it presents the main source of nutrients such as proteins, carbohydrates, lipids, fibre and vitamins, to the most of the world population. Agronomical and nutritionally important status of wheat among the several other cereal crops has obtained because of its large genome size and multifaceted uses. Approximately 734.8 million tons of wheat is produced annually on 247 million hectare of the total cultivated land in the world and supports nearly 35% of the world´s population (http://www.fao.org/worldfoodsituation/csdb/en/).

Enormously growing population and the changing of life style have posed challenges to the wheat breeders to develop newer wheat varieties with high yielding performance, high quality seed and resistance to pests and stress conditions. Modern agronomic practices have reduced the genetic variability of cultivated wheats, which has given great importance in the search for could be useful in contributing genes for wheat improvement **(Jauhar, 1993)**.

Characterization of genetic diversity and genetic relatedness is a fundamental element in crop improvement strategies **(Zhu et al., 2000)**. Like any other crops, the first step of in wheat improvement is full assessment of the local materials, including collection, evaluation and molecular characterization of germplasm lines. Knowledge about morphological and agronomic traits and genetic relationships among breeding materials could be an invaluable aid in crop improvement strategies **(Abbas et al., 2008)**.

A number of methods are currently used for analysis of genetic diversity in germplasm accessions, breeding lines

and segregating populations. These methods were based on pedigree, morphological, agronomic performance, biochemical and molecular (DNA-based) data **(Mohammadi and Prasanna, 2003)**. The diversity patterns allow plant breeders to better understand the evolutionary relationships among accessions and to incorporate useful genotypes in the breeding programs **(Thompson et al., 1998)**.

However, diversity estimates based on pedigree analysis have generally been found inflated and unrealistic **(Fufa et al., 2005)**. Genetic diversity estimates based on morphological traits, on the other hand, suffer from the drawback that such traits are limited in number and are influenced by environment **(Maric et al., 2004)**. Molecular markers are useful tools for estimating genetic diversity as these are not influenced by environment, are abundant and do not require previous pedigree information. Among the biochemical markers, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE) has been widely used due to its simplicity and effectiveness for estimating genetic diversity.

Among the various DNA – based markers, AFLP (Amplified Fragment Length Polymorphism) and RFLP (Restriction Fragment Length Polymorphism) have been used to study genetic diversity. These markers elucidate the phylogenetic relationships among various lines, for their efficient use in breeding and genetic resource management. These methods, however, involve the use of expensive enzymes, radioactive labeling, and are cumbersome and hence, appear unsuitable **(Gajera et al., 2010)**.

On the other hand, RAPD (Random Amplified Polymorphic DNA) markers have offered a valuable opportunity to characterize genetic variation and structure in plant population **(Ayana et al., 2000)** with requiring only small amounts of DNA sample without involving radioactive labels, are simplier as well as faster, and therefore they have been increasingly employed for analysis of genetic diversity **(Ebrahimi et al., 2011)**.

The use of RAPD molecular markers are routine methods for quickly and efficiently estimating relationships between lines and populations of many plant species. It is assumed that these markers are randomly spaced throughout the genome **(Mark et al., 1999)**. RAPD markers have proven to be quite efficient in detecting genetic variations and used for diversity assessment and for identifying germplasm in a number of plant species such as wheat **(Ahmed et al., 2010**; **Mahmood et al., 2011**; **Cifci and Yagdi, 2012**; **Rehman et al., 2013)**, rye **(Petrovičová et al., 2014)**, flax **(Bežo et al., 2005)**, castor **(Vivodík at al., 2015)**, amaranth **(Štefúnová et al., 2013)** or Echinacea **(Kapteyn et al., 2002)**, and also these markers have been used for identification and differentiation on other microbial or animal level, such as in the yeast microbiota grown on grapes **(Drozdz et al., 2015)** or in fishery food products **(Bajzík et al., 2010)**.

The present study is focused on estimation of genetic distance between 24 European wheat genotypes, included the genotype with probably novel high – molecular – weight glutenin subunits identified by SDS – PAGE **(Kuťka Hlozáková et al., 2015)** based on 6 RAPD markers. Although the information gathered here would be helpful in future for genomic mapping studies leading to development of wheat cultivars with broader genetic background to obtain improved crop productivity.

MATERIAL AND METHODOLOGY

Plant material: Twenty – four genotypes of hexaploid wheat (*Triticum aestivum* L.) grain originating from five different geographical areas (Slovakia, Czech Republic, Hungary, Germany and France) of Europe were obtained from the collection of genetic wheat resources of the Gene Bank of Slovak Republic in Piešťany.

Genomic DNA Isolation: DNA of 24 genotypes of wheat was extracted from the endosperm of intact, dry and mature single seeds using the Gene JET Plant Genomic DNA Purification Mini Kit (Thermo Scientific) supplemented with 2% polyvinylpyrrolidone (PVP) in lysis buffer.

RAPD Analysis: Amplification of RAPD fragments was performed according to **Cifci and Yagdi (2012)** using decamer arbitrary primers (Operon technologies Inc, USA; SIGMAD, USA). Amplifications were performed in a 25 µL reaction volume containing 5 µL DNA (100 ng), 12.5 µL Master Mix (Promega), and 1 µL of 10 pmol of primer. Amplification was performed in a programmed thermocycler (Biometra, Germany) with initial denaturation at 94 °C for 3 min., 40 cycles of denaturation at 94 °C for 30 sec., primer annealing at 38 °C for 1 min., extension at 72 °C for 2 min., and final extension at 72 °C for 10 min. Amplified products were separated on 1.2% agarose in 1 × TBE buffer. The gels were stained with ethidium bromide, visualised under ultraviolet (UV) light and documented using gel documentation system Grab-It 1D for Windows. The molecular weight of amplified fragments was estimated with the help of Thermo Scientific FastRuler Middle Range DNA Ladder (MBI, Fermentas).

Data analysis: The RAPD bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands and to prepare a dendrogram. A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the software package SPSS professional statistics version 17 was constructed. For the assessment of the polymorphism in the wheat genotypes using RAPD markers in their differentiation we used diversity index (DI) **(Weir, 1990)**, the probability of identity (PI) **(Paetkau et al., 1995)** and polymotphic information content (PIC) **(Weber, 1990)**, which were calculating according to formulas:

Diversity index (DI)

$$DI = 1 - \sum p_i^2$$

Probability of identity (PI)

$$PI = \sum p_i^4 + \sum_{i=1}^{i=n-1} \sum_{j=i+1}^{n} \left(2 p_i p_j\right)^2$$

information content (PIC)

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2 \right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 \cdot p_j^2$$

(where p_i and p_j are frequencies of i^{th} and j^{th} fragment of given genotypes)

RESULTS AND DISCUSSION

Efficient and effective crop improvement program depends on the extent of genetic diversity either existing or created. The breeding of wheat has achieved hallmark progress that is able to feed ever increasing population in the world **(Rehman et al., 2013)**. Genetic diversity is one of the key factors for the improvement of many crop plants including wheat **(Ahmed et al., 2010)**. The efficiency of genetic gain by selection can be improved if the patterns of genetic diversity within a population of breeding lines are known. Genetic similarity or distance estimates among genotypes are helpful in the selection of parents to be used in the breeding program **(Van Becelaere et al., 2005)**.

In this work, 6 primers were screened for PCR amplification of DNA and RAPD analysis in 24 wheat genotypes. Table 1 shows codes and sequences of these primers, total number of amplified fragments from 24 wheat genotypes, the diversity index, the polymorphic information content and the probability of identity for each primer. All the primers produced 29 DNA fragments (Figure 1) with an average of 4.833 bands per primer. From these six primers, primer SIGMA-D-P was the most polymorphic, where 7 polymorphic amplification products were detected. The lowest number of different fragments (3) was detected in primer OPB-08. Of the 29 amplified bands, all 29 were polymorphic, with an average of 4.83 polymorphic bands per primer. The size of amplified products varied from 300 bp (OPE-07) to 3000 bp (SIGMA-D-P).

To determine the level of polymorphism in the analysed group of wheat genotypes, diversity index DI, probability of identity PI and polymorphic information content PIC were calculated. All three indicators were applied for all six RAPD primers and for their calculation, the individual frequences of fragments of each marker were used.

The diversity index (DI) of the applied RAPD markers ranged from 0.528 (OPB-07) to 0.809 (SIGMA-D-P) with

Table 1 List of RAPD primers, total number of bands and the statistical characteristics og the used RAPD markers.

Primers	Primer sequence (5´-3´)	Total number of bands	DI	PIC	PI
OPA-02	TGCCGAGCTG	5	0.761	0.736	0.016
OPA-03	AGTCAGCCAC	5	0.741	0.712	0.023
OPA-13	CAGCACCCAC	4	0.708	0.668	0.033
OPB-08	GTCCACACGG	3	0.528	0.469	0.165
OPE-07	AGATGCAGCC	5	0.779	0.768	0.014
SIGMA-D-P	TGGACCGGTG	7	0.809	0.798	0.009
Total	-	**29**	-	-	-
Avarage	-	**4.833**	**0.721**	**0.692**	**0.043**

Note: DI – diversity index, PIC – polymorphic information content, PI – probability of identity.

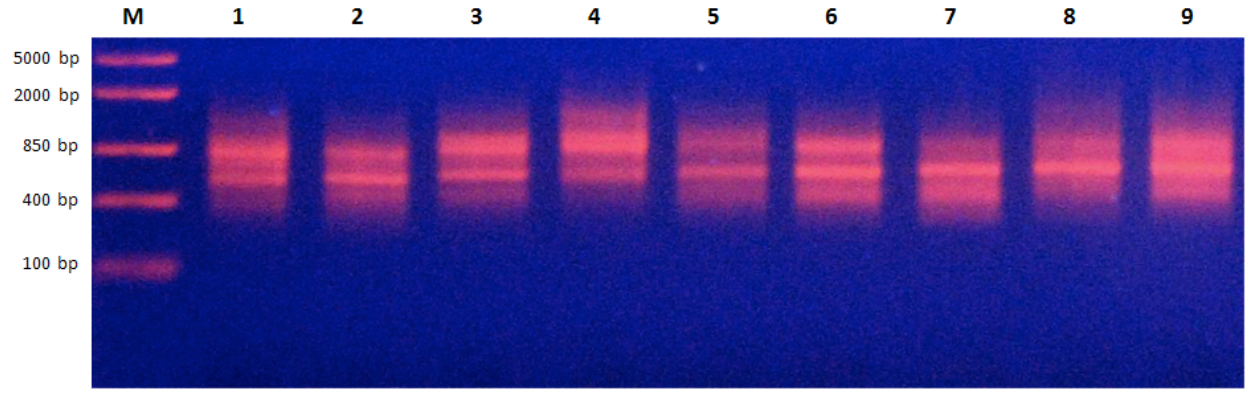

Figure 1 PCR amplification products of nine wheat genotypes with SIGMA-D-P primer: Lane M – Thermo Scientific FastRuler Middle Range DNA Ladder, 1 – Banquet (CZE), 2 – Kalif (FRA), 3 – Bonpain (FRA), 4 – Verita (SVK), 5 – Hana (CZE), 6 – MV Optima (HUN), 7 – Balthasar (FRA), 8 – Bagou (FRA), 9 – Ilona (SVK).

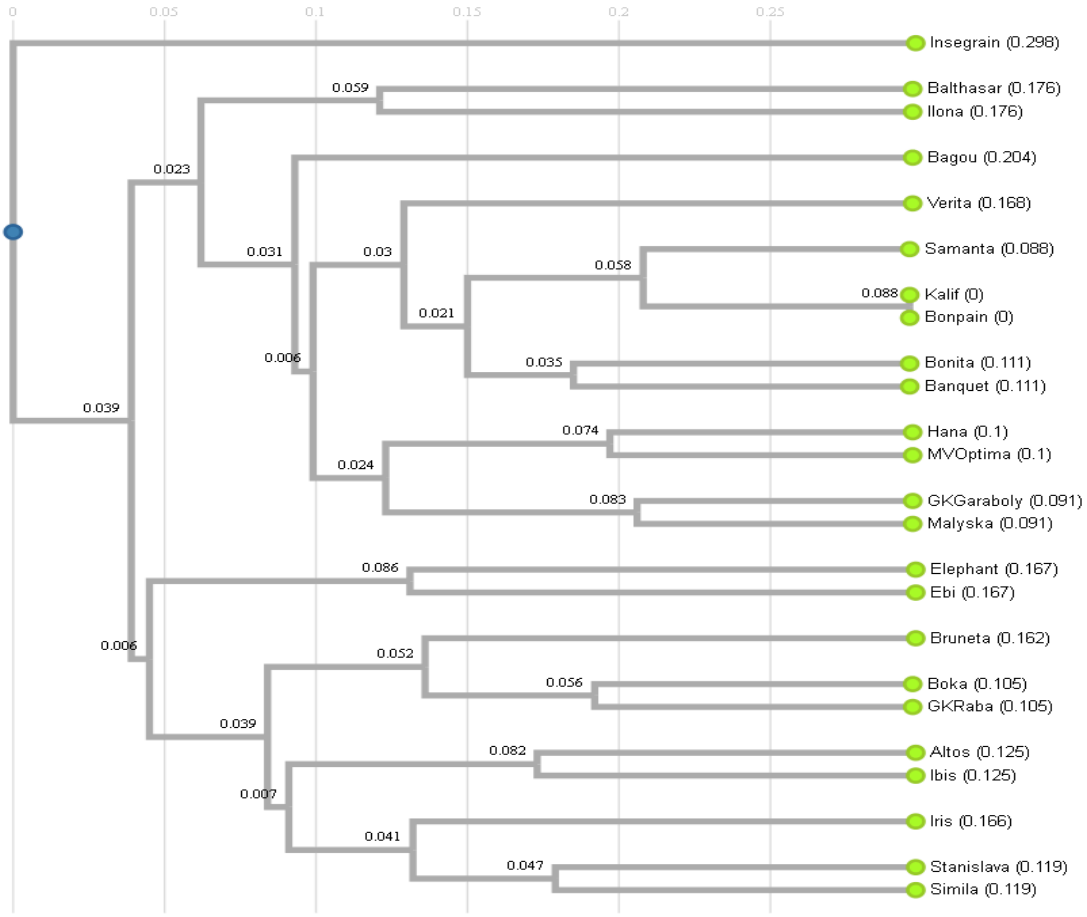

Figure 2 Dendrogram based on 29 RAPD fragments in 24 wheat genotypes.

an avarage of 0.721. The polymorphism information content (PIC) of the markers varied from 0.469 (OPB-07) to 0.798 (SIGMA-D-P) with an average 0.692. 83% of used RAPD markers had PIC and DI values higher than 0.6 that means high polymorphism of chosen markers used for analysis **(Vivodík et al., 2015)**. Probability of identity (PI) was ranged from 0.009 (SIGMA-D-P) to 0.165 (OPB-07) with an average 0.043. Cause of that, it is necessary to use a higher number of RAPD markers.

For the detection of genetic diversity, the dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared (Figure 2). This dendrogram separated unique genotype Insegrain (FRA, cluster I) from other 23 genotypes (cluster II) that were further subdivided into 2 subclusters. In the first subcluster were grouped 13 genotypes from Slovakia (38.5%), France (30.8%), Czech Republic (15.4%) and the same number (15.4%) from Hungary. Genotypes Kalif and Bonpain from France could not be distinguished because of their close genetic similarity. This subcluster also included the genotype Bagou from France, in which were detected novel high – molecular – weight glutenin subunits using SDS – PAGE. The second subcluster involved 10 genotypes from Slovakia (30%), Czech Republic (30%), Germany (20%), France (10%) and the same number (10%) from Hungary.

Lower polymorphism using RAPD analysis was detected by **Cifci and Yagdi (2012)** who used 17 markers to describe genetic similarity of 16 Turkish wheat genotypes. PIC values ranged from 0.11 to 0.92 with an average 0.59. **Ahmed et al., (2010)** also used 15 RAPD markers to analyse the genetic diversity of 32 wheat breeding lines and reached an average 4.1% polymorphism per primer.

On the other hand, high polymorphism was detected in set of amaranth **(Štefúnová et al., 2013)**, rye **(Petrovičová et al., 2014)** or castor genotypes **(Vivodík et al., 2015)**. Also higher polymorphism for RAPD was detected in Pakistan wheat landraces, where **Mahmood et al., (2011)** reached an average 7.8 % polymorphism per primer using 10 RAPD markers.

CONCLUSION

The present study was aimed to determine the genetic variation among wheat genotypes grown in Europe. Our results showed that RAPD markers are useful for exploring genetic diversity of raw material for developing new varieties. The dendrogram prepared based on UPGMA algorithm separated the unique genotype Insegrain (FRA) from the rest of 23 genotypes which were further subdivided into two subclusters. The first subcluster also included the genotype Bagou from France, in which were detected novel high – molecular – weight glutenin subunits using SDS-PAGE. Only two wheat genotypes have not been distinguished using these 6 RAPD markers. For better resolution of the analysed wheat genotypes, it is necessary to use a higher number of RAPD markers.

Despite that, the information gathered here would be helpful in genomic mapping studies and for the development of wheat cultivars with wider and diverse genetic background to obtain improved crop productivity.

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Contact address:

Tímea Kuťka Hlozáková, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xhlozakova@is.uniag.sk.

Zdenka Gálová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biotechnology and Food Sciences, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zdenka.galova@uniag.sk.

Edita Gregová, National Agriculture and Food Centre, Research Institute of Plant Production, Bratislavská cesta 122, 921 01 Piešťany, Slovakia, E-mail: gregova@vurv.sk.

Martin Vivodík, Slovak University of Agriculture, Faculty of Biochemistry and Biotechnology, Department of Biotechnology and Food Sciences, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: martin.vivodik@uniag.sk.

Želmára Balážová, Slovak University of Agriculture, Faculty f Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zelmira.balazova@uniag.sk.

Dana Miháliková, Slovak University of Agriculture, Faculty f Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xmihalikovad@is.uniag.sk.

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COLONIZATION OF GRAPES BERRIES BY *ALTERNARIA* sp. AND THEIR ABILITY TO PRODUCE MYCOTOXINS

Dana Tančinová, Zuzana Mašková, Ľubomír Rybárik, Soňa Felšöciová, Miroslava Císarová

ABSTRACT

Our research focused on identify the *Alternaria* species from grapes (surface sterilized berries and non-surface sterilized berries) of Slovak origin and characterize their toxinogenic potential in *in vitro* conditions. We analyzed 47 samples of grapes, harvested in years 2011, 2012 and 2013 from various wine-growing regions. For the isolation of species, the method of direct plating berries and surface-sterilized berries (using 1 % freshly pre-pared chlorine) on DRBC (Dichloran Rose Bengal Chloramphenicol agar) was used. For each analysis was used 50 berries. Only undamaged berries have been used for analysis. The cultivation was carried at 25 ±1°C, for 5 to 7 days in dark. After incubation, the colonies of *Alternaria* were transferred on PCA - potato-carrot agar and CYA - Czapek-yeast extract agar and cultured for 7 days at room temperature and natural light. A total 4 species-groups of the genus *Alternaria* were isolated from grapes berries: *Alternaria alternata* (1369 isolates), *Alternaria arborescens* (734 isolates), *Alternaria infectoria* (143 isolates), and *Alternaria tenuissima* (3579 isolates). According to European Union legislation mycotoxins produced by species genus *Alternaria* are not monitored in foods and food commodities. Mycotoxins such as alternariol and alternariol monomethylether are mutagenic and genotoxic in various *in vitro* systems. Selected strains were tested for production of altenuene, alternariol monomethylether and alternariol. In neither case of *A. infectoria* species-group isolates was confirmed the production of tested mycotoxins in *in vitro* conditions by TLC method. The ability to produce altenuene, alternariol monomethylether and alternariol in *in vitro* conditions was detected in isolates of *Alternaria alternata*, *Alternaria arborescens* and *Alternaria tenuissima* species-groups. Isolates of *Alternaria alternata* species-group (44 tested isolates) were able to produce altenuene (24 isolates), alternariol monomethyleter (42 isolates) and alternariol (43 isolates). Only one isolate did not produce any mycotoxins. Isolates of *Alternaria arborescens* species-group (38 tested isolates) were able to produce altenuene (24 isolates), alternariol monomethyleter (33 isolates) and alternariol (36 isolates). Only two isolates did not produce any mycotoxins. Isolates of *Alternaria tenuissima* species-group (87 tested isolates) were able to produce altenuene (42 isolates), alternariol monomethyleter (41 isolates) and alternariol (73 isolates). Thirteen isolates did not produce any mycotoxins.

Keywords: *Alternaria*; altenuene; alternariol; alternariol monomethylether; grape

INTRODUCTION

Grapes have a complex microbial ecology including filamentous fungi, yeasts and bacteria with different physiological characteristics and effects upon wine production **(Barata et al., 2012)**. The black mould genus *Alternaria* Ness is ubiquitously distributed and includes various saprophytic, endophytic and pathogenic species. Many of the genus *Alternaria* Ness commonly cause spoilage of various food crops in the field or post-harvest decay **(Ostrý, 2008**; **Logrieco et al., 2009)**. *Alternaria* species are pathogenic and saprophytic fungi widely distributed in soil. They are widespread in both humid and semiarid regions and can infect growing plants in the field. They are the principal contaminating fungi in wheat, sorgum and barley. In addition to cereal crops, *Alternaria* species have been reported to occur in oilseeds such as sunflower and rapeseed, tomato, apples, citrus fruits, olives and several other fruits and vegetables. *Alternaria* species grow at low temperature; hence they are generally associated with extensive spoilage during refrigerated transport and storage **(Ostrý, 2008)**. *Alternaria* genus is the main component of the wine grape mycobiota at harvest time **(Serra et al., 2005**; **Prendes et al., 2015**; **Tančinová et al., 2015)**. The most common fungi spoiling grapes were *Alternaria*, *Botrytis cinerea* and *Cladosporium* **(Tournas, et al., 2005)**. Moreover, several *Alternaria* species are known to produce toxic secondary metabolites, *Alternaria* mycotoxins **(Rotem, 1994**; **Prendes et al., 2015)**. Mycotoxins are secondary metabolites produced by filamentous fungi that have been detected in food commodities, including grapes and wine **(Serra et al., 2005)**. *Alternaria* species have the ability to produce a variety of secondary metabolites, which plays important roles in food safety **(Andresen, et al., 2015)**. The major *Alternaria* mycotoxins belong to three structural classes: tetramic acid derivate, tenuazonic acid; the dibenzopyrone derivates, alternariol, anternariol methylether and altenuene; and the perylene derivates, the altertoxins **(Andersen et al., 2002)**. Food relevant *Alternaria* species are able to produce many more metabolites **(Ostrý, 2008**; **Logrieco et al., 2009)**. *Alternaria* toxins occurred regularly in cereals, tomato sauces, figs, wine and sunflower seeds. Only incidental occurrence of the *Alternaria* toxins was

observed in fresh apples, fresh citrus, fresh tomatoes and olives **(López et al., 2016)**.

Our research focused on the identify the *Alternaria* species from grapes of Slovak origin and characterize their toxinogenic potential in *in vitro*.

MATERIAL AND METHODOLOGY

Samples

Forty-seven samples of grapes, harvested in years 2011, 2012 and 2013 from various wine-growing regions of Slovakia, from small and medium-sized vineyardswere analyzed. White and red grape variety were analyzed. *White grape*: Müller Thurgau (1), Velsch Riesling (4), Grüner Veltliner (5), Pálava (1), Pinot blanc (2), Pinot gris (2), Sauvignon (2), Tramin (1), Zala gyöngye (1). *Red grape*: Alibernet (1 sample), André (2 samples), Blaufrankise (8), Cabernet Sauvignon (2), Pinot noir (2), Saint Laurent (1). Samples (3 kg) were collected at the time of technological ripeness. Picked grapes were stored at 4 ±1 °C and analyzed within 24 h after harvest.

Mycological analysis

For the isolation of *Alternaria* sp. was used the method of direct plating berries: surface-sterilized berries and non-sterilized berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar) **Samson et al., (2002)**.

The endogenous mycobiota was determined by the method of direct placing of superficially sterilized berries on agar plates **(Samson et al., 2002)**. More than 50 pieces of undamaged berries from each sample were superficially sterilized (using 1% freshly pre-pared chlorine). Sterilization was carried out for 2 minutes. Berries were rinsed 3 times with sterile distilled water and dried on sterile filter paper. Exactly 50 berries from each sample were placed on DRBC plates (agar with dichloran, rose bengal and chloramphenicol) **(Samson et al., 2002)**. Cultivation lasted from 5 to 7 days in darkness at 25 ±1 °C. For each analysis was used 50 berries. Only undamaged berries have been used for analysis. After incubation, the colonies of *Alternaria* were transferred onto appropriate identification media.

Identification of *Alternaria* species-groups. Grown micromycetes were classified into the genera and then isolated by re-inoculation on the identification nutrient media and identified by accepted mycological keys and publications. Isolates of the genus *Alternaria* were re-inoculated on PCA - potato-carrot agar and CYA - Czapek-yeast extract agar **(Samson et al., 2002)** and cultured for 7 days at room temperature and natural light.

In order to improve study of sporulation pattern we proceeded as follows. The colonized agar (piece of approx. size 0.5 x 1.0 cm) was cut and transferred to the agar surface, outside the colony. The growth was observed as early as one to two days of cultivation on the edge of the removed part. Main used identification keys were **Andersen et al., (2001)**; **Andersen et al., (2002)**; **Simmons, (1994)**; **Simmons, (2007) and Simmons and Roberts (1993)**.

Obtained results were evaluated and expressed in isolation frequency (Fr) at the species level. The isolation frequency (%) is defined as the percentage of samples within which the species occurred at least once **(Gautam et al., 2009)**. These values were calculated according to **González et al., (1996)** as follows:

$$Fr\ (\%) = (ns / N) \times 100$$

where ns = number of samples with a species; N = total number of samples.

Toxinogenity analysis

Toxinogenity of selected isolates was analysed by means of thin layer chromatography (TLC) by **Samson et al., (2002)**. This method was performed with modifications according to **Labuda and Tančinová (2006)**. Testing was focused on determination of the ability to produce mycotoxins altenuene (ALT), alternariol (AOH) and alternariol monomethylether (AME).

The colonies grown on yeast extract sucrose agar (YES) (7, respectively 14 days, in darkness at 25 ±1°C) were cut into squares of approximate size 2 cm x 2 cm and placed in an Eppendorf tube with 0.5 mL of extraction solution (chloroform: methanol - 2:1; Reachem, SR). The content of the tubes was stirred for 5 minutes by Vortex Genie ® 2 (MO BIO Laboratories, Inc. - Carlsbad, CA). The obtained extracts were applied to silica gel chromatography plate (Alugram ® SIL G, Macherey - Nagel, Germany) and plates were put into the TEF solvent (toluene: ethyl acetate: formic acid – 5 :4 :1; toluene - Mikrochem, SR; ethyl acetate and formic acid - Slavus, SR). After elution and drying, the mycotoxins identity was confirmed by visual comparison with the standards of mycotoxins (AME, ALT and AOH - Merck, Germany) under UV light with a wavelength of 254 nm and 366 nm.

RESULTS AND DISCUSSION

In the current study from all samples were isolated *Alternaria* species (from superficially sterilized berries and berries without sterilization, too). The cosmopolitan fungal genus *Alternaria* consists of multiple saprophytic and pathogenic species. Based on phylogenetic and morphological studies, the genus is currently divided into 26 sections. *Alternaria* section *Alternaria* contains most of the small-spored species with concatenated conidia, including important plant, human and postharvest pathogens **(Woundenberg et al., 2015)**. A total of 4 species-groups (Table 1) of the genus *Alternaria* (*Alternaria* section *Alternaria*) were isolated from grapes berries, namely *Alternaria alternata* group, *Alternaria arborescens* group, *Alternaria infectoria* group, and *Alternaria tenuissima* group. Isolates, which could not be closer specified or contaminated another species were specified as *Alternaria* sp., Sporulation patterns of *Alternaria* species-group are listed according to **Simmons, (2007).** The typical sporulation pattern of *Alternaria alternata* group (Figure 1) comprises a single suberect conidiophore and an apical cluster of branching chains of small conidia separated by short secondary donidiophores. Long, well-defined primary conidiophores of *Alternaria arborescens* group (Figure 2) characteristically bear a few terminal and subterminal branches. Each conidiophore branch bears a branching chain of conidia, giving a relatively tall, three-dimensionally arborescent apperarance to the suberect system.

Table 1 Species-groups of *Alternaria* isolated from berries of Slovak origin determinated by using plate direct method on DRBC agar from 47 samples.

Alternaria groups	**Superficially sterilized berries**		**Berries without sterilization**	
	Number of isolates	Isolation frequency (%)	Number of isolates	Isolation frequency (%)
Alternaria alternata	662	78.7	707	78.7
Alternaria arborescens	405	34.0	329	57.4
Alternaria infectoria	109	31.9	34	29.78
Alternaria tenuissima	1644	93.6	1935	89.36
Alternaria sp.	144	53.2	94	46.81

Note: DRBC - Dichloran Rose Bengal Chloramphenicol agar.

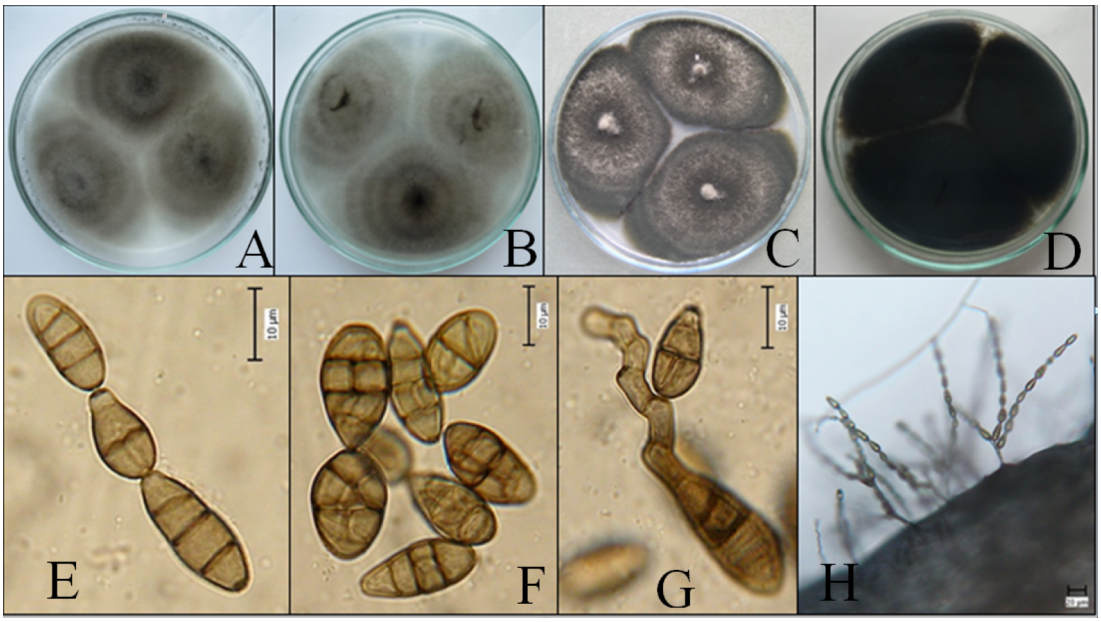

Figure 1 *Alternaria alternata* group 7 days of incubation. **A-B** colonies on PDA (A – top, B – reverse), **C-D** colonies on CYA (C – top, D – reverse), **E-F** conidia (scale bar = 10 µm), **H** – conidium sporulation pattern (scale bar = 20 µm) Photo: Mašková.

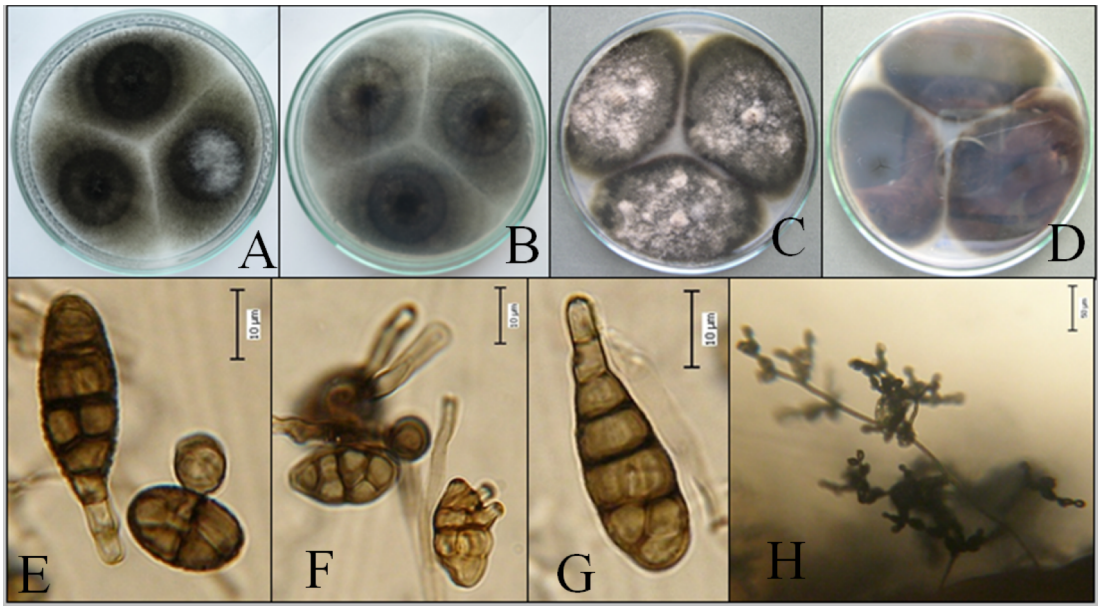

Figure 2 *Alternaria arborescens* group 7 days of incubation. **A-B** colonies on PDA (A – top, B – reverse), **C-D** colonies on CYA (C – top, D – reverse), **E-F** conidia (scale bar = 10 µm), **H** – conidium sporulation pattern (scale bar = 50 µm), Photo: Mašková.

Table 2 Potential ability of *Alternaria* species groups isolates to produce mycotoxins in *in vitro* conditions, tested by TLC method.

Species groups of *Alternaria*	Number of tested isolates	Number of isolates without the production of mycotoxins	Mycotoxins		
			Altenuene	Alternariol monomethylether	Alternariol
Al. alternata	44	1	24	42	43
Al. arborescens	38	2	24	33	36
Al. infectoria	15	15	0	0	0
Al. tenuissima	87	13	42	41	73

Note: TLC - thin layer chromatography, *Al. – Alternaria.*

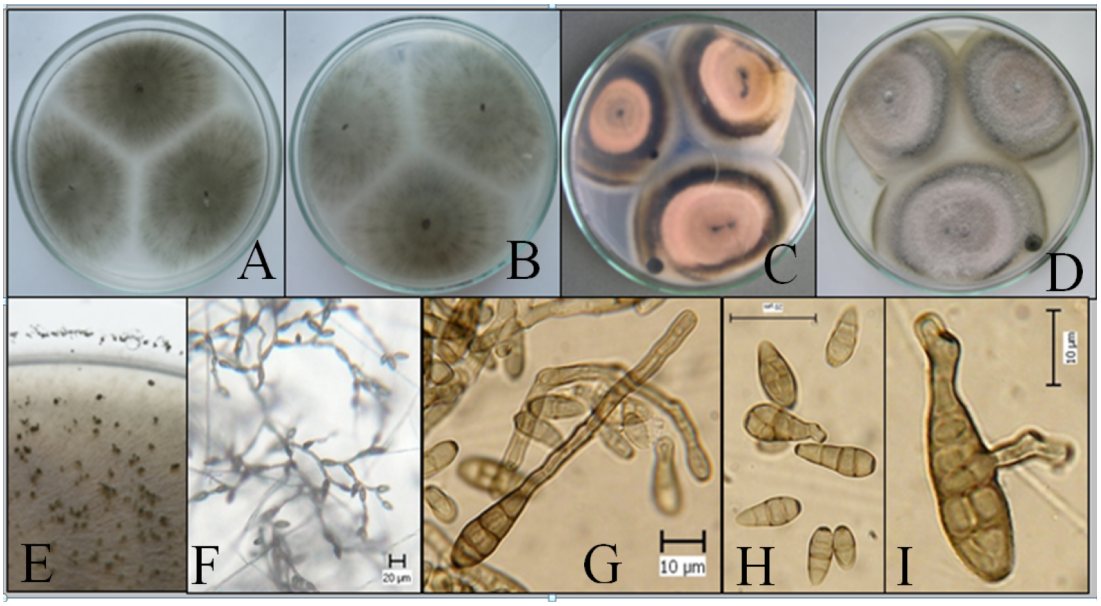

Figure 3 *Alternaria infectoria* group 7 days of incubation. **A-B** colonies on PDA (A – top, B – reverse), **C-D** colonies on CYA (C – top, D – reverse), **E** – granular look of colnies, **F** – conidium sporulation pattern (scale bar = 20 µm), **G-I** conidia (scale bar = 10, 20, 10 µm), Photo: Mašková.

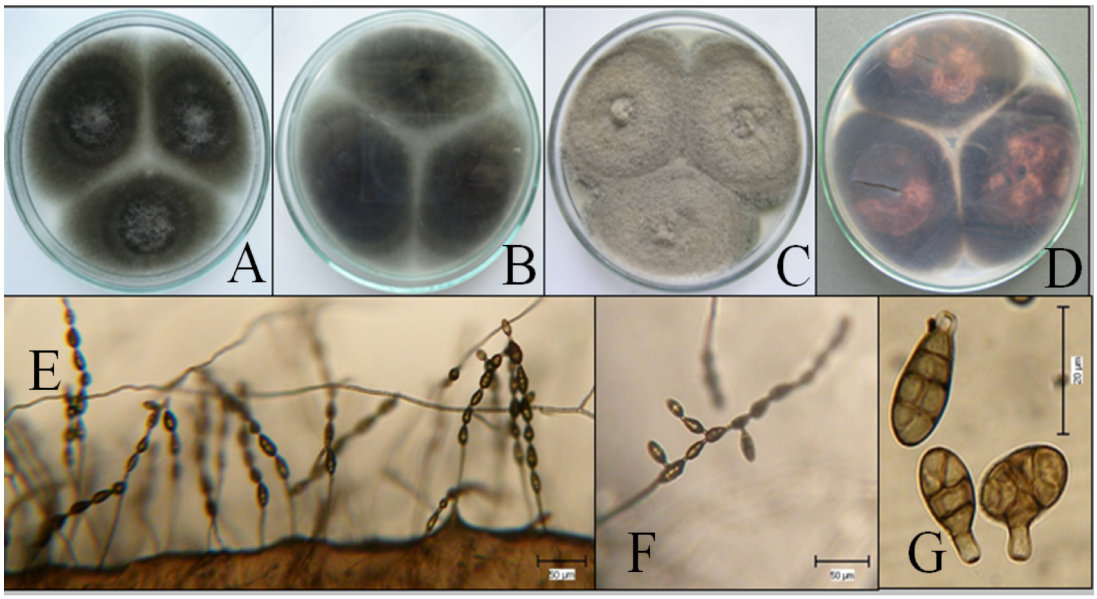

Figure 4 *Alternaria tenuissima* group 7 days of incubation. **A-B** colonies on PDA (A – top, B – reverse), **C-D** colonies on CYA (C – top, D – reverse), **E-F** – conidium sporulation pattern (scale bar = 50 µm), **G** – conidia (scale bar = 20 µm), Photo: Mašková.

Conidiophores of *Alternaria infectoria* group (Figure 3) that sporulate in the surface mass commonly are unbranched but have 1 – 3 geniculate extensions and conidiogenous loci incorporated in a total length of 50 – 100 µm. *Alternaria tenuissima* group (Figure 4) – produce uncrowded chains of up to 12 conidia on branching hyphae. The intial 1 – 2 and sometimes even 4 –5 lowest conidia of a chain usually have only transverse septa; only one or two mature conidia in a chain have the helpfully diagnostic median, subconstricting transverse septum that is such a striking feature of field conidia. The occurrence of the genus *Alternaria* in grape berries reported: **Serra et al., (2005, 2006)**; **Tournas et al., (2005)**; **Ostrý et al., (2007)**; **Polizzotto et al., (2012)**; **Chunmei et al., (2013)**; **Mašková et al., (2013)**; **Lorenzini and Zapparoli (2014)**; **Roseusseaux et al., (2014)**; **Prendes et al., (2015)**; **Tančinová et al., (2015)** and other authors. According **Bau et al., (2005)** predominant mycobiota of grape berries belonged to *Alternaria* spp., *Cladosporium* spp. and *Aspergillus* spp. These three genera were isolated from 75.6%, 22.5% and 17.3% of plated berries, respectively. **Magnoli et al., (2003)** reported that *Alternaria* genus was the most frequent (80% positive samples) from the surface-disinfected berries from Argentina. *Alternaria alternata* was the only species identified from this genus. **Ostrý et al., (2007)**; **Diguta et al., (2011)**; **Prendes et al., (2015**) recorded incidence of *Alternaria alternata*, also. Other authors mentioned the occurrence of *Alternaria* on the grape berries as follows: *Alternaria alternata* and *Alternaria tenuissima* **(Rousseaux et al., 2014)**; *Alternaria alternata* and *Alternaria arborescens* **(Lorenzini et al., 2014)**; *Alternaria arborescens* species-group and *Alternaria tenuissima* species-group **(Polizzotto et al., 2012)**. Isolation of *A. infectoria* species-group mentioned **Mašková et al., (2013)**, from Slovakian samples of grapes, too. In our sample was dominant *Alternaria tenuissima* group (1644 isolates – berries superficially sterilized and 1935 isolates – berries without sterilization), follow by *Alternaria alternata* group (662, respectively 707 isolates), *Alternaria arborescens* group (405 and 329 isolates) and Alternaria infectoria (109 and 34 isolates).

Mycotoxins are abiotic hazards produced by certain fungi that can grow on a variety of crops **(Marin et al., 20013).** According to European Union legislation mycotoxins produced by species genus *Alternaria* are not monitored in foods and food commodities. Mycotoxins such as alternariol and alternariol monomethylether are mutagenic and genotoxic in various *in vitro* systems. In addition, it has been suggested that in certain areas in China *Alternaria* toxins in grains might be responsible for oesophageal cancer. Hence, due to their possible harmful effects, *Alternaria* toxins are of concern for public health **(EFSA, 2011)**. According to **Prendes et al., (2015)**, *Alternaria*, one of the most mycotoxigenic genus commonly found in wine grapes, could represent a high risk for the wine consumer´s health. Representative isolates were selected for analysis to produce mycotoxins in *in vitro* conditions randomly from all obtained isolates. The results are presented in Table 2. A total of 184 isolates were tested. Production of selected secondary metabolites demonstrated the toxinogenity of isolates and on the other hand, it also served as an auxiliary indicator for identification (chemotaxonomy), mainly to distinguish the *Alternaria infectoria* species-group from the others **(Mašková et al., 2012)**. Production of mycotoxins by any of *Alternaria infectoria* strains still has not been demonstrated **(Andersen et al., 2002**; **Labuda et al., 2008**; **Piovarčiová et al., 2007)**. Conversely, *Alternaria alternata* and *Alternaria tenuissima* are known to produce several types of mycotoxins **(Andersen et al., 2002**; **Piovarčiová et al., 2007)**, which were confirmed in our study (Table 2). In neither case of the 15 tested isolates of *Alternaria infectoria* species-group we confirmed the production of mycotoxins ALT, AOH and AME. Although, the reputation of "nontoxigenic" strains of the *Alternaria infectoria* species-group has been undermined in recent years by isolation unknown metabolites **(Mašková et al., 2012)**. Conversely, isolates of other tested species-groups proved to be highly toxigenic (Table 2). Only one isolates of *Alternaria alternata* species-group and two isolates of *Alternaria arborescens* species-group did not produce tested mycotoxins in *in vitro* conditions detectable by TLC method. Robiglio and Lopez were tested eleven *Alternaria alternata* strains, isolated from Red Delicious apples in cold storage in Argentina, for alternariol and alternariol methyl ether production in laboratory media and in whole fresh fruits. Most of them were able to produce both toxins in all media. They were detected also in mycelium free filtrates from liquid cultures and in asymptomatic tissues from inoculated fruit. Thus, in the evaluation of mouldy core incidence in apples, the presence of *Alternaria alternata* toxins in tissues should be considered even in the absence of mycelia **(Robiglio and Lopez, 1995)**.

Small-spored *Alternaria*, such as *Alternaria alternata* group, *Alternaria arboresces* group, *Alternaria infectoria* group and *Alternaria tenuissima* group are important producers of mycotoxins, or other unknown metabolites but they were dominant fungal consortium in grapes berries in our samples. Considering that literature reported about the effectiveness of *Alternaria* endophytes against important grapevine pathogens, it should be interesting to elucidate the chemical structure of *Alternaria* unknown metabolites and to evaluate them as new biological method in the control of grapevine diseases **(Polizzotto et al., 2012)**.

CONCLUSION

From the 2350 surface-sterilized (47 samples) grape berries have been isolated 2964 strains of genus *Alternaria* and from the same number of non-sterilized berries 3099 isolates of this genus. Isolates were identified acording to sporulation patterns to four species groups: namely *Alternaria alternata* (1369 isolates), *Alternaria arborescens* (734), *Alternaria infectoria* (143), and *Alternaria tenuissima* (3579) and 238 isolates were not identified to species group. There were found out the ability to produce following mycotoxis: altenuene, alternariol and alternariol monomethylether in *in vitro* conditions by TLC method of chosen strains of genus *Alternaria*. In another research would be advisable to follow occurrence of these mycotoxins in grapes, must, wine and another grape products.

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Contact address:

Dana Tančinová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: dana.tancinova@uniag.sk.

Zuzana Mašková, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zuzana.maskova@uniak.sk.

Soňa Felšöciová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: sona.felsociova@uniag.sk.

Ľubomír Rybárik, Báb 49, 951 34 Báb, Slovakia, E-mail: alcedo1245@gmail.com

Miroslava Císarová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.cisarova@gmail.com.

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RESEARCH AND PRACTICE: QUANTIFICATION OF RAW AND HEAT-TREATED COW MILK IN SHEEP MILK, CHEESE AND BRYNDZA BY ELISA METHOD

Lucia Zeleňáková, Radoslav Židek, Margita Čanigová, Jana Žiarovská, Peter Zajác, Lenka Maršálková, Martina Fikselová, Jozef Golian

ABSTRACT

The aim of this study was to test the reliability of commercial ELISA tests (RC-bovino) within raw and heat treated cow milk detection in sheep milk and cheese in order to obtain a high-quality, reliable and economically beneficial method suitable for routine application in practice. These tests were subsequently used for quantification of cow milk in commercial "Bryndza". Raw sheep milk, cow milk and heat-treated cow milk (pasteurisation at 72 °C for 15 sec or at 85 °C for 3 sec) were mixed in precisely defined proportions (0 – 100% cow milk in sheep milk). The milk mixtures were sampled to detect adulteration and subsequently cheese was made. By ELISA tests was possible to determine these amounts of raw cow milk in sheep milk: 0.5% (0.2%), 5 % (4.81%), 50% (42.08%) and 75% (56.52%). The pasteurized samples in different combinations gave lower optical density responses than those prepared from raw milk (by approximately 60%). In context with the above mentioned, the relationship between the real and detected amount of cow milk (%) in different production stages (milk, cheese) using a regression analysis was examined. However, a lower reliability of the detection was indicated by R^2 values, which ranged from 0.4058 (cheese) to 0.5175 (milk). In practice this means that although individual percentage (%) of cow milk in the sample can be detected, but in the unknown sample it can not be clearly confirm whether the cow milk was raw or heat-treated. In this context, the results can be inaccurate and may not correspond to the real situation. Within monitoring phase of this research, 9 samples of bryndza were analysed with the results of detected cow milk ranged from 11.56% to 14.3%. The obtained results confirm that the appropriate selection of ELISA tests can become an important factor in the setting of analytical capabilities for the detection of milk and cheese adulteration.

Keywords: ELISA; milk; cheese; adulteration; reliability

INTRODUCTION

Consumption of fresh dairy products is the important motive factor for their production in European Union **(Habánová et al., 2010)**.

The unknown mixture of milk from different species is a common fraud in dairy sector. Milk with high economic value is commonly adulterated with milk from species of lower cost. This adulteration is especially important for cheese makers, due to unknown milk mixtures produce changes in the final sensory properties and reduce the product quality. Sheep milk is more expensive than goat or cow milk and tends to be adulterated with those of lower cost **(Puchades and Maquieira, 2013; Mayer et al., 2012)**.

Fraudulent incorporation of nondeclared kind of milk during technological processing is a common practice that can cause a problem for reasons related to intolerance or allergy, religious, ethical or cultural objections, and legal requirements. Therefore, accurate evaluation of the milk species used in dairy products is needed, especially for high-grade cheeses made exclusively with sheep or goat milk, many of which are registered by European law with a Protected Designation of Origin (PDO) **(Zeleňáková et al., 2008)**.

Traditional bryndza is sharp, salty, grayish, grated and pin-rolled, crumbly, semi-spreadable 100% sheep cheese. There is no close equivalent in taste and texture among sheep, cow, or goat cheeses. Most modern commercially available bryndza is milder, bleached creamy white, and two of its three varieties can legally contain up to 49% cow cheese. The European Commission registered the latter as *Slovenská bryndza* on its food list of Protected Geographical Indications on 16 July 2008 **(Commission regulation (EC) No 676/2008)**.

For legal reasons and for consumer protection and confidence many analytical techniques for detecting mixtures of milks from different species have been developed in last decades **(Zachar et al., 2011; Zeleňáková et al., 2011)**.

The official EU reference method which is based on the IEF of γ-caseins **(Commission regulation (EC) No 273/2008)** is an appropriate tool to detect cow milk in products made from milk of other species (detection limit ≤0.5%). A high number of other analytical techniques (e.g. electrophoretic, chromatographic, immunological and molecular biological methods) have also been used for qualitative (and partly also quantitative) species

authentication in dairy products **(Bobková et al., 2009; Mayer et al., 2012; Pizzano et al., 2011; Asensio et al., 2008; Xue et al., 2010; Costa et al., 2008; Suhaj et al., 2010; Stanciuc and Rapeanu, 2010 etc.)**.

Zeleňáková et al., (2009) described current situation in adulteration of the sheep milk and sheep milk products in Slovakia as well as in some countries in the EU. The results were evaluated according to the requirements of the valid legal standards. From the total number 70 samples 20 were adulterated with nondeclared cow milk.

ELISA is the most widely used form of immunoassay in milk analysis and has advantages of high sensitivity, low cost and fast application. It is easy to use, reliable, rapid and readily automated **(Song et al., 2011; Costa et al., 2008)**.

The development of immunoenzymatic methods and their practical use depends mainly on the selection of the immunogenes, experimental animals, way of immunization, quality of used antiserum, or possibly used antibodies and specificity as well as sensitivity of the evidencing system **(Yeung, 2006)**.

An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection and quantification of bovine milk adulteration in goat's milk. The polyclonal antibodies have been modified by mixing with goat's milk for the assay purposes. The absorbance at 450 nm in indirect ELISA revealed a linear relationship with the concentration of adulterated bovine milk at the range of 4% – 50% **(Xue et al., 2010)**.

Zarranz and Izco (2007) applied a protocol in order to validate a specific ELISA test for cow milk quantification in sheep milk, studying the main analytical properties displayed. The method was applied to analyze sheep milk samples collected from farms and it was found that 10% samples were adulterated with cow milk.

The aim of the study was to test the reliability of commercial ELISA tests for raw and heat-treated cow milk detection in the sheep milk and cheese and subsequently to quantify cow milk in commercial "Bryndza".

MATERIAL AND METHODOLOGY

1. Analysis of the samples in research part of the study:

Cow and sheep milk were obtained from a local dairy farm, refrigerated at 4 °C and tested for their quality. Both types of milk were mixed in the various alternatives, including heat treatment and subsequently cheese was made. In this research 32 samples were analysed what corresponded to 16 combinations of cow and sheep milk mixtures. At first, the intra assay and interassay were performed in terms of laboratory testing of results accuracy and repeatability. The sample extracts were pipetted into wells in duplicates.

Samples preparation:

Milk composition was performed at Lactoscan device. The working principle is based on measuring the speed of the *ultrasound* in milk. Observed parameters: Density ($kg.m^{-3}$), Fat content (g/100 g), Proteins (g/100 g), Lactose (g/100 g), Ash determined by calculation (g/100 g), Solids-non-fat (g/100 g), Freezing point of milk (°C). Other parameters: Calcium content (mg/100 g) by the complexometric titration method, Clotting activity (s), Titratable acidity of milk (°SH) by the method of Soxhlet-Henkel and Active acidity of milk by pH meter.

Raw sheep milk, cow milk and heat-treated cow milk (pasteurisation at 72 °C for 15 sec and at 85 °C for 3 sec) were mixed in precisely defined proportions (0, 0.5; 5; 50; 75; 100% cow milk in sheep milk). The milk mixtures were sampled to detect adulteration and subsequently cheese was made. At first the cheesiness test was performed and then 1 – 2.5 mL $CaCl_2$ per 1 liter was added to individual samples (depending on the level of heat treatment). The cheese production process included: cheesing of milk, processing of cheese curd, turning of cheese curd surface, its cutting, harping and mixing and finally formation of cloddish cheese. Subsequently the created clods were treated with 2% NaCl solution and left to mature at temperatures corresponding to the technological requirements (23, 19 and finally 8 °C). The temperature and pH in individual clods had been measured for 12 days. Subsequently they were processed and analysed according to the ELISA manufacturer instructions.

2. Analysis of the samples in monitoring and control part of the study:

The samples of bryndza (9 samples) were obtained in the grocery stores as well as from small sellers who product various sheep cheese. All the samples were refrigerated in the 30 mL boxes until the beginning of analysis. Subsequently they were processed and analysed according to the ELISA manufacturer instructions. The absorbance of the samples in research and monitoring part of the study was measured photometrically at 450 nm (STAT FAX 321/plus microwell reader - Awareness Technology, Palm City, FL). Comparisons of trends has been calculated with linear regression methods and visualized in graphs.

3. ELISA test characteristic:

ELISA tests RC-bovino (ZEU-INMUNOTEC, S.L, Spain) were used in our analysis. These tests are an enzyme immunoassay for the detection of cow milk in sheep or goat milk and their cheese. All reagents required for the enzyme immunoassay are contained in these test kits. The test kits are sufficient for 48 or 96 determinations (including standards). Detection limit is 0% cow milk. Assay time is approximately 90 minutes. The principle of the test is based upon the antigen-antibody reaction. The presence of cow milk in given sample is determined by the immunological detection of bovine IgG. The wells of the microtiter strips are coated with a specific antibody against bovine IgG. In the case of adulterated products, the antibodies contained in the cow milk will bind to the immobilized antibody. Any unbound components are removed in a washing step. By adding an antibody peroxidase-conjugate directed against bovine IgG, bound antigen is detected. Any unbound conjugate is removed in a washing step. Enzyme substrate and chromogen are added to the wells and incubated. The bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop reagent leads to a color change from blue to yellow. The measurement of the absorbance is made photometrically at 450 nm.

RESULTS AND DISCUSSION

In accordance with the ELISA instructions, within research part of the study, laboratory analysis of 32 samples of sheep milk and cheeses, adulterated with the addition of raw and heat-treated cow milk was performed. Prior to the analysis of these samples, quality control of ELISA tests was done. C.V. of results (n = 10) for inter and intra assay was 5.8% and 4.95%. As the basis for the evaluation, calibration curves were made by plotting % of cow milk in standard samples in a Y-axis and absorbance values in the X-axis. Values for the creation of calibration curves are shown in Table 1.

Table 1 The values for the creation of calibration curve for the detection of cow milk in samples by ELISA tests.

Standards	Concentration of cow milk in standards (%)	Absorbance at 450 nm	
		Analysis of milk samples	Analysis of cheese samples
1	0	0.369	0.401
2	1	0.492	0.526
3	5	0.973	1.036
4	10	1.483	1.528

The calibration curve should be linear in the range of 0 – 10% cow milk. It can then pass through the linear regression. These calibration curves were completed with trend lines of linear and polynomial function of the 2nd grade. Individual percentages of cow milk in the samples were calculated using regression equations or by interpolating the absorbance values obtained into the calibration curve. The obtained concentration data were the real values. They didn´t need any conversion factor. An example of calibration curve with regression equation for the detection of cow milk in mixed milk samples is shown in Figure 1. Numerous producers and sellers offer their own softwares for imunoanalysis data processing and these are also the part of fotometric analysers (four-parametric logistic model and spatial comparison method).

Czerwenka et al., (2010) have studied the calibration relationships in frame of chromatographic detection of buffallo milk adulteration by cow milk. β-Lg was the main marker and the results pointed that no effect was obtained in detection reliability when comparing the linear and the polynomic regression. The R^2 for linear regression was 0.9973 and for polynomic regression 0.9951.

García et al., (1994); Hurley et al., (2004 a, b); Zarranz and Izco (2007); Asensio et al., (2008) and many others also reported the very comparable calibration curves used for the detection of cow, goat and sheep milk and cheese adulteration. The degree of the variability calibration samples expressed R^2 was not less than 0.9 in all samples.

The above mentioned regression models were used in our data processing, too. The R^2 values ranged from 0.9981 up to 0.9956 for the linear regression and R^2 was 1 in two datasets for the polynomial regression models.

The important prerequisite for results evaluation was an adequate preparation of samples in which the series of dilutions was realized. The samples showing optical density over the valuef of highest standard were further diluted and tested again. The percentage of cow milk was calculated multiplying by diluting factor. The absorbancies that either exceeded the detection limit or were under it were not suitable for the quantitative analysis.

The lowest dilution amounts that possess the detectable absorbancies are summarized in the Table 2. All the absorbancies were analysed in the detection range of used ELISA kits. The absorbance values, that exceed the detection limit or were lower, were not possible to quantify. Based on the results, the dilution of samples in the range from 10^0 to 10^{-2} was used for the analysis 0 – 75% cow milk in sheep milk or cheese. The quatification was possible in the range from 10^0 to 10^{-1}. The only exceptions were the cow milk samples without

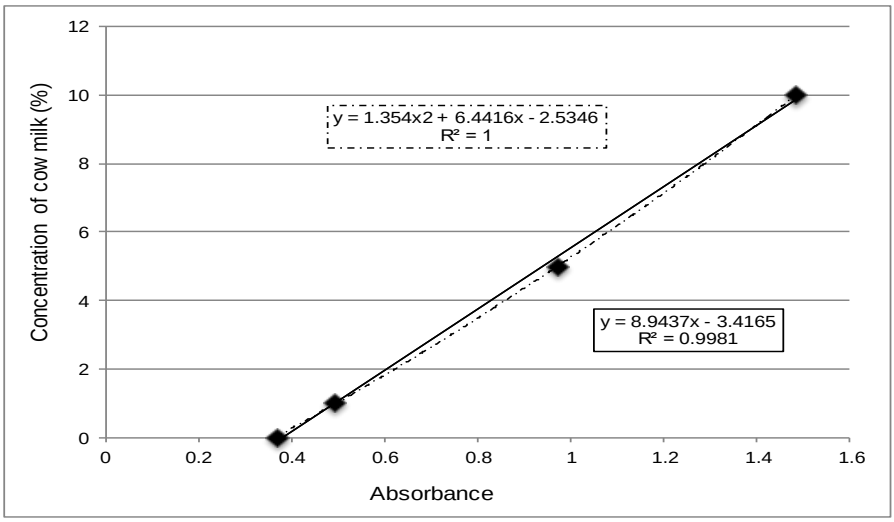
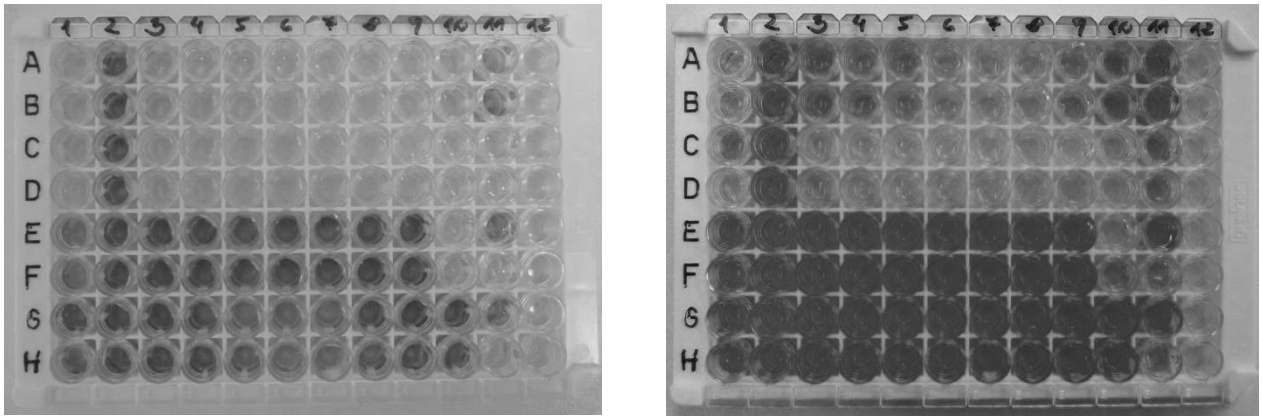


Figure 1 Calibration curve for the detection of cow milk in sheep milk.



A B

Picture 1 Visualization of ELISA test after addition of Substrate (A) and Stop solution (B).

sheep milk (14 M – 16 M and 14 CH – 16 CH). The absorbance values of these samples were similar instead of increased dilution what has influenced the final value of cow milk percentage. All these samples have no exact values in the Table 2 (!) and were not analysed further. For some samples (<75%), similar calculated concentrations were obtained, when two subsequently prepared decimal dilutions (from 10^0 to 10^{-1}) were used for the analysis. As an example is sample 9 CH. The absorbance 0.867 was detected for the dilution 10^0 what corresponds to the calculated concentration 3.95%. In the case of 10^{-1} dilution a lower absorbance was detected (0.452) what corresponds to the calculated concentration 3.98%. It was confirmed that the samples 1 M and 1 CH did not contain cow milk.

Regarding the choice of regression analysis model it can be said that with the increasing amount of cow milk, the higher values were calculated using linear regression equation. Nevertheless, producer of the used ELISA tests recommended analyze the obtained data by linear regression. The calculated values are reported in the Figures 2 – 4. ELISA tests of this producer are primarily designed to detect the adulteration of sheep and goat milk by raw cow milk. These amounts of raw cow milk in sheep milk it was possible to determine by ELISA tests: 0.5% (0.2%), 5% (4.81%), 50% (42.08%) and 75% (56.52%).

The amount of cow milk up to 10% (what is the detection range for these ELISA tests) can be analysed only by calibration curve including regression equations, without dilution of the samples. However, in a concentration range between 0 – 0.5%, quantification is more sensitive to imprecision. Therefore, it is important to prepare appropriate reagents, standards (especially in the concentration range from 0 to 1%) and keep a good laboratory practice. The producer also recommended creating a curve or curves with a specific detection range.

These curves were also used in analysis performed in study by **Zeleňáková et al., (2008)**. They found out that these types of curves can significantly affect the quality and accuracy of individual measurements. The same authors have reported that the results do not sometimes meet the quantitative criteria, especially at higher percentages. That can be caused by the saturation of the amount of specific antigens that are fixed in the microtitration plate and subsequently tight on the antigen surface.

ELISA is considered to be good quality when it can detect less than 1% foreign milk additives **(Song et al., 2011; Luis et al., 2009)**.

The next phase of the results analysis was focused on the evaluation of ELISA kits reliability within detection of different raw and heat-treated cow milk amounts in sheep milk and cheese. The results are reported in the Figure 2. The pasteurized samples in different combinations (including the cheese manufacturing) gave lower optical density responses than those prepared from raw milk. The detected amount of cow milk was in some samples (0.5 – 5%) under the detection range.

The main advantages are processing of a large number of samples, creation of calibration curve and measuring of blind samples simultaneously on one microtitration plate, which eliminates the impact of the changing conditions during the determination. ELISA has also disadvantages, for example in that it detects unimpaired proteins, but the protein hydrolysates need not react immunologically **(Hurley et al., 2006b; Taylor et al., 2009)**.

The caseins feature advantage in being more or less stable under high temperature conditions. Therefore, they can be successfully used as the main antigens in heat treatment (pasteurization, UHT) of milk and milk products. Their major disadvantage is weak immunogenicity and higher sensitivity to protheolytic degradation. The whey proteins are much better immunogens and they are protheolytically degradable only in minimal quantity. In respect of high temperatures the whey proteins are less resistant **(Lowe et al., 2004)**.

In context with the above mentioned, the relationship between the real and detected amount of cow milk (%) in different production stages (milk, cheese) using a regression analysis was examined. Four detection trends were setf for the analysed ranges from 0 to 75%. All of them were characterized by the linear functions with the appropriate regression equations.

Table 2 Comparison of assay sensitivity by two regression models.

Sample - cow milk in sheep milk (M) and cheese (CH)	Absorbance at 450 nm	Dilution	Detected amount of cow milk (%)	
			Linear function	Polynomial function
1 M (0% raw)	0.309	a	——	——
2 M (0.5% raw)	0.404	a	0.197	0.289
3 M (0.5% low pasteurized)	0.334	a	——	——
4 M (0.5% high pasteurized)	0.327	a	——	——
5 M (5% raw)	0.919	a	4.806	4.529
6 M (5% low pasteurized)	0.37	a	——	——
7 M (5% high pasteurized)	0.34	a	——	——
8 M (50% raw)	0.853	b	42.08	39.409
9 M (50% low pasteurized)	0.534	b	13.597	12.913
10 M (50% high pasteurized)	0.458	b	6.797	6.997
11 M (75% raw)	1.014	b	56.524	53.894
12 M (75% low pasteurized)	0.609	b	20.257	18.865
13 M (75% high pasteurized)	0.528	b	13.058	12.44
14 M (100% raw)	!	!	!	!
15 M (100% low pasteurized)	!	!	!	!
16 M (100% high pasteurized)	!	!	!	!
1 CH (0% raw cow)	0.204	a	——	——
2 CH (0.5% raw)	0.409	a	——	0.114
3 CH (0.5% low pasteurized)	0.407	a	——	0.101
4 CH (0.5% high pasteurized)	0.398	a	——	——
5 CH (5% raw)	0.634	a	1.98	1.768
6 CH (5% low pasteurized)	0.411	a	——	0.128
7 CH (5% high pasteurized)	0.405	a	——	0.088
8 CH (50% raw)	0.569	b	13.254	12.177
9 CH (50% low pasteurized)	0.867	a	3.945	3.55
10 CH (50% high pasteurized)	0.637	a	1.985	1.723
11 CH (75% raw)	0.646	b	20.023	17.866
12 CH (75% low pasteurized)	1.025	a	5.334	4.435
13 CH (75% high pasteurized)	0.648	a	2.024	1.805
14 CH (100% raw)	!	!	!	!
15 CH (100% low pasteurized)	!	!	!	!
16 CH (100% high pasteurized)	!	!	!	!

Dilution: 10^0 (a); 10^{-1} (b); differences within individual dilutions (!); outside the detection range (——).

In the Figure 3 it can be seen that individual curves are indeed increasing character that corresponds to the growing amount of cow milk. However, a lower reliability of the detection was indicated by R^2 values, which ranged from 0.4058 (cheese) to 0.5175 (milk). In practice this means that although individual percentage of cow milk in the sample can be detected (%), but in the unknown sample it can not be clearly confirm whether the cow milk was raw or heat-treated. In this context, the results can be inaccurate and may not correspond to the real situation.

Creating the specific regression curves for each way of cow milk heat treatment (Figure 4) was performed in order to asses the relationship between the real and detected amounts of cow milk in sheep milk. The values of determination coefficients (R^2) were higher than 0.82. Reliable detection of the real amount of cow milk can be performed in the praxis by both, interpolation as well as the regression analysis. The basic limitation for the precise detection is to know the way of cow milk heat treatment. Similar regression curves can be provided for the detection of cheese adulteration, too.

As the various processing of milk can negatively affect the reliability of adulteration detection, such type of the analysis has not been applied in the praxis yet and also there is not recommended for the use. Therefore, the use of these ELISA tests is not adequate for routine surveillance of marketed cheese, especially for mixed cheeses, when the amount of milk from different species used for cheese making is unknown.

The detection and quantification of cow milk in the sheep milk and cheese using the commercial ELISAs was performed by **Costa et al., (2008)**, too. The detected value in cheese samples was by 10% lower than the experimental value for QBT ELISA test and by 20 % lower for QGT ELISA test, when more than 40% cow or goat milk was added.

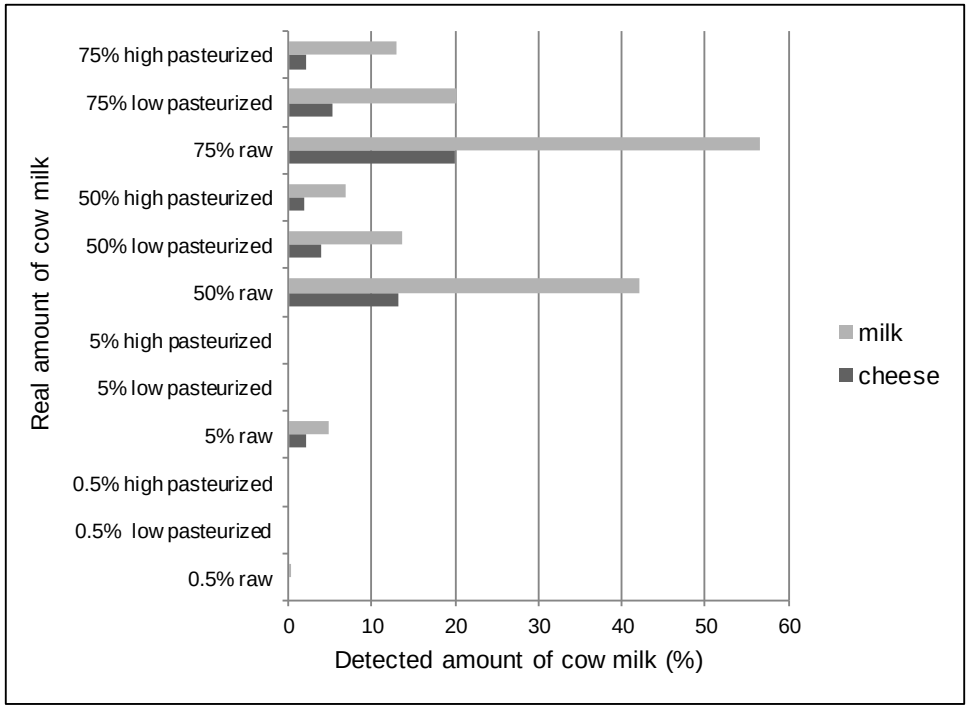


Figure 2 Impact of cow milk heat treatment on its detection in sheep milk and cheese.

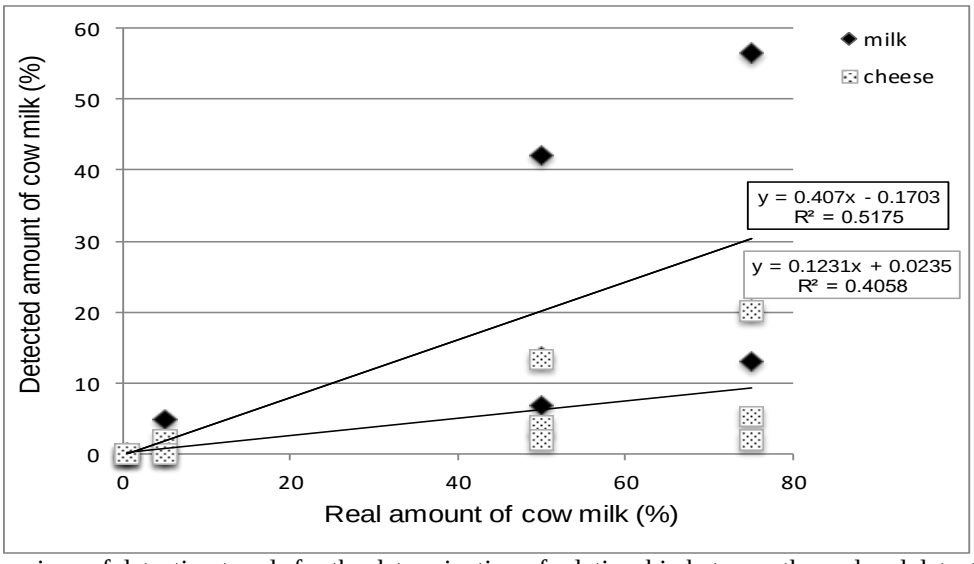


Figure 3 Comparison of detection trends for the determination of relationship between the real and detected percentage of cow milk in sheep milk and cheese (%).

The ELISA tests RC-bovino were subsequently used for quantification of cow milk in 9 samples of commercial "Bryndza". Individual percentage of cow milk in the samples were calculated by interpolating the absorbance values obtained into the calibration curve and using regression equations ($y = 7.3075x - 1.9301$; $R^2 = 0.9995$). The presence of cow milk was confirmed in all analysed samples of bryndza (Table 3). The samples 1 – 8 were evaluated together and the sample 9 was evaluated separately according to the composition differences as given by manufacturers. By ELISA test there were detected from 11.56% (sample 1) to 14.3% (sample 4) cow milk. The coefficient of variation was 9.26% for these 8 samples. The sample 9 „Tatranská bryndza" was specific because of high portion of cow milk. The manufacturer indicates this fact on the labeling (25% of sheep cheese).

In this sample 31.44% cow milk was detected by ELISA. But it can be assumed, that the real addition of cow milk in commercial samples of bryndza was higher than those detected by ELISA. This is based on the previously performed analyses and over mentioned results. Reliability of the ELISA tests and their applicability in the routine analysis was studied by many authors such as **Popelka et al., (2002); Zeleňáková et al., (2008, 2009, 2011); Zarranz and Izco (2007); Costa et al., (2008); Štumr et al., (2008); Brinkhof et al., (2009); Luis et al., (2009); Taylor et al., (2009); Kardar (2010); Sleziaková and Baleková (2010); Xue et al., (2010); Song et al., (2011)** and many others.

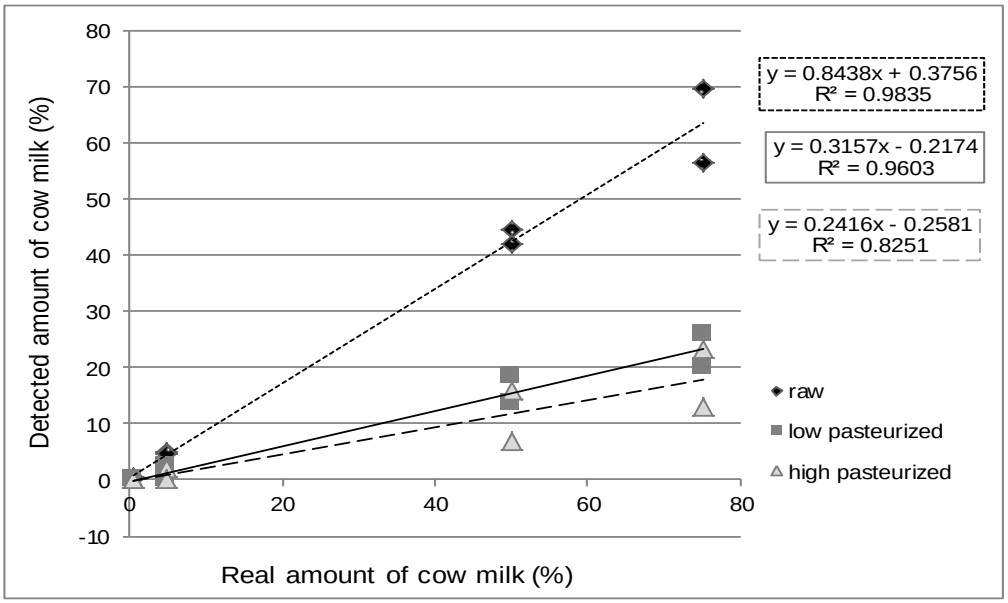


Figure 4 Linear functions with the regression equations for raw and heat-treated cow milk determination in sheep milk (%) amount of cow milk in sheep milk and cheese (%).

Table 3 Samples of the bryndza analysed by the ELISA tests.

Sample number/ manufacturer	Label and composition of bryndza	Quantification of cow milk by ELISA tests
1	Sheep cheese processed from raw milk (min 51%), water, edible salt (max 2.5%), dry matter (min 44%), fat in dry matter (min 48%)	11.56%
2	Stored sheep cheese, cow cheese, edible salt (max 3 %), water, dry matter (min 44%), fat in dry mater (min 48%)	13.91%
3	Stored sheep cheese (min 51%), cow cheese, edible salt (max 3%), water, dry matter (min 44%), fat in dry matter (min 48%)	14.24%
4	Stored sheep cheese (min 51%), cow cheese, edible salt (max 3%), water, dry matter (min 44%), fat in dry matter (min 48%)	14.3%
5	Stored sheep cheese (min 51%), cow cheese, edible salt (max 2%), water, dry matter (min 44%), fat in dry matter (min 48%)	11.95%
6	Sheep cheese processed from raw milk (min 51%), water, edible salt (max 3%), dry matter (min 44%), fat in dry matter (min 4 %)	12.57%
7	Sheep cheese processed from raw milk (min 5 %), cow cheese processed from pasteurized milk, water, edible salt (max 2.5 %), dry matter (min 44%), fat in dry matter (min 48%)	11.63%
8	Mixture of cow and sheep cheese processed from pasteurized milk, water, edible salt (max 2.5%), dry matter (min 44%), fat in dry matter (min 48%)	12.08%
9	Cow cheese, sheep cheese (25%), fat (21%)	31.44%

% – weight percentage, min – minimum, max – maximum.

CONCLUSION

The analyses carried out in laboratory conditions recently, focused on the current situation monitoring of milk and cheese adulteration, have proved the necessity to deal with this issue more thoroughly. Most of the ELISA tests come from abroad (outside Slovakia). Their quality is important for milk producers and processing companies as well as public inspection authorities. The tests should be highly specific, sensitive, reliable, an easy to use, easy to laboratory equipment and of course affordable. As the tests are certified, nobody doubts their quality. Our survey, which we have been performing for a few years, has shown that few milk producers know possibilities of milk and cheese adulteration detection. This situation results in the fact that the producers either don´t do any detection or they use the tests provided by distributors.

The aim of the study was to test the reliability of commercial ELISA tests for raw and heat-treated cow milk detection in the sheep milk and cheese and subsequently to quantify cow milk in commercial "Bryndza". The used ELISA kits are designed for the quantitative determination of cow milk in sheep milk, sheep cheese, goat milk and goat cheese. By ELISA tests was possible to determine these amounts of raw cow milk in sheep milk: 0.5% (0.2%), 5% (4.81%), 50% (42.08%) and 75% (56.52%). The pasteurized samples in different combinations gave lower optical density responses than those prepared from raw milk. The decrease of cow milk amount by 53.53%

and 59.34% (at 5% low and high pasteurized cow milk) and by 62.64% and 66.56% (at 75% low and high pasteurized cow milk) was detected. In next phase of the research, the relationship between the real and detected amount of cow milk (%) in different production stages (milk, cheese) using a regression analysis was examined. However, a lower reliability of the cow milk detection was found and indicated by R^2 values, which ranged from 0.4058 (cheese) to 0.5175 (milk). In practice this means that although individual percentage of cow milk in the sample can be detected (%), but in the unknown sample can´t be clearly confirmed whether the cow milk was raw or heat-treated. In this context, the results can be inaccurate and may not correspond to the real situation. As was noted above, one of the solutions is to set a specific regression curves for each of the heat treatment of analysed milk. The values of determination coefficients were higher than 0.82, which assumes the conditions for the reliable determination of raw or heat-treated cow milk in sheep milk. The only limitation here is the knowledge of cow milk heat treatment.

In total, 9 samples of bryndza were analysed in the monitoring phase of the research with the results of detected cow milk ranged from 11.56% to 14.3%. It can be assumed, that the real addition of cow milk in commercial samples of bryndza was higher than those detected by ELISA.

In conclusion, the analysis has shown that the ELISA tests identified the presence of cow milk, but quantification was not exact because of irreversible changes caused by the manufacturing process. Despite this fact, producer recommended ELISA tests for the detection of sheep milk and cheese adulteration by cow milk. Despite some negatives identified in this study, ELISA tests may find practical application, if they are used only for the qualitative detection of cow milk in other species milks or cheeses. Such detection is important for health, nutritional, technological as well as for economic reasons.

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Contact address:

Lucia Zeleňáková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: lucia.zelenakova@uniag.sk.

Radoslav Židek, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: radoslav.zidek@uniag.sk.

Margita Čanigová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: margita.canigova@uniag.sk.

Jana Žiarovská, Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding , Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: jana.ziarovska@uniag.sk.

Peter Zajác, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra; State Veterinary and Food Institute, Jánoškova 1611/58, 026 01 Dolný Kubín, Slovak Republic, E-mail: zajac@potravinarstvo.com.

Lenka Maršálková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: lenka.marsalkova@uniag.sk.

Martina Fikselová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: martina.fikselova@uniag.sk.

Jozef Čapla, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: jozef.capla@uniag.sk.

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COMPOSITION AND MICROSTRUCTURE ALTERATION OF TRITICALE GRAIN SURFACE AFTER PROCESSING BY ENZYMES OF CELLULASE COMPLEX

Elena Kuznetsova, Svetlana Motyleva, Maria Mertvischeva, Vladislav Zomitev, Ján Brindza

ABSTRACT

It is found that the pericarp tissue of grain have considerable strength and stiffness, that has an adverse effect on quality of whole-grain bread. Thereby, there exists the need for preliminary chemical and biochemical processing of durable cell walls before industrial use. Increasingly used in the production of bread finds an artificial hybrid of the traditional grain crops of wheat and rye – triticale, grain which has high nutritional value. The purpose of this research was to evaluate the influence of cellulose complex (*Penicillium canescens*) enzymes on composition and microstructure alteration of triticale grain surface, for grain used in baking. Triticale grain was processed by cellulolytic enzyme preparations with different composition (producer is *Penicillium canescens*). During experiment it is found that triticale grain processing by enzymes of cellulase complex leads to an increase in the content of water-soluble pentosans by 36.3 – 39.2%. The total amount of low molecular sugars increased by 3.8 – 10.5 %. Studies show that under the influence of enzymes the microstructure of the triticale grain surface is changing. Microphotographs characterizing grain surface structure alteration in dynamic (every 2 hours) during 10 hours of substrate hydrolysis are shown. It is found that the depth and direction of destruction process for non-starch polysaccharides of grain integument are determined by the composition of the enzyme complex preparation and duration of exposure. It is found, that xylanase involved in the modification of hemicelluloses fiber having both longitudinal and radial orientation. Hydrolysis of non-starch polysaccharides from grain shells led to increase of antioxidant activity. Ferulic acid was identified in alcoholic extract of triticale grain after enzymatic hydrolysis under the influence of complex preparation containing cellulase, xylanase and β-glucanase. Grain processing by independent enzymes containing in complex preparation (xylanase and β-glucanase) shows that more significant role in polysaccharide complex composition and grain surface microstructure alteration belongs to xylanase. Grain processing by independent of cellulolytic enzymes may decrease the strength of pericarp tissue of grain and improved sensory characteristics of the bread.

Keywords: triticale; grain; xylanase; microstructure; antioxidant activity

INTRODUCTION

Dietary fiber in cereals is presented by non-starch polysaccharides found in the cell walls and consisting mainly of arabinoxylan and β-glucan **(Jacobs et al., 1998; Gebruers et al., 2008)**. Wheat and rye, as a universal raw material used in baking, became the main objects in studies of the grain pentosans properties. Currently, however, an artificial hybrid of these grains named triticale get wider range of application **(Cauvain et al., 2007)**. Total dietary fiber content of triticale grain is 13 – 16 % depending on the sort. Triticale comprises 6.8% of arabinoxylan, 0.7% of β-glucan and 2.1% of cellulose on average **(Izydorczyk et al., 1995; Barron et al., 2007)**.

Studies show that the molecular structure and the structural organization of arabinoxylans and β-glucans of grain (pentosans) are important determinants of their physical properties, such as solubility in water, viscosity, digestibility. This determines the functionality of said polysaccharides and their physiological functions in the gastrointestinal tract of humans **(Vaikousi et al., 2004; Lazaridou et al., 2007)**. Inclusion of cereals products containing dietary fiber in diet helps reduce cholesterol concentration, that decrease the risk of coronary heart disease **(McIntosh et.al., 1991; Brown et al., 1999)**, improve the glycemic level control for people with type II diabetes **(Lu et al., 2004)**, increases intestinal peristalsis **(Cummings et al., 1992, 2009)**.

Arabinoxylans and β-glucans, along with providing benefits to human health, have the potential to improve the quality of bakery products **(Said et al., 2011)**. There are two types of arabinoxylans: water extractable (about 35% of the total) and non-extractable **(Leggio et al.,, 1999)**. These two fractions differ in physicochemical and functional properties, including water-binding and gel-forming ability **(Courtin et al., 2002)**. Most of the dietary fiber of rye and wheat bran is insoluble **(Grigelmo-Miguel et al., 1999; Van Craeyveld et al., 2009)**. However, water-extractable arabinoxylan is more effective compared to non-extractable in terms of quality improvement and shelf life extension of bread by reducing the effect of staling and starch retrogradation **(Courtin et al., 1999; Said et al., 2011)**.

The pericarp tissue of grain have considerable strength and stiffness, that has an adverse effect on quality of

whole-grain bread **(Antoine et al., 2003)**. There is a growing demand for the usage of sustainable processes of soft biotech processing of plant cell walls, which will replace the chemical treatment **(Ulvskov et al., 2011)**. Usage of a xylanase for the hydrolysis of water-insoluble non-starch polysaccharides of the cell walls leads to improvement in swelling, sensory performance and to deceleration of starch retrogradation process **(Gruppen et al., 1998; Andlaver et al., 2002; Charalampopoulos et al., 2002; Jiang et al., 2005)**.

The purpose of this research was to evaluate the influence of cellulose complex (*Penicillium canescens*) enzymes on composition and microstructure alteration of triticale grain surface, for grain used in baking.

MATERIAL AND METHODOLOGY

Two sorts of triticale grain from different genetic sources were studied. They are «Antaeus» and «Talva 100» (Russian Federation). Dry complex enzyme preparation comprising cellulase, β-glucanase and xylanase, as well as preparations containing individual enzymes (producer is *Penicillium canescens*, The Russian Academy of Sciences' Skryabin Institute of Biochemistry and Physiology of Microorganisms) were used during research. Enzymes had the following activity: cellulase 58711 nkat/g, xylanase 12135 nkat/g, β-glucanase 51317 nkat/g and were given by chemical faculty of Moscow State University **(Sinitsyna et al., 2003)**.

Enzyme preparation in powder was mixed by a magnetic stirrer with a citrate buffer (pH 4.5) for 0.5 hours at a concentration of 0.6 $g.L^{-1}$ before the analysis. This concentration corresponds with the optimum enzyme concentration for bread production from whole triticale grain **(Kuznetsova et al., 2010)**. Whole triticale grain was incubated in enzyme preparation solution with grain-solution ratio of 1:1.5 for 8 hours at 50 °C in thermostat. Duration of cereal substrate hydrolysis determined by the time during which the grain moisture was 40% or more that is required to get the cereal mass with ability to dispersion and allow to use grain raw material for bakery. To save material intact enzyme inactivation wasn't performed after incubation.

Determination of cellulose content, ratio of amorphous and crystalline cellulose and total amount of hemicellulose were carried out according to procedures described by **Ermakov (1972)**. To detect the soluble pentosans, grain sample was analyzed by orcinol-chloride method **(Hashimoto et al., 1987)**.

Concentration determination of low molecular carbohydrates in the grain samples was performed by a chromatographic method with electrochemical detection using liquid chromatograph Agilent 1100 with electrochemical detector ESA Coulochem III. Sugars mixture separation carried out using an anion exchange column with grafted amine phase followed by electrochemical detection.

Microstructural studies were conducted using an electron scanning microscope ZEISS EVO LS. Survey was carried out at an acceleration voltage of 15 kV.

Complex of phenolic compounds was determined by HPLC using MiLiChrome-5 device. Triticale grain ethanolic extract was used, eluent of composition is acetonitrile – water solution of trifluoroacetic acid (pH 2.5, in a ratio of 15:85); elution mode is isocratic, the analysis time is 12 – 25 min, the sample volume – 6.2 ml. Antioxidant activity was determined by spectrophotometric method in alcoholic extract described by **Silva et al., 2005**.

RESULTS AND DISCUSSION

Table 1 shows the research results of dietary fiber content in two sorts of triticale grain.

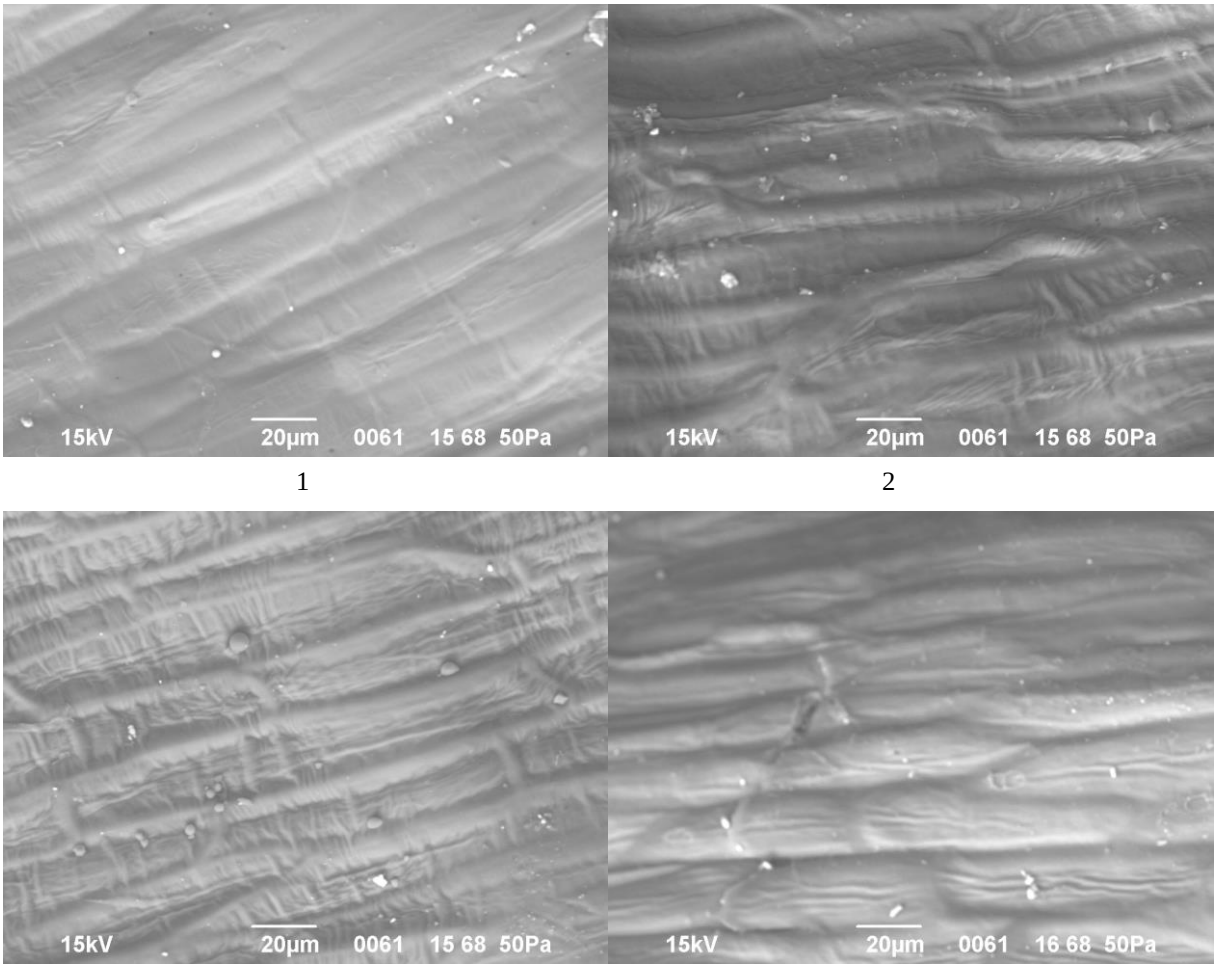
Table 1 Composition of non-starch polysaccharides of dry triticale grain soaked in a citrate buffer and treated with enzyme preparations, in %.

Experiment variation	Cellulose	Ratio of amorphous and crystalline cellulose	Hemicellulose	Soluble pentosans
		Antaeus		
Dry grain	2.20 ±0.04	2.33 ±0.04	7.93 ±0.04	5.46 ± 0.04
Control grain	2.08 ±0.05	2.24 ±0.04	7.24 ±0.05	5.88 ±0.06
Complex preparation	1.96 ±0.03	2.00 ±0.04	6.70 ±0.05	7.60 ±0.03
Xylanase	1.98 ±0.04	2.08 ±0.03	6.96 ±0.04	7.34 ±0.03
β- glucanase	2.04 ±0.03	2.18 ±0.03	7.18 ±0.03	6.66 ±0.05
		Talva 100		
Dry grain	2.14 ±0.04	2.60 ±0.04	7.94 ±0.02	5.84 ±0.03
Control grain	2.02 ±0.03	2.48 ±0.04	7.38 ±0.02	6.15 ±0.02
Complex preparation	1.85 ±0.02	2.21 ±0.04	6.95 ±0.04	7.96 ±0.03
Xylanase	1.90 ±0.05	2.26 ±0.03	7.12 ±0.06	7.68 ±0.03
β- glucanase	2.00 ±0.03	2.34 ±0.03	7.24 ±0.02	6.74 ±0.04

As a result of grain processing by enzyme complex concentration of water-soluble pentosans increased by 36.3 – 39.2%, depending on the sort of grain. Processing of grain by individual enzymes like hemicellulase comprised in complex preparation (xylanase and β-glucanase), showed that more significant role in polysaccharide complex composition of grain surface structures alteration belongs to xylanase. These results are consistent with data

Table 2 Carbohydrate composition of dry triticale grain, soaked in citrate buffer and treated with enzyme preparations, $g.L^{-1}$.

Sugar	Dry grain	Control grain	Complex preparation	Xylanase	β-glucanase
Arabinose	0.00	0.01	0.03	0.02	0.02
Galactose	0.00	0.00	0.00	0.00	0.00
Glucose	0.31	0.35	0.43	0.40	0.37
Xylose	0.00	0.00	0.04	0.02	0.01
Fructose	0.26	0.24	0.22	0.23	0.24
Raffinose	0.00	0.01	0.03	0.03	0.02
Unidentifiedsugar	0.07	0.08	0.12	0.10	0.09
Cellobiose	0.00	0.00	0.00	0.00	0.00
Maltose	1.68	1.98	2.08	2.05	2.02
Total	2.32	2.67	2.95	2.85	2.77



1 2

3 4

Figure 1 Microstructure of triticale grain surface (1 – grain soaked in buffer – control; 2 - treated by complex enzyme preparation; 3 – by xylanase preparation; 4 – by β-glucanase preparation), an increase of x 700. Photo: S. Motyleva, 2014.

from **(Havrlentova et al., 2011)**, where it was found that the level of soluble dietary fiber in wheat bran increases under the influence of enzyme preparations – hemicellulases, especially those containing endoxylanase.

Since varietal differences in non-starch polysaccharides of triticale grain shells content alteration after enzymes processing (cellulase complex *Penicillium canescens*) is not significant, the determination of triticale grain carbohydrate composition by chromatographic method was carried out for the average triticale grain sample, composed of two represented sorts. Hydrolysis of glycosidic linkages in polysaccharides molecules is occurred and partially collapsed matrix carcass nodes,

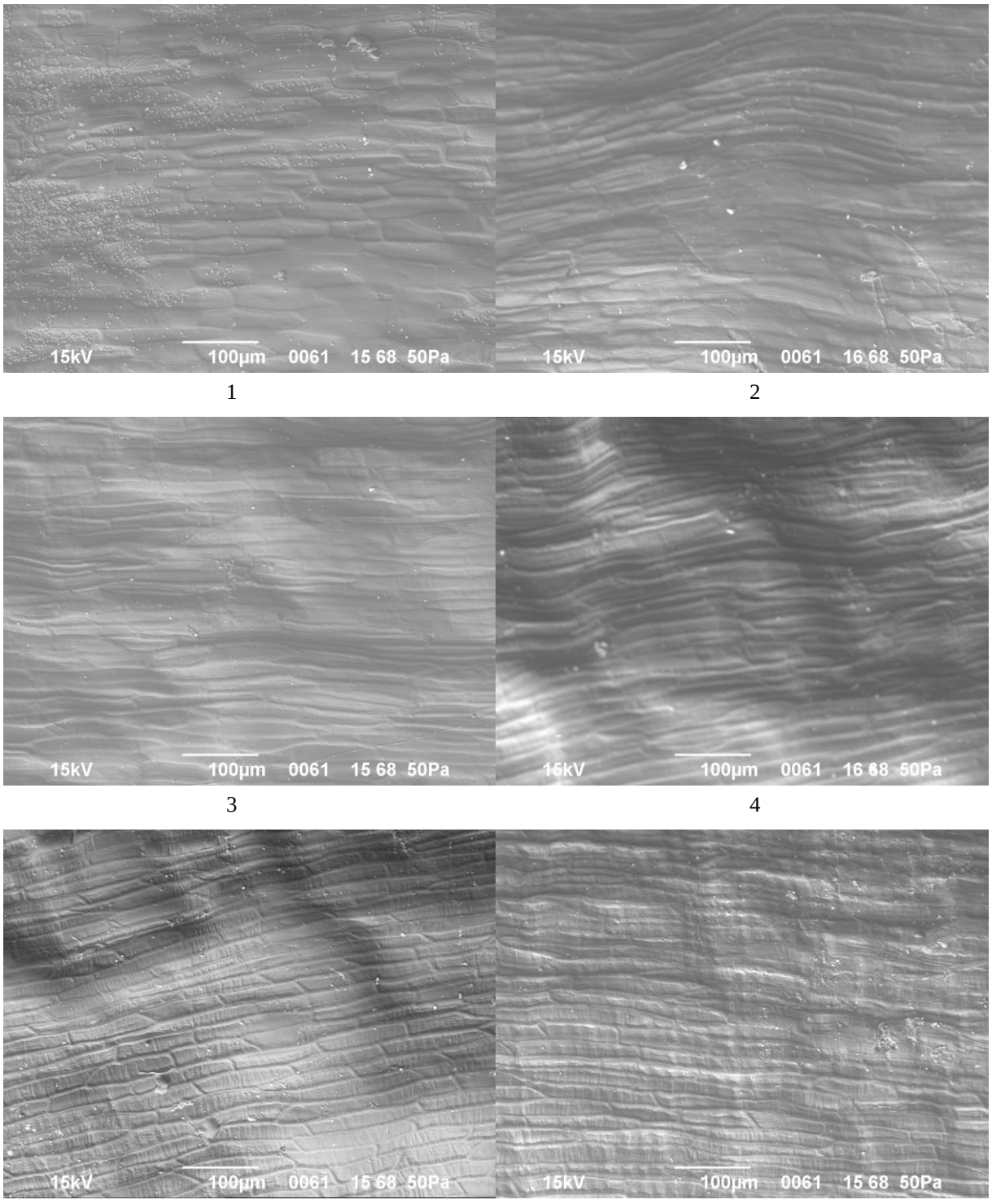


Figure 2 The surface microstructure of triticale grain, soaked in a solution of a complex preparation (cellulase, β-glucanase, xylanase) during different periods (1 – immediately after being placed in a solution; 2 – 2 hours; 3 – 4 hours; 4 – 6 hours; 5 – 8 hours; 6 – 10 hours) x 200 magnification. Photo: S. Motyleva 2014.

wherein substances with low molecular weight and high solubility was formed (see Table 2).

The content of arabinose (0.02 – 0.03 $g.L^{-1}$) and xylose (0.01 – 0.04 $g.L^{-1}$) in grain extracts indicate occurred biochemical processes in arabinoxylan chains. Such processes can be caused by the presence of hydrolyzing glycosidic linkages in the enzyme complex of hemicellulases preparations. Results of chemical composition alteration of the cell walls in wheat grain shells under the influence of xylanase reconciled with scanning electron microscopy **(Tervilä-Wilo et al., 1996; Parkkonen et al., 1997)**. Figure 1 shows microphotographs of dry triticale grain surface structure, soaked in citrate buffer and treated with enzyme preparations.

Xylanase have an influence on both type of hemicellulose fibers with longitudinal and radial orientation. Channels on the surface of the grain shells, having various directions are found. This fact shows that endoxylanases *Penicillium canescens* have much stronger destructive forces for non-starch polysaccharides in outer integument of triticale grain compared to β-glucanase.

Figure 2 shows photographs of the surface microstructure of triticale grain soaked in a solution of a complex preparation for adifferent time.

Microphotographs shows triticale grain surface alteration during hydrolysis by complex enzyme preparation in the dynamics. First of all microfibrils having a longitudinal orientation became bare because hemicellulose shielding layer exposed to degradation influence. Hollows having a radial orientation appear on the surface of the grain shells after 6-8 hours of hydrolysis. It means that deeper processes affecting both arabinoxylan molecules and cellulose matrix microfibrils.

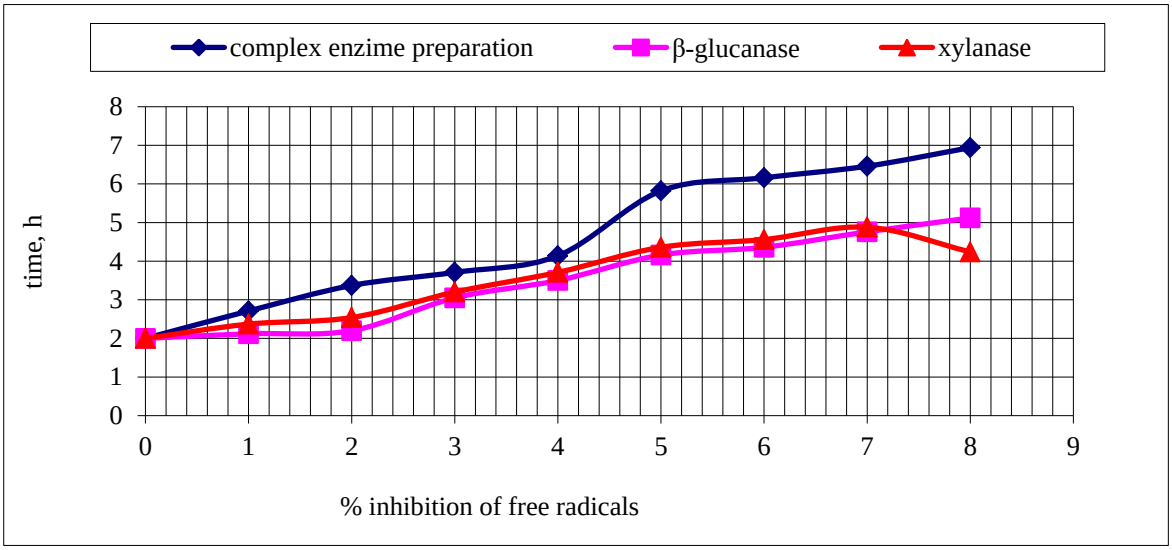


Figure 3 Triticale grain antioxidant activity alteration in the process of enzymatic hydrolysis by cellulase preparations.

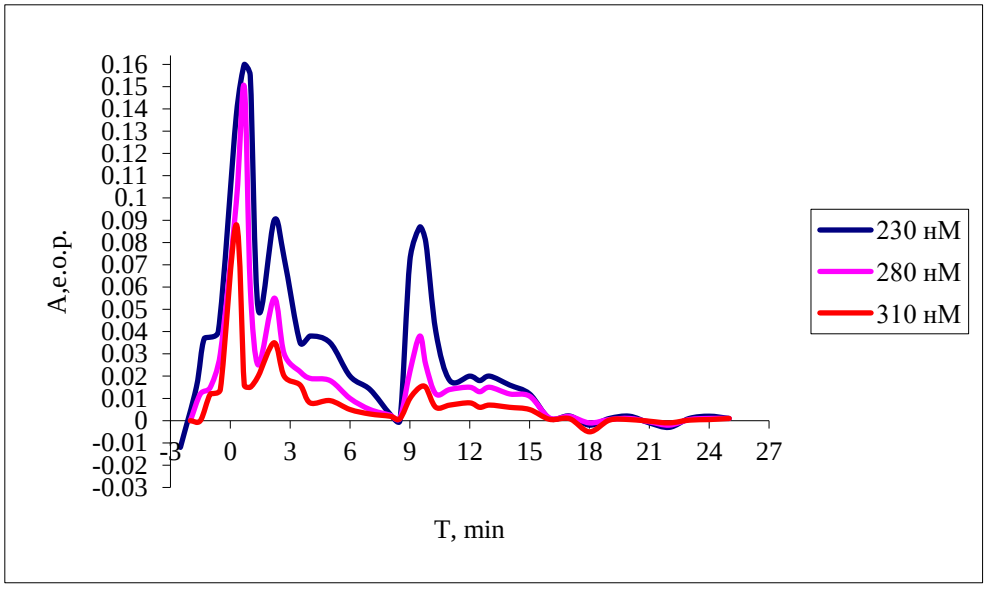


Figure 4 The chromatogram of triticale grain extract after enzymatic hydrolysis with a complex enzyme preparation (cellulase, β-glucanase, xylanase) made of *Penicillium canescens*.

The degradation of xylan from cell walls matrix under the influence of endo-xylanase and β-glucanase leads to destruction of the natural triticale grain shells structure and to increase of the water-soluble pentosans concentration.

Determination of antioxidant activity (Figure 3) for alcoholic extract of triticale grain treated for 8 hours by a complex enzyme preparation, β-glucanase and xylanase, show that the percentage of DPPG free radicals inhibition increasing with extension of hydrolysis duration.

Composition of phenolic compounds in triticale grain extract after enzymatic hydrolysis with a complex enzyme preparation was determined by HPLC method. Chromatogram is shown in Figure 4.

Chromatogram of alcoholic extract allowed to identify organic and hydroxycinnamic acids. Ferulic acid was identified (VR = 9,6; RS = 0,533). These findings are consistent with the results of **(de Vries et al., 2000)**, where stated that after the degradation of xylan chain by endo-xylanase, the antioxidant activity of cereal substrates increases by the release of ferulic acid.

CONCLUSION

During experiment it is found that triticale grain processing by enzymes of cellulase complex leads to an increase in the content of water-soluble pentosans by 36.3 – 39.2% and carbohydrates with a low molecular weight and high solubility. Xylan degradation of the cell walls matrix under the influence of endo-xylanase and β-glucanase leads to the destruction of the natural structure of triticale grain shells, that is consistent with data on the content increase of water-soluble pentosans. Application of cellulase complex enzymes (producer is *Penicillium canescens*) for the treatment of triticale grain increases the content of water-soluble pentosans, low molecular carbohydrates, the antioxidant activity of raw material that has positive implications for the future grain usage in bread baking. Grain surface microstructure alteration leads to modifications of non-starch polysaccharides, that may decrease their strength and improved sensory characteristics of the product.

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Contact address:

prof. Elena A. Kuznetsova, DrSc., State University-Education-Science-Production Complex, 302020, Orel, Naugorskoe shosse, 29, Russian Federation, E-mail: elkuznetcova@rambler.ru.

Ing. Svetlana M. Motyleva PhD. Maria Mertvischeva All-Russian Institute of plant breeding and gardening and nursery, 115598, Moskow, Zagorievskay street, 4, Russian Federation, E-mail: motyleva_svetlana@mail.ru;

Maria Mertvischeva, All-Russian Institute of plant breeding and gardening and nursery, 115598, Moskow, Zagorievskay street, 4, Russian Federation, E-mail: denkviz@yandex.ru.

Vladislav Y. Zomitev, PhD. student, State University-Education-Science-Production Complex, 302020, Orel, Naugorskoe shosse, 29, Russian Federation, E-mail: gz63@mail.ru.

doc. Ing. Ján Brindza PhD., Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Institute of Biological Conservation and Biosafety, Trieda Andrey Hlinku 2, 949 76 Nitra, Slovakia. E-mail: Jan.Brindza@uniag.sk.

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THE CONTENT OF MERCURY IN VARIOUS TYPES OF CEREALS GROWN IN THE MODEL CONDITIONS

Ľuboš Harangozo, Miriama Kopernická, JanneteMusilová, Pavol Trebichalský

ABSTRACT

The consumption of cereals in Slovakia but also worldwide is increasing by every year. From 30000 to 50000 tons of mercury circulates throught the biosphere that gets into the atmosphere degassing of the earth's crust and world oceans. Mercury affects CNS and causes its disorders. The high doses of mercury causes a lot of different changes of personality as well as increased agitation, memory lossorinsomnia. It can also affect other organ systems such as the kidney. The exposure level is reflected in the concentration of mercury in blood and urine. The aim of our work was the evaluation of transfer of mercury from sludge to edible part of chosen cereals. The objectives were achieved in simulated conditions of growing pot experiment. We used agricultural soil from the location of Výčapy – Opatovce for the realization of the experiment. The sludge, which was added atvarious doses, was taken from Central Spiš area from locality of Rudňany near the village where minedironore that contains mainly copperand mercury during last few decades was. We used three types of cereals: barley (*Hordeum sativum L.*) variety PRESTIGE, spring wheat (*Triticum aestivum L.*) variety ISJARISSA and oat (*Avena sativa L.*) variety TATRAN. The length of growing season was 90 days. From the obtained results of two years can be concluded that the accumulation of mercury by seed follows wheat < barley < oat. Even though that the oat is characterized by the highest accumulation of mercuryin the seeds, the content did not exceed the maximum level sspecified by The Codex Alimentarius of Slovak Republic. The results shows that the suitable cultivation of the cereals in localities, which are contaminated with heavy metals, especially by mercury, that the high content of mercury in soil do not pose a risk of accumulation of the metal into the cereal grain.

Keywords: cereals; plants; mercury; heavy metals

INTRODUCTION

The cereals are probably the most important source of food for humans and feed foranimals. Consequently, the low level of contamination can affect the health of consumers. Chemical contamination can occur from growing of cereals to their processing and storage **(Alldrick, 2012)**.

The cereals are the most common crops that are grown on arable land of EU. The fifty percent of cereal production in Southern Europe consists of wheat and than barley and maize. Other cereals as oat and rye are grown to a limited amounth **(Finch et al., 2014)**. The consumption of cereals in Slovakia but also worldwide is increasing by every year. The cereals are particularly very important for its nutritional value. The opinion of many experts is that cereals should constitute from 40 to 60 percent of well - balanced diet. Cereals provide most of the calories and proteins consumed worldwide. The current annual world production is more than 2.5 billion tons. This out put is either directly channeled to the food industry or used as animal feed to provide meats, dairy, and poultry products. Among cereals, rice, wheat, and maize yield approximately 89% ofthe total production and constitute the main stay of practically all cultures. The otherless important cereals are barley, oats, sorghum, rye, triticale, and millets. All cereals are strachy foods and contain protein that does not meet the essential aminoacid balance required by growing infants. They are considered a good source of energy, most B vitamins, and dietary fiber when consumed as whole grains **(Serna Saldivar, 2016)**.

The cereals and cereal products are the main sources of carbohydrates in food for humans and feed for animals. Cereal grains are an important source of energy and nutrients in the form of protein, fat, fiber, minerals and vitamins **(Beverly, 2014)**.

The cereals are as well as the most important source of fructans in our daily diet. Nowadays are hotly discussed and compared a lot of different cereals in the terms of fructans structure. Their degradation during processing of food is considered as a potential health benefit. Recent published data suggest that they may also have a prebiotic effect **(Verspreeta et al., 2015)**.

The cereals and cereal bran obtained a significant position as a functional food. They are a source of carbohydrates (arabinoxylan, beta – glucan), phenolic acids (ferulic acid), flavonoids (anthocyanins), oil (γ - oryzanol), vitamins, carotenoids, folates and sterols. Their physico – chemical properties makes them a necesssary ingredient for food fortification. The bran of rice, wheat, oat, barley, millet, rye and corn contain a huge

amount of health-promoting ingredients. The anti-atherogenic, anti-hypertensive and hypoglycemic properties were verified. Further, it was found the effect against oxidative stress. They reduce insulin resistance, prevent the risk of obesity by inducing the feel of fullness **(Patel, 2015)**.

The importance of fiber as a part of a well - balanced diet, has been known for a decades. Soluble fiber such as β-glucan has a significant glycemic effect. The cereals, especially barley and oat are a perfect source of these functional components. Current research suggests that the efficacy of the beta-glucans is also appreciable in the immune system. They have a positive effect as prebiotics **(Koutinas et al., 2014)**. Recent large-scale epidemiological studies have shown that regular consumption of whole grain cereals can reduce the risk of heart disease and certain cancers by 30 percent. One of the factors that increase the functionality of foods is theso-called in digestible resistant starch **(Duchoňová and Šturdík, 2010)**.

The most common toxic heavy metals include Hg, Cd and Pb. The current state of the environment significantly influences gene pool of plants and animals and through food chain and population health and animals **(Cimboláková and Nováková, 2009)**.

Food consumption has been identified as a major source of contaminants income. There are a lot of elements which have a pathological effect on the organism, and are characterized by high toxicity. These contaminants are mercury and arsenic too **(Melo et al., 2008)**. Lead, mercury and cadmium are the elements which have a harmfule ffects on the central nervous system in the development of the child **(Kippler et al., 2012)**. Mercury, as well as other trace elements, moves between different media (i.e. atmospheric aerosol, dust, soil, plants, sediment, in the gas phase, in aqueous solution and solids **(Charlesworth et al., 2011)**.

Mercury mining and its use in products continue to the present. Consumer products containing mercury are batteries, fluorescent lamps, and some cosmetics **(McKelvey et al., 2011; Streetsetal, 2011)**. Mercury and its compounds are toxic to humans and the environment. Mercury is found in various chemical forms and is able to cause a wide variety of clinical effects **(Bernhoft, 2012)**.

The toxicity of mercury and its compounds to humans, such as a taxy, narrow vision, hearing loss and death were firstly described in 1865 **(Grandjean et al., 2010)**. Man receives the highest concentrations of mercury through the food chain and the largest sourceof food consists of animal origin **(Tóth et al., 2012)**.

The high doses of mercury can be fatal to humans, but even relatively low doses can have a serious effect on the nervous system and the development. Nowadays it has been disscused a lot about the harmful effectson the cardiovascular, immune and reproductive systems. Mercury also slow down microbiological activity in soil and under the regulation of classification of ground and surface water is one of the most hazardous substances to health. Mercury is persistent and can change the environment into methylmercury, the most toxic form. The phytotoxicity of mercury depends on its form and sorption. Elemental mercury is a potential source of highly toxic gases. Plants possess different degrees of tolerance of mercury **(Samešová, 2012)**. The plants may be exposed to either direct effect of mercury as antifungal agents, particularly through the crop seed treatment or foliar spray or by an accident. The exposure to mercury may occur through soil, water and air pollution.The concentration of mercury in above – ground parts of plants depends largely on foliar uptake Hg^0 volatilisation from the soil. Wilde dible fungiare characterized by high bioaccumulative ability – they are able to take from substrate and then aggregated up to several tens of its concentrationin soil **(Árvay et al., 2014; Árvay et al., 2015)**.

The factors that are affecting the accumulation of mercury by plants are organic matter contentin the soil or sediments, organic carbon content, redox potential and total metal content. Generally, mercury up takein plants could be related to the degree of soil pollution **(Patra and Sharma, 2000, Tomáš et al., 2012, Árvay et al., 2013; Tomáš et al., 2014)**.

MATERIAL AND METHODOLOGY

The aim of our work was the evaluation of transfer of mercury from sludge to edible part of chosen cereals. The objectives were achieved in simulated conditions of growing pot experiment. We used agricultural soil from the location of Výčapy – Opatovce for the realization of the experiment. The sludge, which was added at various doses, was taken from Central Spiš area from locality of Rudňany near the village where was mined iron ore that contains mainly copperand mercury during last few decades. We used three types ofcereals: barley (*Hordeum sativum L.*) variety PRESTIGE, spring wheat (*Triticum aestivum L.*), variety ISJARISSA and oat (*Avena sativa L.*) variety TATRAN. The length of growing season was 90 days.

Before the establishment growing pot experiment we performed all necessary analyses in soil and sludge. We determined soil reaction, content of nitrogen by Kjeldahl method, phosphorus content, potassium and magnesium contentby Mehlich II solution. Subsequently, we determined the content of heavy metals in the acid mixture HNO_3 and HCl (decomposition by aqua regia) by AA Swith Varian AA240FS (Australia). For analysis of each elelment, we used the multi-element standard SigmaAldrich (Germany).

To every one of all tested pots was weighed 5 kg of soil with 1 kg of silica sand, while the bottom of the container was filled with a small drainage layer of gravel. In each pot was applied the calculated dose of sludge.

Crop shave been harvested at full maturity time and after drying were assessed by mercury by AAS for AMA254 (Czech Republic). Seed samples are analyzed directly without modification.

For statistical evaluation of obtained results was used a statistical program STATISTICA 6.0 Cz. Wetested the resultson the level of descriptive statistical evaluation, and overall visual indication of the level factor, variability and the deviation was expressed in text. For statistical evaluation we used T-test at the level $p \leq 0.05$.

RESULTS AND DISCUSSION

Soil from the locality of Výčapy – Opatovce has analcalic soil reaction with medium level of humidity. It is characterized by good content of phosphorus and potassium and a high content of magnesium.The contents of heavy metals do not exceed the limit values **(Act No. 220/2004 Coll.)**.

Sludge from the locality of Rudňany has a strongly alkaline soil reaction. It is characterized by a very low content of phosphorus and potassium. Mercury content (57.81 $mg.kg^{-1}$) exceeds the maximum permissible amount by 5.78 times **(Act No. 188/2003 Coll.)**.

Mercury content in barley seeds in D variant has increased by 7.9 times in 2013 and 14.2 times in 2014 compared to variant A (soil without addition of sludge). Escalating amount of sludge added to the soilis proportionally reflected in mercury content in the seeds of barley. In 2013 the mercury content in the seeds of barley was almost a half lower than in 2014.

Mercury contentin barley seeds in all variants exceeded the maximum permissible amount specified by The Codex Alimentarius of Slovak republic (CA SR).

Statically significant difference between 2013 and 2014 in mercury content in the seeds of barley was obtained in the variant D. In other variants was not statistically significant difference.

The highest mercury content of seeds of wheat was obtained in the variant D in 2014 where the Hg content in the seeds was higher by 5.7 times than in variant A. The differences between the mercury content of variants C and D was not as significant as in the case of barley. The difference between the highest mercury content of seeds of wheat in variant D in the year 2013 and 2014 had not a

Table 1 Variants of the experiments.

variants	
A	soil 100%
B	soil 90%, sludge 10%
C	soil 80%, sludge 20%
D	soil 70%, sludge 30%

Table 2 The content of microelements in the soil.

MEHLICH II ($mg.kg^{-1}$)			
K	**Ca**	**Mg**	**P**
287.5	6948.0	392.0	587.5

Table 3 The contents of heavy metals in the soil (decomposition by aqua regia) and comparison of **Act No. 220/2004 Coll. of Slovak Republic** ($mg.kg^{-1}$).

	Cd	**Pb**	**Cu**	**Zn**	**Cr**	**Ni**
soil	0.70	18.2	18.4	55.6	17.2	30.6
Act No. 220/2004	0.70	70	60	150	70	50

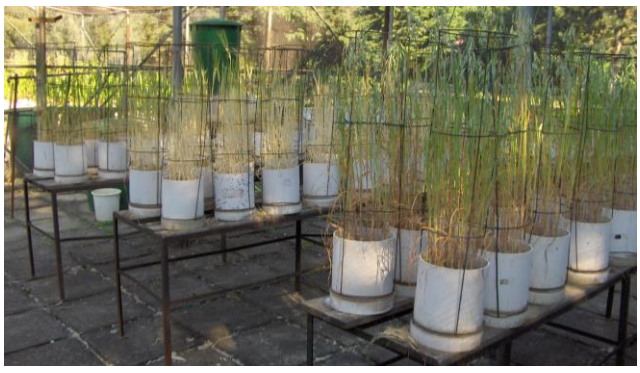

Figure 1 Simulated conditions of growing pot experiment.

Table 5 Mercury content in the seeds of wheat variety Jarissa in 2013 and 2014 ($mg.kg^{-1}$) and the comparison of mercury content with Codex Alimentarius of Slovak republic (CA SR).

	WHEAT	
variants	**2013**	**2014**
A	0.001821	0.001395
B	0.003241	0.001866
C	0.006582	0.004729
D	0.006809	0.007966
CA SR	0.05	0.05

Table 6 Mercury contentin the seeds of oat variety Tatran in 2013 and 2014 ($mg.kg^{-1}$) and the comparison of mercury content with Codex Alimentarius of SR (CA SR) .

	OAT	
variants	**2013**	**2014**
A	0.002971	0.002288
B	0.007252	0.003753
C	0.009468	0.010078
D	0.019722	0.040756
CA SR	0.05	0.05

high level of significance.

Mercury content in wheat seeds in all variants was not exceeded the maximum permissible amount specified by Codex Alimentarius of SR (CA SR) .

Regarding the differences in mercury content in wheat seeds was not obtained statistically significant difference beetween different years.

The largest in take of mercury was obtained in oat seeds. Mercury contentin variant D (maximum addition of sludge) was higher by 17.8 times than in variant A in 2014. In 2013 the mercury content in the seeds of oat was almost a half lower than in 2014. The most significant increase in mercury conten tin the seeds of the oat option C and D was recorded in 2014.

Although oat is characterized by the highest accumulation of mercury in the seeds, the content was not exceeded the maximum permissible amounts specified by Codex Alimentarius of SR (CA SR).

Statically significant difference between 2013 and 2014 in mercury contentin the seeds of oat was obtained in the variants B and D.

Bajčan et al., (2010) measured mercury concentrations in the samples taken agricultural crops grown on all uvial soils in the region of Hont. Hg content in the grains wer in a range of less than 0.0001 mg.kg-1 to 0.0198 1 $mg.kg^{-1}$, what is significantly less mercury as maximum allowable limit for Food (0.05 $mg.kg^{-1}$).

Hg content corresponds to the content in the soil, the greater Hg content in thes oil, the higher the Hg content in the grain cereals. The highest Hg content in the grain weset in the grain of the barley from area Markušovce and 0.2006 $mg.kg^{-1}$, what It represents four times the limit value **(Šabo, 2013).**

In general, the acceptability of the soil for the plants Hg low, tending accumulation in the roots, but the aerial parts of the plants absorbed from the atmosphere directly Hg. To of total Hg in plants has, according to some authors direct deposition up to 90% share. The natural average concentration of Hg in the plants are moved between 0.005 - 0.17 mg of Hg. kg^{-1}, with values of 1 - 3 mg $Hg.kg^{-1}$ are considered phytotoxic **(Toman et al., 2000).**

The following figures show the comparison of the mercury contentin the seeds of commodities in different variants.

Figure 2 shows that the mercury content of the seeds of each cereal in variants B, C and D increases with increasing addition of sludge intot he soil. The smallest storage capacity of mercury was recorded in spring wheat variety ISJARISSA. In a variant D was 0.006809 $mg.kg^{-1}$ of mercury content, which represents almost a half of the amount that has been accumulated by seeds of barley and by 3 times smaller than accumulated amount in the case of seeds of oat in the same variant.

Figure 3 shows a similar situation as Figure 2, thus increasing doses of sludge into the soil affected also by increased mercury content in the seeds of varieties of all crops.The results obtained from the two years can therefore say that in terms of accumulation of mercury seeds equence is as follows wheat < barley < oat.

Increased mercury content in soil in each variant due to the addition of sludge had a statistically significant effect on mercury content in the seeds of all variants ata significance level of $p<0.05$.

For all crops the additions of sludge into the soil have a statistically significant effect on mercury content in different variants ata significance level of $p <0.05$.

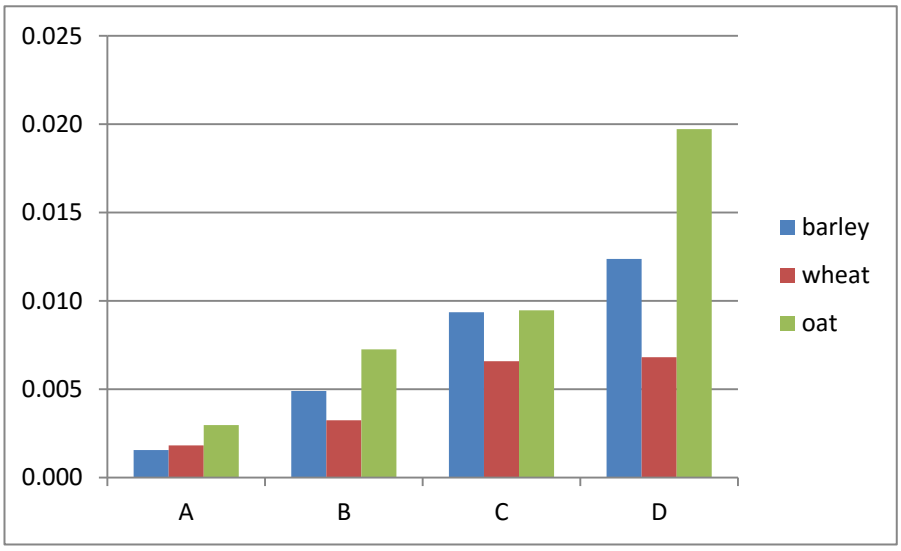

Figure 2 Mercury content in the seeds of various cereals in all variants (mg.kg^{-1}) that were grown in the year 2013.

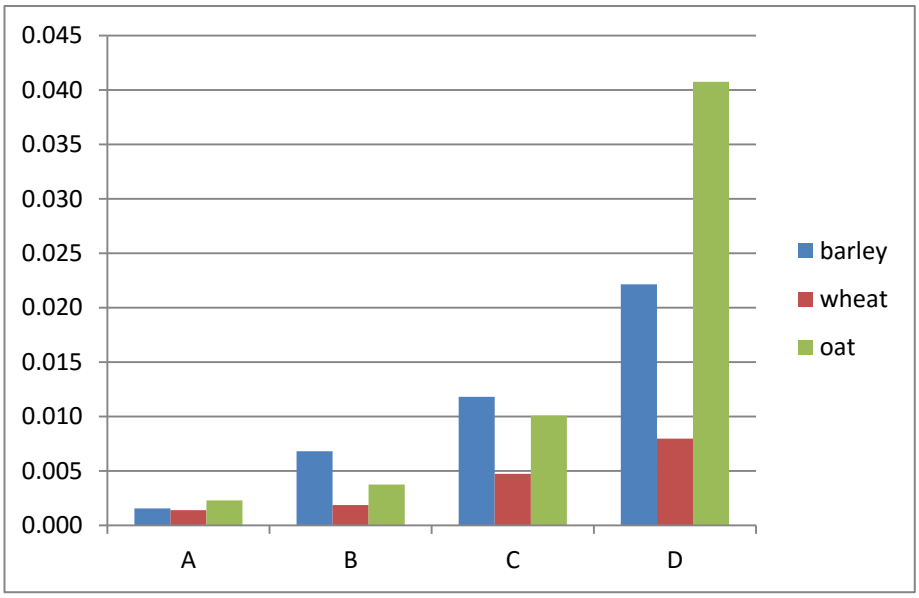

Figure 3 Mercury content in the seeds of various cereals in all variants (mg.kg^{-1}) that were grown in the year 2014.

CONCLUSION

The results showed that the amount of sludge added in specified amounts into the soil increases mercury content in seeds of crops.

Although oat was characterized by the highest accumulation of mercury in the seeds, the content was not exceeded the maximum permissible amount specified by Codex Alimentarius of SR.

From the obtained results of two years can be concluded that the accumulation of mercury by seed follows wheat < barley < oat.

The results showed that the suitable cultivation of the cereals in localities, which are contaminated with heavy metals, especially by mercury, that the high content of mercury in soil do not pose a risk of accumulation of the metal into the cereal grain.

Increasing number of toxic metals in soil leads to an increased content of the emetals in crops and subsequently in animal products. This may have adverse effects on people who consume these products.

There are two main reasons why the contamination of the environment with heavy metals causes concern. First, it can reduce the productivity of plants used as human food and animal feed. Second, it affects the quality of agricultural products.

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Contact address:
Ľuboš Harangozo, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: harangozolubos@gmail.com.
Miriama Kopernická, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: m.kopernicka@gmail.com.
Jannete Musilová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jannete. musilova@uniag.sk.
Pavol Trebichalský, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: palotre@atlas.sk.

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HEAVY METALS DETERMINATION IN EDIBLE WILD MUSHROOMS GROWING IN FORMER MINING AREA – SLOVAKIA: HEALTH RISK ASSESSMENT

Zuzana Záhorcová, Július Árvay, Martin Hauptvogl, Ján Tomáš, Ľuboš Harangozo

ABSTRACT

The aim of the paper is to assess a contamination level of forest substrates and aboveground parts of edible wild mushroom (*M. procera* (Scop.) Singer, *B. recitulatus* Schaeff., *C. cibarius* Fr., *S. grevillei* (Klotzsch) Singer, *A. campestris* L., *R. xerampelina* (Schaeff.) Fr., *L. salmonicolor* R. Heim & Leclair, *C. gibba* (Pers. Ex Fr.) Kumm., *X. chrysenteron* (Bull.) Quél., *M. oreades* (Bolton) Fr.; n = 70) by heavy metals (Cd, Cu, Pb and Zn). The studied location was a broader surroundigs of the historical mining and metal processing area of Banská Bystrica. The collected mushroom samples and underlying substrate samples were analysed using Flame Atomic Absorption Spectrofotometry and Flame Absorption Spectrophotometry with graphite furnace. Bioaccumulation factors (BAF) for individual species and their anatomical parts were calculated from the results obtained. In order to assess a health risk resulting from regular consumption of the mushrooms, provisional tolerable weekly intake (PTWI) was calculated from the results of the monitored heavy metal concentration. Limit values for the studied contaminants (Cd: 0.49 $mg.kg^{-1}$ and Pb: 1.75 $mg.kg^{-1}$ for an individual with an average weight of 70 kg) are defined by FAO and WHO. Our results indicate that *S. grevillei* has a high bioaccumulation ability of Cd. It was confirmed by bioaccumulation factors (BAF_H = 3.47 and BAF_{RFB} = 2.30). The $PTWI_{Cd}$ value was exceeded by 4.11 times. A similar situation occurred in the case of Pb where the highest bioaccumulation factor (BAF_H = 0.24 and BAF_{RFB} = 0.19) was also recorded in the samples of *S. grevillei* and the $PTWI_{Pb}$ value was exceeded by 1.35 times. In general, it can be stated that a consumption of edible wild mushrooms represent a relatively small risk of negative impact on the health of consumers.

Keywords: edible wild mushroom; heavy metal; contamination; bioaccumulation; health risk assessment; Slovakia

INTRODUCTION

Heavy metals are ubiquitous environmental components, the origin of which is natural or anthropogenic **(Jiang et al., 2006; Feng et al., 2003)**. Environmental contamination with heavy metals is increasingly coming to the fore and it is one of the most serious problems of modern society nowadays. Their riskiness arise from the substantial persistence, toxicity and ability to bioaccumulate into environmental components and consequently into the food chain **(Burges et al., 2015; Douay et al., 2013; Roman and Popiela, 2011)**. Long-time industrialization of society and subsequent rapid urbanization lead to an increased amount of xenobiotics and thus also heavy metals in the urban environment **(Szolnoki et al., 2013; Luo et al., 2012)** but also in non-urban areas **(Luo et al., 2014)**, which represents a significant risk to the global ecosystem and the health of human populations **(Siciliano et al., 2009)**.

Some heavy metals (Hg, Cd, Cr, Ni, Pb), arsenic and esential trace elements (Cu, Zn) pose a significant risk to the quality of the environment, which influences on the health of the human population **(Alloway, 2013; Jomová and Valko, 2011)**. They enter the environment via natural activities (volcanic activity, weathering, etc.) and anthropogenic activities (e. g. extraction and processing of minerals, combustion of fossil fuels and waste, etc.). **(Hooda, 2010)**. Cadmium and lead belong to non-essential trace elements and are classified as toxic metals that are harmful to plants, animals and human body even at very low concentrations. They are introduced to the body mostly by inhalation and/or resorption and consequently damage individual systems of the human body **(Timorácká et al., 2011; Silva et al., 2003)**. However, high amounts of the heavy metals can get into the body also by food. Zinc and copper are classified as essential trace elements **(Wuan and Okieimen, 2011; John et al., 2010)**, however they can be toxic to humans in higher concentrations **(Licata et al., 2012)**. They participate in the regulation of various physiological functions, including inflammatory and oxidative processes **(Mocchegiani et al., 2012; Malavolta et al., 2010)**. For example, increased concentration of copper has adverse effects on the activity of the central nervous system and certain physiological processes **(Grandner et al., 2013; Cappuccio et al., 2011)**.

Edible wild mushrooms represent a natural part of forest ecosystems and play an important role in the cyclic pathways of elements and organic matter **(Petkovšek and Pokorny, 2013)**. They are able, together with micro-organisms, to biodegrade substrate and thus utilize waste from agricultural production and/or human activities

(Ouzouni et al., 2009). Some mushroom species are considered as a delicacy in many countries, including countries of Central and Eastern Europe. Fruiting bodies of the mushrooms are popular not only for their texture and flavor, but also for their nutritional properties **(Cheung, 2013; Kalač, 2013)**. They are characterized by low energy value and high concentration of essential biologically valuable elements, specific β-glucans and antioxidant substances **(Kalač, 2013; Kalač, 2009)**. Moreover, they provide a valuable source of fiber, vitamins and minerals such as thiamin, riboflavin, vitamin D, potassium, phosphorus, iron and calcium **(Wang et al., 2014; Falandysz and Borovicka, 2013)**. It has been known for long time that mushrooms are able to accumulate large amounts of heavy metals **(Zhang et al., 2008)**, what makes them ideal for biomonitoring of environmental pollution – particularly contamination of forest ecosystems **(Radulescu et al., 2010)**. There are many factors that influence the presence of metals in mushrooms, for example climate, environmental conditions and concentration of macromolecules in the cell wall of each specific species **(Ostos et al., 2015)**. Studies of the interaction of heavy metals in the system soil/substrate – mycelium showed that mushrooms have several fold higher bioaccumulation capacity to uptake xenobiotics – heavy metals from the substrate compared to higher plants (intake from the atmosphere is negligible) **(Falandysz, 2015; Saba et al., 2015; Zhu et al., 2011; Gursoy et al., 2009)**.

Under natural conditions, the concentration of heavy metals in certain species of edible mushrooms can be higher, even if the soil contamination level is low **(Falandysz et al., 2003)**. The highest concentrations of trace elements are mostly found in the hymenophore, lower values are in the spores and the lowest values are in the stem **(Árvay et al., 2015a; Krasińska and Falandysz, 2015; Falandysz et al., 2007; Alonso et al., 2003)**.

The aim of the paper is to determine the level of transition of the studied heavy metals (Cd, Cu, Pb and Zn) from the substrate into the aboveground parts of edible macroscopic mushrooms collected in the broader area of Banská Bystrica, which is characterized by historic mining and metalworking activity (mining and processing of ore rich in precious metals, copper, lead and associated components: mercury, cadmium, etc.). Bioaccumulation factors (BAF) for individual anatomical parts of mushrooms (hymenophore - H and rest of fruit bodies - RFB) were calculated. Due to the popularity of collecting wild edible mushrooms in Central Europe **(Árvay et al., 2014; Kalač, 2009)**, a health risk arising from their regular consumption was investigated.

MATERIAL AND METHODOLOGY

Study area, sampling and pre-analytical procedure

For the needs of our work, 10 species of the most commonly collected wild mushrooms, which generally represent the most frequently collected mushrooms in Slovakia were chosen. The samples of edible wild mushrooms and substrate (N = 70) were collected in 2014 in the broader area of Banská Bystrica, in the cadastral areas of villages Ľubietová, Radvaň, Malachov, Selce, Nemce, Hrochoť and Podkonice that are characteristic by historical mining and metalworking activity. Identification of the sampling points was made using GPS coordinates (Figure 1). The concentration of heavy metals (Cd, Cu, Pb and Zn) was studied in individual parts of edible wild growing mushrooms. Studied species and their respective sampling frequencies are inculede in Table 1.

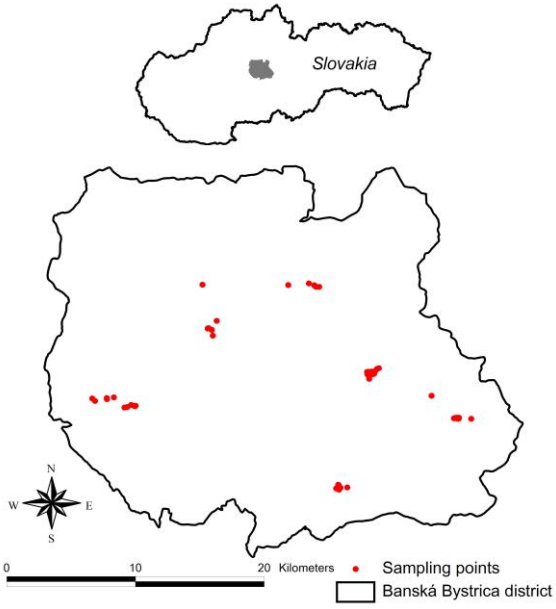

Figure 1 Map of the studied area with sampling points.

Table 1 The heavy metals cencentration (mg.kg^{-1} DM) in substrate.

Species*	N	Cd	Pb	Cu	Zn
		Median ±SD (range)			
Macrolepiota procera (Scop.) Singer	11	2.43 ±0.80 (0.58 – 3.66)	45.3 ±15.7 (28.5 – 88.8)	23.3 ±16.0 (10.0 – 66.9)	97.8 ±31.0 (58.6 – 155)
Boletus reticulatus Schaeff.	9	2.89 ±1.60 (1.76 – 6.94)	49.4 ±12.1 (36.6 – 76.6)	19.4 ±9.0 (11.6 – 38.5)	122 ±66.9 (59.4 – 278)
Cantharellus cibarius Fr.	3	2.68 ±0.60 (2.24 – 3.37)	47.2 ±3.15 (43.7 – 49.8)	20.3 ±8.02 (13.0 – 28.9)	112 ±24.1 (95.2 – 139)
Suillus grevillei (Klotzsch) Singer	11	2.49 ±0.68 (1.66 – 3.51)	41.9 ±16.2 (25.3 – 75.1)	18.1 ±9.91 (3.10 – 38.8)	41.9 ±16.2 (25.3 – 75.1)
Agaricus campestris L.	3	2.74 ±0.19 (2.53 – 2.90)	49.8 ±25.6 (34.4 – 79.3)	31.1 ±17.0 (14.8 – 48.7)	155 ±65.5 (97.0 – 226)
Russula xerampelina (Schaeff.) Fr.	8	2.44 ±0.54 (1.72 – 3.40)	58.2 ±14.5 (41.4 – 76.6)	27.0 ±11.9 (11.3 – 48.1)	58.2 ±14.5 (41.4 – 76.6)
Lactarius salmonicolor R. Heim & Leclair	10	2.82 ±0.41 (2.25 – 3.50)	61.1 ±23.9 (42.7 – 113)	23.2 ±8.84 (13.0 – 43.1)	101 ±24.4 (70.7 – 141)
Clitocybe gibba (Pers. Ex Fr.) Kumm.	3	2.52 ±0.13 (2.34 – 2.66)	43.5 ±4.65 (37.8 – 49.2)	19.8 ±11.7 (11.2 – 36.4)	108 ±28.7 (67.9 – 131)
Xerocomus chrysenteron (Bull.) Quél.	7	2.03 ±0.38 (1.37 – 2.38)	39.3 ±5.23 (31.2 – 47.1)	18.7 ±6.49 (8.40 – 25.8)	39.3 ±5.23 (31.2 – 47.1)
Marasmius oreades (Bolton) Fr.	5	2.22 ±0.43 (1.81 – 2.94)	59.1 ±11.5 (44.1 – 73.6)	25.5 ±10.1 (16.2 – 38.6)	144 ±58.9 (84.9 – 241)

N, number of samples; **SD**, standard deviation; ***Index fungorum (2015)**

Organic and inorganic debris was removed mechanically by ceramic knife and the cap (hymenophore) was separated from the rest of fruit body immediately after collecting of the mushroom samples. Later, the samples were sliced and dried at 45 °C to constant weight. The dried samples were homogenized in a porcelain mortar and then stored in polyethylene bags. After the collection of the mushroom samples, substrate samples were taken from the same spot to a depth of 10 cm. In the laboratory, the substrate samples were dried to a constant weight, and afterwards they were sieved through a sieve with mesh width of 2 mm.

One gram (1 g.) of dried mushroom samples (accuracy to 4 decimal places) were mineralized by 5 cm^3 of concentrated HNO_3 (Merck, Germany) and the same volume of deionized water using microwave mineralization system in MARS X-press 5 (CEM, USA). Afterwards, the sample was filtered through filter paper 390 Filtrak (Munktell, Germany) and filled with deionized water to 50 cm^3. The substrate samples were mineralized the same way as the mushroom samples in the mixtures of HNO_3 and HCl (Merck, Germany) in the ratio 1:1. After the mineralization, the digest was filtered through filter paper 390 Filtrak (Munktell, Germany) and diluted with deionized water to a total volume of 100 cm^3 **(Árvay et al., 2015b; Árvay et al., 2014)**.

Analytical procedure

Quantitative determination of the concentration of the studied trace elements (Cd, Cu, Pb, Zn) was carried out in the mineralized samples by flame atomic absorption spectrometry (F-AAS) in Varian AA 240 FS apparatus (Varian, Australia), by method published in **Árvay et al. (2014)**.

Statistical analysis and risk assessment

All data on the concentration of the studied contaminants in the samples were processed by descriptive statistical analysis at the level of the minimum and maximum values, median values and standard deviation in Statistica 12 software (StatSoft, USA).

Due to the popularity of the collection and subsequent consumption of edible wild mushrooms in Slovakia **(Árvay et al., 2015a; Árvay et al., 2015b; Árvay et al., 2014; Kalač, 2009)**, tolerable weekly intake (PTWI) was calculated, based on the data obtained on the concentration of the studied heavy metals, for a standardized person weighing 70 kg with a consumption of 300 g of fresh edible wild mushrooms per day. The parameter is defined by FAO/WHO (1993) for cadmium and lead separately. The value for cadmium is 0.007 mgkg^{-1} of body weight of a consumer. The value for lead is 0.025 mg.kg^{-1} **(JECFA, 2010; WHO, 1993)**. The legislation does not state PTWI values for the zinc and copper. Due to the high water concentration (which is dependent on weather conditions), generally accepted value of 90% was used for conversion of the water concentration in the mushroom samples **(Kalač, 2009)**.

RESULTS AND DISCUSSION

Table 2 The heavy metals concentration in hymenophore (mg.kg^{-1} DM) and hymenophore and rest of fruit bodies bioaccumulation factors.

Species		Cd	Pb	Cu	Zn
		Median ±SD (range)			
Macrolepiota procera (Scop.) Singer	H	3.98 ±6.48 (0.48-22.9)	6.46 ±3.38 (2.45-13.2)	81.1 ±56.4 (23.7-207)	106 ±63.6 (42.3-247)
	BAF_H	1.64	0.14	3.48	1.08
	BAF_{RFB}	1.34	0.11	2.44	0.83
Boletus reticulatus Schaeff.	H	5.08 ±7.14 (0.66-21.9)	6.58 ±3.63 (2.32-15.0)	57.5 ±34.3 (23.6-122)	226 ±157 (86.9-585)
	BAF_H	1.76	0.13	2.97	1.86
	BAF_{RFB}	1.44	0.11	2.21	1.51
Cantharellus cibarius Fr.	H	0.56 ±0.20 (0.39-0.78)	4.07 ±1.32 (2.55-4.90)	52.1 ±6.57 (47.5-59.6)	75.3 ±3.79 (71.6-79.2)
	BAF_H	0.21	0.09	2.56	0.67
	BAF_{RFB}	0.07	0.06	1.80	0.52
Suillus grevillei (Klotzsch) Singer	H	8.64 ±9.87 (1.72-29.7)	10.1 ±10.0 (1.33-30.0)	42.2 ±36.1 (12.8-138)	118 ±35.2 (71.7-174)
	BAF_H	3.47	0.24	2.33	1.35
	BAF_{RFB}	2.30	0.19	1.69	1.09
Agaricus campestris L.	H	1.44 ±1.12 (0.69-2.72)	6.20 ±5.08 (2.73-12.0)	43.1 ±20.6 (21.3-62.1)	113 ±51.8 (69.7-170)
	BAF_H	0.52	0.12	1.39	0.73
	BAF_{RFB}	0.41	0.09	1.25	0.51
Russula xerampelina (Schaeff.) Fr.	H	2.97 ±3.09 (0.86-10.2)	6.02 ±5.11 (0.97-13.1)	43.8 ±25.3 (15.1-96.5)	93.9 ±40.0 (51.9-182)
	BAF_H	1.22	0.10	1.62	0.84
	BAF_{RFB}	0.83	0.07	0.97	0.57
Lactarius salmonicolor R. Heim & Leclair	H	1.11 ±1.01 (0.50-3.47)	3.17 ±1.08 (1.50-5.67)	15.7 ±7.22 (8.30-31.8)	184 ±132 (45.6-419)
	BAF_H	0.39	0.05	0.67	1.83
	BAF_{RFB}	0.34	0.04	0.46	1.16
Clitocybe gibba (Pers. Ex Fr.) Kumm.	H	5.29 ±5.64 (1.91-11.8)	2.93 ±0.88 (1.93-3.62)	49.6 ±12.6 (37.9-62.9)	131 ±44.7 (83.1-171)
	BAF_H	2.10	0.07	2.50	1.21
	BAF_{RFB}	1.73	0.05	1.99	1.04
Xerocomus chrysenteron (Bull.) Quél.	H	1.84 ±0.78 (0.67-3.22)	6.77 ±3.76 (2.85-12.0)	35.8 ±15.7 (17.5-63.6)	177 ±132 (98.5-448)
	BAF_H	0.90	0.17	1.91	2.10
	BAF_{RFB}	0.71	0.14	1.09	1.61
Marasmius oreades (Bolton) Fr.	H	2.45 ±1.14 (1.08-3.86)	6.93 ±5.86 (1.81-16.9)	34.3 ±36.8 (4.59-96.0)	99.2 ±29.9 (72.6-145)
	BAF_H	1.10	0.12	1.35	0.69
	BAF_{RFB}	0.95	0.08	0.92	0.61

SD, standard deviation; **H**, hymenophore; **BAF_H**, bioaccumulation factor in hymenophore; **BAF_{RFB}**, bioaccumulation factor in rest of fruit bodies.

Heavy metals in the substrate samples

All concentrations of the studied contaminants in the samples of substrates and edible wild mushrooms are given per dry matter (DM). The concentrations of the studied contaminants in the substrate represent an important factor that influences the bioaccumulation ability of individual species of edible wild mushrooms. Therefore, a variable level of translocation of heavy metals into macroscopic mushrooms can be assumed **(Chudzyński *et al.*, 2011)**. The total concentration of the contaminants in the substrate varied within wide ranges (Table 1). The total cadmium concentration in the substrate samples (N = 70) ranged from 0.58 to 6.94 mg.kg^{-1} DM, with the highest concentrations (6.94 mg.kg^{-1} DM) recorded in the substrate samples of *B. recitulatus* (N = 9). The total concentration of lead in the substrate samples (N= 70) ranged between 25.3 – 113 mg.kg^{-1} DM and the highest concentration (113 mg.kg^{-1} DM) was recorded in the substrate samples of *L. salmonicolor* (N = 10). The copper concentration in the samples (N = 70) ranged between 3.10 – 66.9 mg.kg^{-1} DM. The highest concentration (66.9 mg.kg^{-1} DM) was recorded in the substrate samples of *M. procera* (N = 11). The last studied element was zinc, the concentration of which ranged from 25.3 – 278 mg.kg^{-1} DM in all samples, with the highest concentrations (278 mg.kg^{-1}) recorded in the substrate

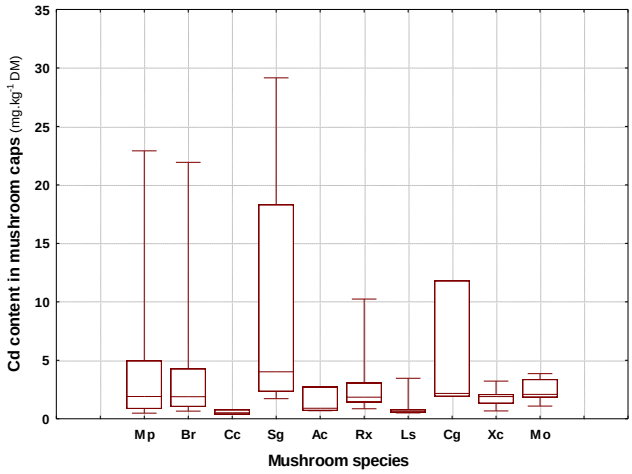

Figure 2 Range (min. – max.) and median, upper and lower quantile values of the cadmium concentration in the caps (mg.kg^{-1} DM) of individual species of edible wild mushrooms. ***MP,*** *M. procera*; ***BR,*** *B. recitulatus*; ***CC,*** *C. cibarius*; ***SG,*** *S. grevillei*; ***AC,*** *A. campestris*; ***RX,*** *R. xerampelina*; ***LS,*** *almonicolor*; ***CG,*** *C. gibba*; ***XC,*** *X. chrysenteron*; ***MO,*** *M. oreades*.

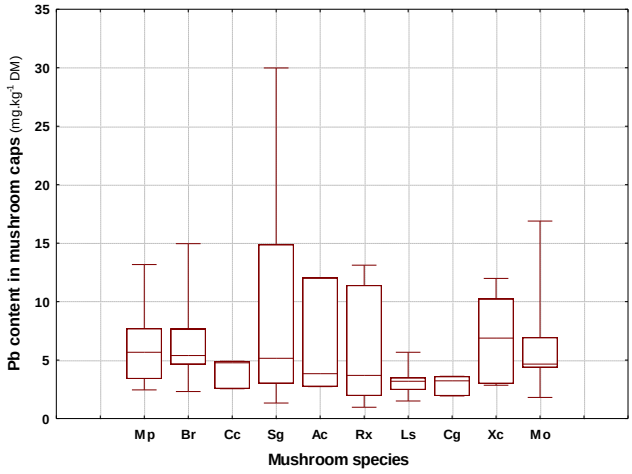

Figure 3 Range (min. – max.) and median, upper and lower quantile values of the lead concentration in the caps (mg.kg^{-1} DM) of individual species of edible wild mushrooms. ***MP,*** *M. procera*; ***BR,*** *B. recitulatus*; ***CC,*** *C. cibarius*; ***SG,*** *S. grevillei*; ***AC,*** *A. campestris*; ***RX,*** *R. xerampelina*; ***LS,*** *L. salmonicolor*; ***CG,*** *C. gibba*; ***XC,*** *X. chrysenteron*; ***MO,*** *M. oreades*.

samples of *B. recitulatus* (N = 9). High variability of the zinc concentration in the substrate indicates significant heterogenity of the zinc concentration in the studied sites.

Heavy metals in mushroom samples

Macroscopic mushrooms are considered to be an important bioaccumulator of xenobiotics (especially heavy metals) **(Árvay *et al.*, 2015a; Islam *et al.*, 2014)**, which was reflected in the concentration of the contaminants in individual anatomical parts of the studied mushroom species. The highest concentration of cadmium was recorded in the samples of *S. grevillei*, where the values in the hymenophore ranged from 8.64 ±9.87 mg.kg^{-1} DM (Figure 2). The ability of the species to bioaccumulate cadmium is the highest among all species (BAF_H = 3.47 and BAF_{RFB} = 2.30). It was confirmed by the findings of **Árvay *et al.*, (2014)**. The cadmium concentration in the hymenophore of individual species was in the following order: *S. grevillei* > *C. gibba* > *B. recitulatus* > *M. procera* > *R. xerampelina* > *M. oreades* > *X. chrysenteron* > *A. campestris* > *L. salmonicolor* > *C. cibarius*.

Similarly, in the case of the lead concentration the maximum values were recorded in the samples of *S. grevillei* (10.1 ±10.0 mg.kg^{-1} DM, 1.33 – 30.0 mg.kg^{-1} DM) (Figure 3). This species had also the highest bioaccumulation factor BAF_H = 0.24 and BAF_{RFB} = 0.19. The lead concentration in the hymenophore of individual species was in the following order: *S. grevillei* > *M. oreades* > *X. chrysenteron* > *B. recitulatus* > *M. procera* > *A. campestris* > *R. xerampelina* > *C. cibarius* > *L. salmonicolor* > *C. gibba*.

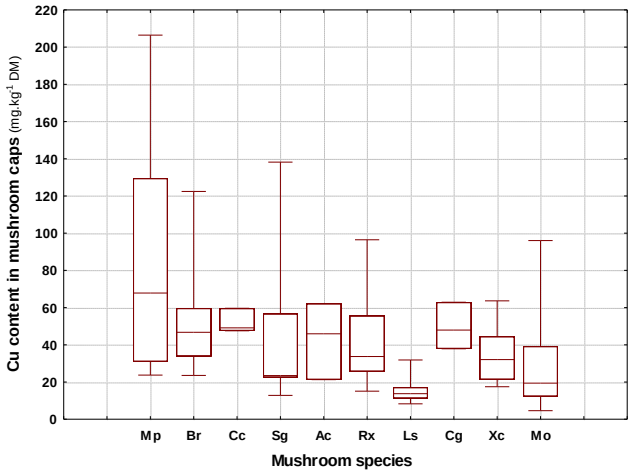

Figure 4 Range (min. – max.) and median, upper and lower quantile values of the copper concentration in the caps (mg.kg^{-1} DM) of individual species of edible wild mushr ooms. ***MP,*** *M. procera*; ***BR,*** *B. recitulatus*; ***CC,*** *C. cibarius*; ***SG,*** *S. grevillei*; ***AC,*** *A. campestris*; ***RX,*** *R. xerampelina*; ***LS,*** *L. salmonicolor*; ***CG,*** *C. gibba*; ***XC,*** *X. chrysenteron*; ***MO,*** *M. oreades*.

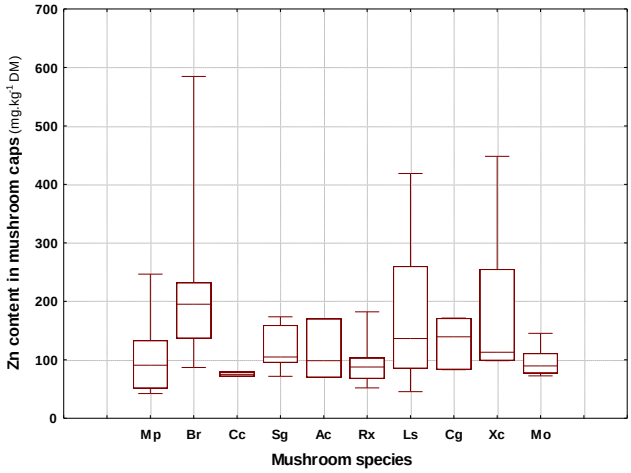

Figure 5 Range (min. – max.) and median, upper and lower quantile values of the zinc concentration in the caps (mg.kg^{-1} DM) of individual species of edible wild mushrooms. ***MP,*** *M. procera*; ***BR,*** *B. recitulatus*; ***CC,*** *C. cibarius*; ***SG,*** *S. grevillei*; ***AC,*** *A. campestris*; ***RX,*** *R. xerampelina*; ***LS,*** *L. salmonicolor*; ***CG,*** *C. gibba*; ***XC,*** *X. chrysenteron*; ***MO,*** *M. oreades*.

Although copper is considered an essential trace element for almost all organisms, its high levels may have a negative impact on physiological processes in the body **(Árvay *et al.*, 2014; Wuana and Okieimen, 2011)**. The highest copper concentration in the samples was recorded in the hymenophore samples of *A. procera* (Scop.) Singer, where the copper concentration was 81.1 ±56.4 mg.kg^{-1} DM (23.7 – 207 mg.kg^{-1} DM) (Figure 4). This species had the highest ability to bioacumulate copper among all species tested (BAF_H = 3.48 and BAF_{RFB} = 2.44). The concentration of copper in individual species was in the following order: *M. procera* > *B. recitulatus* > *C. cibarius* > *C. gibba* > *R. xerampelina* > *A. campestris* > *S. grevillei* > *X. chrysenteron* > *M. oreades* > *L. salmonicolor*.

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Zinc, like copper, is considered an essential trace element. Individual mushroom species showed significant ability to bioaccumulate zinc, with higher accumulation values on locations with lowest zinc cencentration in substrate. The highest zinc concentration was recorded in the samples of *B. recitulatus* Schaeff., with the concentration around 226 ±157 mg.kg^{-1} DM (86.9 – 585 mg.kg^{-1} DM) (Figure 5). The highest ability to bioaccumulate zinc was recorded in the samples of *X. chrysenteron* (BAF_H = 2.10 and BAF_{RFB} = 1.61). The zinc concentration in the hymenophore of individual mushroom species was in the following order: *B. recitulatus* > *L. salmonicolor* > *X. chrysenteron* > *C. gibba* > *S. grevillei* >

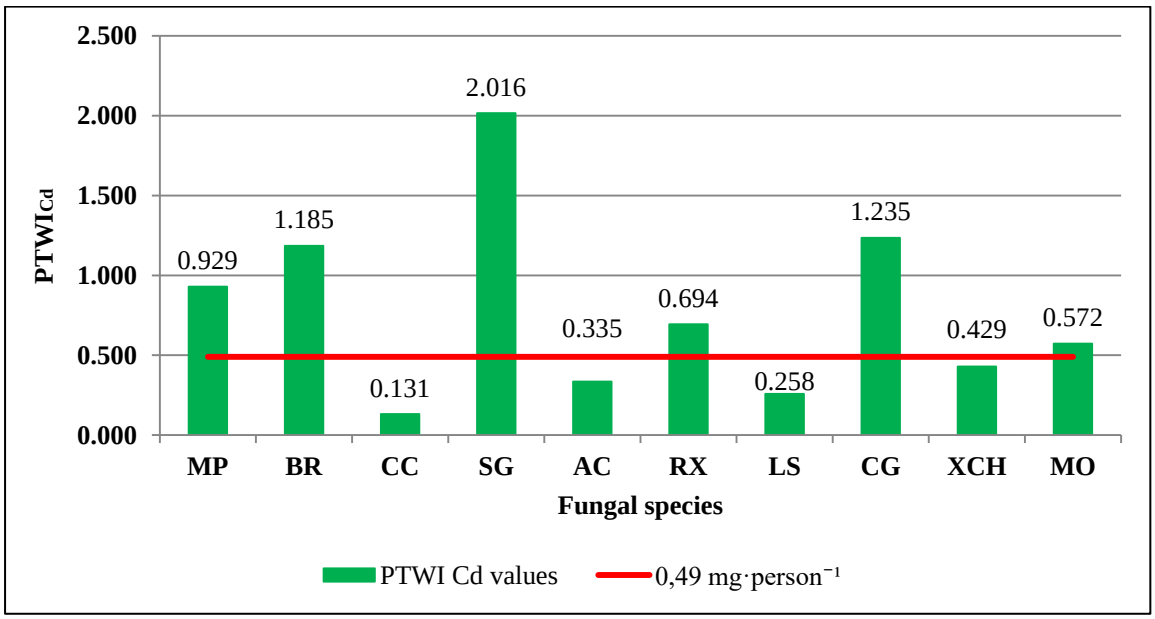

Figure 6 The comparison of weekly intake of cadmium with 300 g of various mushroom species per day to $PTWI_{Cd}$ limit for adult person (0.490 $mg.kg^{-1}$). ***MP,*** *M. procera*; ***BR,*** *B. recitulatus*; ***CC,*** *C. cibarius*; ***SG,*** *S. grevillei*; ***AC,*** *A. campestris*; ***RX,*** *R. xerampelina*; ***LS,*** *L. salmonicolor*; ***CG,*** *C. gibba*; ***XC,*** *X. chrysenteron*; ***MO,*** *M. oreades*.

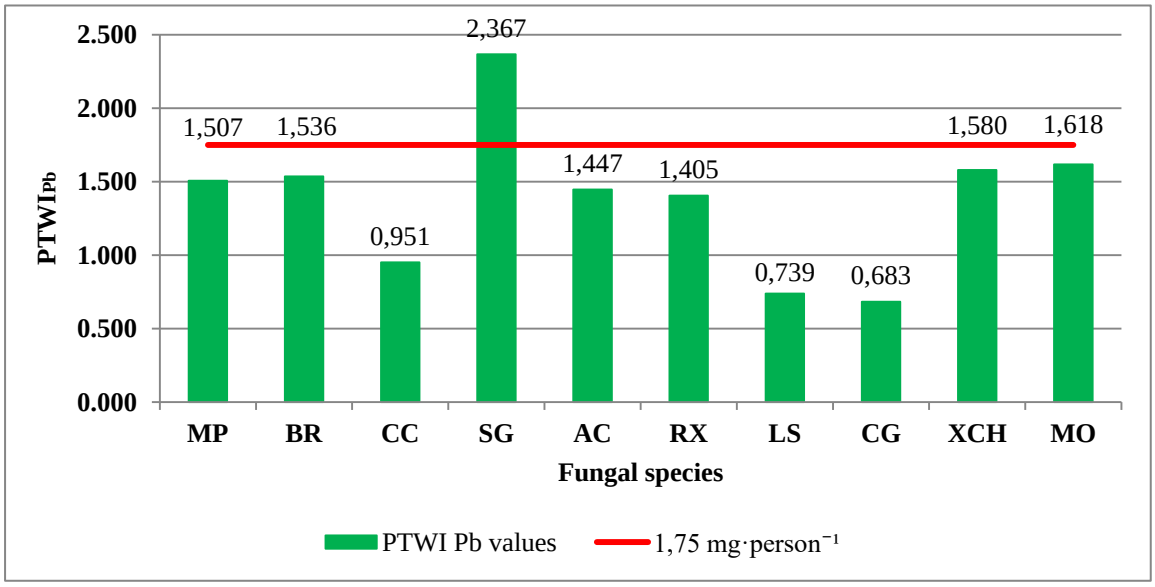

Figure 7 The comparison of weekly intake of lead with 300 g of various mushroom species per day to $PTWI_{Pb}$ limit for adult person (1.750 $mg.kg^{-1}$). ***MP,*** *M. procera*; ***BR,*** *B. recitulatus*; ***CC,*** *C. cibarius*; ***SG,*** *S. grevillei*; ***AC,*** *A. campestris*; ***RX,*** *R. xerampelina*; ***LS,*** *L. salmonicolor*; ***CG,*** *C. gibba*; ***XC,*** *X. chrysenteron*; ***MO,*** *M. oreades*.

A. campestris L. > *M. procera* > *M. oreades* > *R. xerampelina* > *C. cibarius*.

All data on the concentration of the studied contaminants in the substrate and individual anatomical parts of mushrooms are shown in Tables 1 and 2.

Health risk assessment

Provisional tolerable weekly intake (PTWI) is a value set by the FAO and WHO **(JECFA, 2010)** and defined as the maximum quantity of contaminants that may a consumer weighing 70 kg intake per one week. We assumed that the person consumes 300 g fresh mushrooms or 30 g of dried mushrooms per day. The legislation states the following PTWI indices for individual heavy metals: Cd: 0.007 $mg.kg^{-1}$ of bodyweight (0.490 mg $Cd.person^{-1}$) and Pb: 0.025 $mg.kg^{-1}$ of bodyweight (1.750 mg $Pb.person^{-1}$). For the evaluation of the PTWI values of the studied contaminants, their median concentration in the hymenophore were used. The median values were multiplied by the weight of 70 kg. The result was the maximum amount of the contaminants that a consumer can intake per week (Cd: 0.49 mg and Pb: 1.75 mg). The $PTWI_{Cd}$ values were exceeded in several samples. The highest exceedance was recorded in the samples of *S. grevillei* (4.11 fold). In the case of lead, the $PTWI_{Pb}$ values

were exceeded only in the samples of *S. grevillei* (1.35 fold). It indicates a potential risk of intoxication, since it is often collected and consumed species, characterized by significant bioaccumulation ability. The comparison of the calculated $PTWI_{Cd}$ and $PTWI_{Pb}$ values with the defined limit values are shown in Figures 6 and 7.

CONCLUSION

The aim of this study was to assess the contamination level of the substrate and the aboveground part of the edible wild mushroom species collected in the surrounding area of Banská Bystrica characterized by significant mining activity in the past. Macroscopic mushrooms represent a part of the environment that is sensitive to the increased amount of contaminants, which is reflected by their increased concentration in the aboveground parts of wild mushrooms. The results showed that the health risk resulting from the consumption of the studied mushroom species decreases as follows: *M. procera* (Cd) > *R. xerampelina* (Cd) > *S. grevillei* (Cd, Pb) > *B. recitulatus* (Cd, Pb) > *C. gibba* (Cd) > *M. oreades* (Cd, Pb) > *X. chrysenteron* (Cd, Pb) > *A. campestris* (Pb) > *L. salmonicolor* > *C. cibarius*.

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Contact address:

Zuzana Záhorcová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zuzana.zahorcova@uniag.sk.

Július Árvay, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: julius.arvay@gmail.com.

Martin Hauptvogl, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Sustainable development, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: martin.hauptvogl@uniag.sk.

Ján Tomáš, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jan.tomas@uniag.sk.

Ľuboš Harangozo, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: lubos.harangozo@uniag.sk

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REDISTRIBUTION OF MINERAL ELEMENTS IN WHEAT GRAIN WHEN APPLYING THE COMPLEX ENZYME PREPARATIONS BASED ON PHYTASE

Elena Kuznetsova, Ludmila Cherepnina, Svetlana Motyleva, Ján Brindza

ABSTRACT

Biogenic minerals play an important role in the whole human nutrition, but they are included in the grain of the phytates that reduces their bioavailability. Whole wheat bread is generally considered a healthy food, but the presence of mineral elements in it is insignificant, because of weak phytate degradation. From all sources of exogenous phytase the most productive are microscopic fungi. To accelerate the process of transition hard mineral elements are mobilized to implement integrated cellulolytic enzyme preparation based on the actions of phytase (producer is *Penicillium canescens*). Phytase activity was assessed indirectly by the rate of release of phosphate from the substrate. It has been established that the release rate of the phosphoric acid substrate is dependent on the composition of the drug and the enzyme complex is determined by the presence of xylanase. The presented experimental data shows that a cellulase treatment of the grain in conjunction with the β-glucanase or xylanase leading to an increase in phytase activity could be 1.4 – 2.3 times as compared with the individual enzymes. As a result of concerted action of enzymes complex preparation varies topography grain, increase the pore sizes in seed and fruit shells that facilitate the penetration of the enzyme phytase in the aleurone layer to the site of phytin hydrolysis and leads to an increase in phytase activity. In terms of rational parameters of enzymatic hydrolysis, the distribution of mineral elements in the anatomical parts of the grain after processing complex enzyme preparation with the help of X-ray detector EMF miniCup system in a scanning electron microscope JEOL JSM 6390 were investigated. When processing enzyme preparation wheat trend in the distribution of mineral elements, characteristic of grain - the proportion of these elements in the aleurone layer decreases, and in the endosperm increases. Because dietary fiber and phytate found together in the peripheral layers of fiber-rich grains, it is difficult to separate the effects of degradation processes nonstarch polysaccharides and fiberphytate redistribution of polyvalent metal ions. However, studies have shown that phytase - an effective mechanism for regulating mineral nutrient diet. Application of phytase in grain bakery technology will increase the biological value of the product.

Keywords: phytin; phytase; complex enzyme preparation; microstructure; mineral element; grain of wheat

INTRODUCTION

Phytic acid is found in many plant systems. In beans and cereal grain, it is approximately 5.1% by weight. This compound is of vital importance for the successful development of the seed and growth of plants. However, phytic acid is a strong chelator and in the interaction with the polyvalent cations and the formation of complexes with proteins - phytate - reduces the bioavailability of many vital mineral compounds **(Cheryan, 1980; Bergman et al., 1997)**. Studies in animal and human as subjects showed that a diet high in phytic acid leads to a deficiency of zinc, calcium, magnesium, phosphorus. This can cause immunodeficiency and lead to cognitive and growth disorders **(Erdman, 1979)**. At the same time, as anti-nutrients in the human diet, phytates can carry a positive role in the nutrition as antioxidants and anti-cancer drugs **(Graf et al., 1987; Lott et al., 2000; Urbano et al., 2000; Hyun-Joo et al., 2004)**.

Reduction of phytate content in the diet is one way to improve nutrient absorption of mineral elements. This may be achieved through use of cooking methods that lead to activation of the endogenous phytase, through the action of microorganisms, such as yeast in baking, with the proviso that the pH and other environmental conditions are favorable, or by application of exogenous phytase technology **(Lonnerdal, 2002; Lestienne et al., 2005; Eklund-Jonsson et. al., 2006)**. Wholewheat bread, usually considered more healthy food than of high-grade flour because different high content of dietary fiber, vitamins (especially B and E) and biogenic minerals. However, whole-grain bread also contains large amounts of phytate. A reduction in the level of phytate in whole meal flour, rye, oats and wheat after fermentation, a figure only slightly dependent on the temperature of the process **(García-Estepa et al., 1999; Buddrick et al., 2014)**. When making bread from whole grain wheat in establishing pH 5.0 during fermentation phytate level was reduced by 64% **(Türk et al., 1996)**. Phytate degradation to the free ends of phytic acid in the production of almost rye bread with a long fermentation time, but if the bread is made from whole grains, minor degradation of phytate **(McKenzie-Parnell and Davies, 1986; Nielsen et al., 2007)**.

From all sources of exogenous phytase, which have been studied (plants, animals, microorganisms), the most

productive are microscopic fungi **(Wodzinski and Ullah, 1996)**. All commercial phytase preparations containing enzymes of microbial origin, produced by fermentation **(Haefner et al., 2005)**. The main application of phytase are found in feeding monogastric animals **(Madrid et al., 2013)**, but it is also used for the treatment of raw materials destined for human nutrition. This enzyme has already found use in breadmaking, the production of vegetable protein isolates, the wet milling of corn, bran fractionation **(Greiner and Konietzny, 2006)**.

The use of phytase in the art from the bread wheat results in a significant increase in its specific volume and improves the texture and shape. Phytase in baking as improver has two benefits: it improves the nutritional status by reducing the phytate content and promotes activation of endogenous a- amylase, which improves the quality of the product **(Haros et al., 2001)**.

The purpose of the presented work was to study the redistribution of trace elements within the grain by the enzymes of the cellulase complex and phytase (producer *Penicillium canescens*).

MATERIAL AND METHODOLOGY

For the study, we took winter wheat varieties obtained in Moscow 139 Moscow Research Institute of Agriculture "Nemchinovka". Dry using a complex enzyme preparation comprising cellulase, β- glucanase, xylanase, phytase, as well as formulations containing the individual enzymes of the complex or combination thereof (P-215, producing *Penicillium canescens*, IBPM RAS). Enzymes had the following activity: cellulase 58711nkat/g, xylanase 12135 nkat/g, β- glucanase 51317 nkat/g, phytase 205268 nkat/g and were given laboratory physical and chemical transformation of polymers chemical faculty of Moscow State University. MV Lomonosov **(Sinitsyna et al., 2003)**.

Enzyme preparations in powdered form were mixed using a magnetic stirrer with citrate buffer (pH 4.5) for 0.5 hours at a concentration of 0.6 $g.L^{-1}$ in the solution before placing grain. This concentration corresponds to the optimum enzyme in the production of bread from whole grain **(Kuznetsova et al., 2007; 2013).** Whole grain incubated enzyme preparation in solution at the ratio of grains: 1 : 1.5 solution for 8 hours at 50 ±2 °C in an incubator. Modes hydrolysis (t = 50 °C, pH 4.5) are optimum for the operation of the enzymes studied. Duration of cereal substrate hydrolysis determined by the time during which the grain moisture is 40% or more, which is necessary to obtain the grain mass, the ability to undergo dispersion and allow the use of grain raw material for the production of grain bakery. After incubation, the inactivation of enzymes not performed.

Microstructural studies were conducted using an electron scanning microscope ZEISS EVO LS. Survey was carried out at an accelerated voltage of 15 kV.

Phytase activity was assessed indirectly by the rate of release of phosphate from the substrate spectrophotometrically. To a 1 cm^3 of fluid was poured keyhole 1 cm^3 of 10% trichloroacetic acid solution and 2 cm^3 reagent "C" (3.66 g iron sulfate (II) was dissolved in a solution of ammonium molybdate (2.5 g of ammonium molybdate was dissolved in pre- 8 cm^3 of sulfuric acid and adjusted to 250 cm^3 with distilled water). Absorbance of the test solution after 30 minutes of soaking at room temperature for CK-3 for a wavelength of 750 nm in a cuvette with a distance of 1 cm between the faces against distilled water. Calibration curve found the mass concentration of phosphorus using standard aqueous solutions of known concentration of KH_2PO_4. Phytase activity was calculated using the formula:

$$FA = ([PO_4] * 106 * Rrs * Rs) / (M * 103 * t_p), \quad (1)$$

where Rrs - dilution of the enzyme preparation in the reaction mixture;

Rs - pre-dilution of the enzyme preparation (before adding to the reaction mixture);

M - molecular weight phosphate;

t_p - the reaction time.

Determination of trace performed after dry digestion in a muffle furnace at 450 °C and dissolving the ash in the mixture of 10% hydrochloric acid and nitric acid by atomic absorption spectrophotometry, the air-acetylene flame device firm HITACHI 180-80 with deuterium background corrector. For calibration using standard solutions of elements of the company (Merck).

Analysis of the distribution of mineral elements in the anatomical parts of grains and the relative content of mineral elements in the washings were performed using X-ray detector EMF miniCup system in a scanning electron microscope JEOL JSM 6390.

RESULTS AND DISCUSSION

Cereal products provide delivery 20 – 30% minerals (Cu, Zn, Mg, Mn, etc.) in the human diet **(Gyori et al., 1996)**. Table 1 shows the results of the determination of certain mineral element nutrients in wheat.

A number of studies on the processes of distribution of manganese and iron in plant tissues. These elements exhibit a strong affinity for moving organic chelates and complexes. However, when the supply of manganese in

Table 1 Mineral content in wheat grain.

Mineral element	Content [$mg.kg^{-1}$ DM]
Zinc	22.43 ±1.23
Copper	2.13 ±0.13
Manganese	37.50 ±2.10
Iron	64.30 ±4.50
Cobalt	0.04 ±0.01

small plants, its mobility is very limited in tissues. Transfer of iron in plant tissues is difficult. Manganese is a specific component of two enzymes - arginase and phosphotransferase, moreover it increases the activity of certain oxidases. Iron - an essential metal involved in the transformation of the energy required for synthetic processes in the cells. Zinc is part of multiple enzymes - dehydrogenase, peptidases, proteinases and fosfohydrolase. Basic functions related to zinc metabolism of carbohydrates, protein and phosphate. Copper is part of

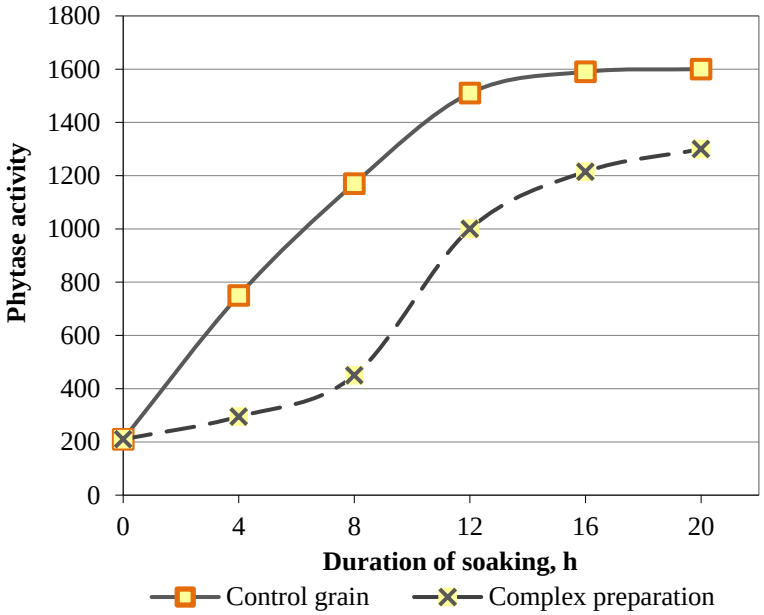

Figure 1 The change in phytase activity of the substrate in the processing of wheat complex enzyme preparation.

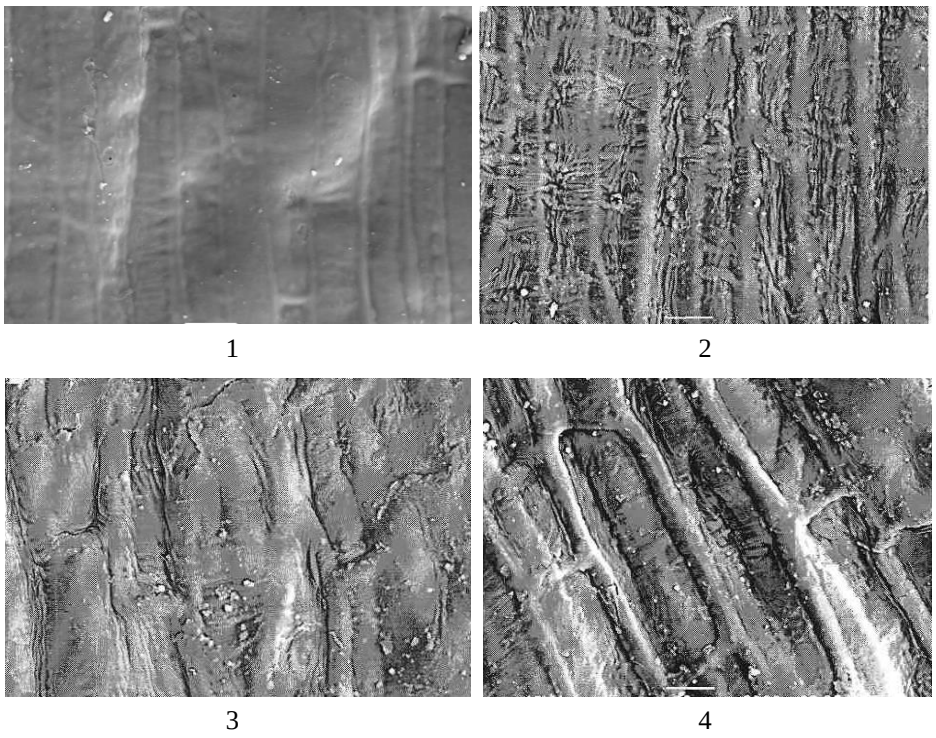

Figure 2 Photomicrographs of the surface of wheat treated with enzymes of the cellulase complex (1 – control without enzyme, 2 – β-glucanase-phytase, 3 – xylanase+phytase, 4 – complex enzyme preparation. An increase of x700. Photo: S. Motyleva, 2013).

enzymes that regulate the processes of respiration, redistribution of carbohydrates, protein metabolism **(Kabata-Pendias and Pendias, 1989)**. Therefore, these trace nutrients plays a key role in the processes of waking up and swelling of the grain as a result of the activation of metabolic processes need to move the movable element in the form increases.

It is known **(Betchel et al., 1981; Jacobsen et al., 1981)**, that it is located in special phytates aleurone grains of the aleurone layer and the embryo and associated biogenic minerals in remote systems. To accelerate the process of transition hard mineral elements are mobilized to implement integrated cellulolytic enzyme preparation based on the actions of phytase.

Treatment of wheat grains complex enzyme preparation during 20 hours was observed changes in the activity of phytase substrate. The experimental data is presented in Figure 1.

Studies have shown that in the first 8 hours of soaking the grain in buffer solution pH 4.5, the activity of phytase substrate is slowly increased as the grains swell and increased by 1.7 times compared to the control. In the period from 8 to 12 hours of hydrolysis observed maximum phytase activity values increase. In the next 8 hours of exposure to the substrate preparation phytase activity in the grain did not undergo significant changes. Phytase activity, the values presented is total value of the substrate activity of endogenous and exogenous phytase.

Table 2 shows the values of the phytase activity of the substrate after 8 hour treatment wheat individual enzymes that are part of a complex enzyme preparation, and their combination in the obligatory presence of exogenous phytase.

From the experimental data presented shows that

Table 2 Effect of enzyme complexes to change the phytase activity of the substrate.

The composition of the enzyme complex	Phytase activity [unit activity]
Cellulase + phytase	685 ±12
β- glucanase + phytase	880 ±21
Xylanase + phytase	940 ±22
Cellulase + β-glucanase + phytase	1050 ±33
Xylanase + cellulase + phytase	1180 ±27

Table 3 Distribution of mineral elements in the anatomical parts of the grain after processing complex enzyme preparation in mass%.

Chemical element	Morphological parts of the grain													
	Germ		The surface of the fruit shell		Fruit shell		Seed coat		Aleurone layer		Endosperm		Barb	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
C+N+O	93.39	98.37	96.77	97.48	99.18	98.95	98.98	99.42	90.08	98.11	99.00	95.86	93.02	94.95
Na	0.03	–	0.01	–	0.03	0.06	0.01	–	–	–	0.02	0.05	0.03	–
Mg	0.10	0.19	0.12	0.22	0.06	0.11	0.08	0.04	2.03	–	0.05	0.08	0.20	0.13
Al	0.06	0.04	0.06	–	0.03	0.05	–	0.01	–	–	0.01	0.01	0.33	-
P	0.31	0.54	0.15	0.13	0.06	0.06	0.04	0.09	3.95	0.25	0.02	0.78	0.30	0.91
S	0.16	0.24	0.18	0.21	0.07	0.10	0.13	0.12	0.13	0.14	0.03	0.93	0.06	0.51
K	0.55	0.21	0.10	0.13	0.08	0.13	0.21	0.02	2.71	0.31	0.09	0.12	0.03	0.32
Ca	0.15	0.06	0.33	0.34	0.10	0.20	0.18	0.10	0.05	0.19	0.01	0.47	0.49	0.61
Cr	0.01	–	0.12	0.14	0,01	0.03	0.01	0.06	–	–	0.05	0.07	0.84	–
Mn	0.92	0.02	–	0.13	–	–	0.01	0.02	0.02	0.23	–	0.24	–	0.32
Fe	0.89	0.03	0.04	0.03	0.02	0.06	0.01	0.02	0.11	0.11	–	0.38	–	0.01
Co	0.02	–	0.08	–	–	0.03	0.01	–	0.03	0.04	0.03	0.13	1.19	0.26
Ni	0.02	0.05	0.03	0.24	0.01	0.01	0.06	–	0.05	–	0.07	0.09	0.14	–
Cu	1.71	–	0.12	0.12	0.10	0.06	0.15	0.03	0.07	0.16	0.10	0.19	2.36	0.45
Zn	1.41	0.06	0.05	0.20	0.03	0.01	0.03	–	0.01	0.24	–	0.46	–	0.28
Se	0.17	0.19	0.20	0.46	0.05	0.05	0.05	–	0.11	0.17	0.06	0.09	0.88	0.46

1 - The distribution of chemical elements in the morphological parts of wheat (control variant), mass%;
2 - Distribution of chemical elements in morphological parts of wheat treated complex enzyme preparation, mass%

a cellulase treatment of the grain in conjunction with the β-glucanase or xylanase leading to an increase in phytase activity could be 1.4 – 2.3 times as compared with the individual enzymes.

This indicates a synergistic effect on the action of enzyme complex which is caused by the action of enzymes on successive substrates entering into the matrix of the cell walls, which is a complex composition of the substrate.

Figure 2 shows photomicrographs of the surface of the wheat treated with the enzyme solution of the test drug under the optimum conditions of hydrolysis.

Pictures were made with a scanning electron microscope at 700x magnification.

Under the action of biocatalysts based cellulases has changed the surface topography of grain. In control variant the relief of the grain surface is parallel strands of cellulose fibrils, hemicellulose polysaccharides layer overlain nature (1). Under the action of the enzyme β-glucanase grain surface topography changes. Denudation observed parallel strands of cellulose microfibrils of varying thickness and tortuosity. Probably destroy exposed top layer of hemicellulose (2).

Xylanase enzyme action causes destruction layer hemicelluloses tissue depth direction. Modification of the surface structures occurs both in longitudinal and radial direction (3). Under the action of the complex enzymes cellulase, β-glucanase and xylanase (4) masonry surface relief grains are formed deep enough, they are represented by parallel strands almost devoid of cuticular crosslinks. As a result of concerted action of enzymes complex preparation varies topography grain, increase the pore sizes in seed and fruit shells that facilitates the penetration of the enzyme phytase in the aleurone layer to the site of phytin hydrolysis and leads to an increase in phytase activity. The article **Haraldsson et al. (2004)** also points to the possibility of combining the degradation of phytate degradation β-glucan under the joint action of phytase and β-glucanase during malting, which is of interest for the production of cereal products with high nutritional value. However, according to our studies listed, phytate degradation to a greater extent due to the presence in the complex enzyme preparation comprising xylanase and phytin hydrolysis, intensity is a maximum when the cereal substrate operates complex enzyme preparation comprising cellulase, β-glucanase, xylanase.

To analyze the distribution of trace elements P, K, Mg, Ca, Fe, Mn in the outer layers of the wheat grain has been used and the X-ray structure analysis. It has been found that the studied elements are concentrated in the aleurone layer. In particular, P, Mg, K were concentrated in the aleurone layer of subcellular particles and outer layers of the wheat grain; Ca was found in abundance in the tissues of the pericarp **(Tanaka et al., 1974)**.

In terms of rational parameters of enzymatic hydrolysis, investigated the distribution of mineral elements in the anatomical parts of the grain after processing complex enzyme preparation with the help of X-ray detector EMF miniCup system in a scanning electron microscope JEOL JSM 6390.

Gained data relative content of mineral nutrient elements are presented in Table 3.

The studies showed that after soaking in solution of the enzyme preparation on the basis of phytase migrates within the mineral grains.

Reduces the number of elements studied in the aleurone layer and significantly increased in the endosperm.

Under the influence of enzyme preparations polysaccharides constituting the matrix of the cell walls are modified, the system is broken native intermolecular bonds between the main structural components of the polysaccharide complex, the process of maceration and partial structures shells fragmentation polymers themselves. This ensures destruction of intercellular substance, leading to the separation of cells, solubilization hydrolysis products. Electrostatic forces arising due to the functional group having affinity for the metal ions at the micelle surface terminate. The process is accompanied by desorption of ions, molecules associated with non-starch polysaccharides. Experimental studies of morphological parts of wheat showed that under the action of biocatalysts based cellulases distribution of chemical elements in the caryopsis changed. There is a tendency in the distribution of mineral elements, characteristic of grain - the proportion of these elements in the aleurone layer (5) is reduced, and in the endosperm (6) - increases. The relative content of nutrients that are part of metalloenzymes and biologically active compounds increased in the endosperm, where during swelling grain intensified oxidative decomposition processes of high- replacement compounds. The chemical elements which have a high mobility, potassium and sodium are moved from the central portion to the peripheral weevil. Because dietary fiber and phytate found together in the peripheral layers of fiber-rich grains, it is difficult to separate the effects of degradation processes nonstarch polysaccharides and fiber phytate redistribution of polyvalent metal ions **(Torre M. et al., 1991)**.

Distribution of chemical elements in the morphological parts of the grain shows that activation occurred own enzyme systems. This is evidenced by the increase in the relative content of sulfur, which is part of the proteins, enzymes and free amino acids, as well as phosphorus, participating in all the processes of metabolism. Increase in the relative amount of phosphorus, sulfur, potassium, magnesium, selenium in the bud indicates the activation of the synthesis of organic compounds necessary for the construction of the developing plant tissues. Reduction in the relative content of trace elements in the aleurone layer speaks about embryonic germ awakening, intensifying the process of synthesis and migration of enzymes in the endosperm. The preferential increase in the endosperm fraction trace indicates that after 8 hours of soaking wheat germination basic feature consists in the biochemical processes in direction towards hydrolysis. The appearance of selenium, known for their antioxidant properties, morphological parts of grains indicates the incorporation of plant protection from the negative effects of oxidation products - free radicals, peroxides and hydroperoxides.

These data confirm that in the hydrolysis of phytin complexes decompose exogenous phytase, phytin formed with mineral elements: calcium, magnesium, iron, copper and zinc. These chemicals migrate into the endosperm where the basic seed and nutrients included in the modification process and replacement of biological

polymers. These experimental data are in agreement with the findings of previous studies that during imbibition of wheat mineral elements (magnesium, calcium and potassium) redistributed from the aleurone layer and mobilized for the development of seedlings **(Eastwood and Laidman, 1971)**.

CONCLUSION

As a result of a complex enzyme preparation based on phytase (producer *Penicillium canescens*) for the treatment of wheat has been a change of the surface microstructure of grain. Microstructural changes and phytase activity indicator substrate, characterizing the rate of release of phosphate caused enzyme complex composition of the drug and determined the presence of xylanase enzyme. Availability phytin phytase is associated with the degree of degradation of the hemicellulose. It has been established that the release rate of the phosphoric acid substrate is dependent on the composition of the drug and the enzyme complex is determined by the presence of xylanase. When processing enzyme preparation wheat trend in the distribution of mineral elements, characteristic of grain - the proportion of these elements in the aleurone layer decreases, and in the endosperm - increases. These data confirm the results of microstructural studies of chemical analysis.

Thus, studies have shown that phytase - an effective mechanism for regulating mineral nutrient diet. Application of phytase in grain bakery technology will increase the biological value of the product.

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Contact address:

prof. Elena A. Kuznetsova, DrSc., State University-Education-Science-Production Complex, 302020, Orel, Naugorskoe shosse, 29, Russian Federation, E-mail: elkuznetcova@rambler.ru,

Ing. Ludmila Cherepnina, PhD, State University-Education-Science-Production Complex, 302020, Orel, Naugorskoe shosse, 29, Russian Federation, E-mail: lvcherepnina@rambler.ru;

Ing. Svetlana Motyleva, PhD. All-Russian Institute of plant breeding and gardening and nursery, 115598, Moskow, Zagorievskay street, 4, Russian Federation, E-mail: motyleva_svetlana@mail.ru;

doc. Ing. Ján Brindza PhD., Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Institute of Biological Conservation and Biosafety, Trieda Andrey Hlinku 2, 949 76 Nitra, Slovakia. E-mail: Jan.Brindza@uniag.sk.

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MOLECULAR CHARACTERIZATION OF RYE CULTIVARS

Želmíra Balážová, Lenka Petrovičová, Zdenka Gálová, Martin Vivodík

ABSTRACT

The results of molecular analysis of 45 rye taxa (*Secale cereale* L.) represented by agricultural varieties originated from Central Europe and the Union of Soviet Socialist Republics (SUN) are presented. The genetic diversity of rye cultivars by 6 SSR markers was evaluated. Six specific microsatellite primer pairs produced 58 polymorphic alleles with an average of 9.7 alleles per locus. The number of alleles ranged from 6 (*SCM2*) to 14 (*SCM86*). Genetic polymorphism was characterized based on diversity index (DI), probability of identity (PI) and polymorphic information content (PIC). The diversity index (DI) of SSR markers ranged from 0.5478 (*SCM2*) to 0.887 (*SCM86*) with an average of 0.778. The lowest value of polymorphic information content was recorded for *SCM2* (0.484) and the highest value for *SCM86* (0.885) of PIC was detected in *SCM86* with an average of 0.760.The dendrogram of genetic similarity was constructed, based on UPGMA algorithm. The hierarchical cluster analysis divided rye genotypes into 4 main clusters. The first cluster of 14 genotypes was subdivided in two subclusters (1a and 1b) where 50% of genotypes were Czechoslovak origin. The second cluster contained four genotypes were three (75%) of them had Czech or Czechoslovak origin. In the third subcluster separated three rye genotypes of different origin. The rest (24) of rye genotypes in the fourth cluster were divided into two subclusters (4a and 4b) where clearly separated group of Polish (4aa) and Czech and Czechoslovak (4ab) genotypes. Two genotypes of 4aa subcluster (Wojcieszyckie and Dankowskie Nowe) from Poland were genetically the closest. In the dendrogram alle genotypes were differentiated and clustering partially reflects geographic origin of studied rye genotypes. In this experiment, SSRs markers proved to be a high informative and usefull tool in genetic diversity research for the distinguishing and characterization of close related varieties.

Keywords: *Secale cereale* L.; polymorphism; microsatellite; PCR; dendrogram

INTRODUCTION

Common rye (*Secale cereale* L.) is one of the most important cereal crops cultivated in Eastern and Northern Europe **(Targońska et al., 2015)**. Rye (*Secale cereale* L.) is a diploid (2n = 2x = 14) annual, cross-pollinated cereal with an effective gametophytic self-incompatibility system. On a global scale rye (*Secale cereale* L.) is a minor crop, its production being about 5% that of wheat or rice. However, in northern European countries with extreme climatic and poor soil conditions, rye may occupy up to 30% of the acreage **(Altpeter and Konzun, 2007)**. The main advantages of rye over other winter cereals are its excellent tolerance to low temperatures and the ability to realize relatively high grain yields under environmental conditions in which other crops perform poorly. Rye is also known to have the lowest requirements for chemical treatments like fertilizers or pesticides, which makes it an ecologically and economically sound crop for specific regions **(Korzun et al., 2001)**. Moreover, rye offers high contents of many nutritionally favorable compounds such as a whole suite of minerals (Zn, Fe, P), beta-glucans, resistant starch, and bioactive compounds. Rye products are characterized by a high level of dietary fiber **(Andersson et al., 2009)** that may contribute to positive health effects **(Rosén et al., 2011)**.

Molecular markers can provide an effective tool for efficcient selection of desired agronomic traits because they are based on the plant genotypes and thus, are independent of environmental variation. Nowadays, several molecular markers are developed, of which simple sequence repeats (SSRs) or microsatellites are the most widely used types **(Jenabi et al., 2011; Maršálková et al., 2014)**.

Simple sequence repeat (SSR) markers show a relatively good transferability between closely related species **(Botes and Bitalo, 2013)** and they are one of the most promising molecular marker types to identify or differentiate genotypes within a species **(Salem et al., 2008)**. They were successfully used in many plant species, e.g. triticale **(Kuleung et al., 2004; Odrouškova and Vyhnanek, 2013)**, **wheat (Röder et al., 1995; Huang et al., 2002)**, rye **(Khlestkina et al., 2004)**, rice **(Jiang et al, 2010)**, maize **(Ignjatovic-Micic et al., 2015)**, and amaranth **(Žiarovská et al., 2013)**.

Rye SSR markers were first developed over 10 years ago **(Saal and Wricke, 1999; Hackauf and Wehling, 2002,)** and have also been used in studies on genetic diversity **(Shang et al. 2006; Bolibok et al., 2005)**.

The aim of our study was to detect genetic variability among the set of 45 rye genotypes using 6 microsatellite markers.

MATERIAL AND METHODS

Forty five rye (*Secale cereale* L.) genotypes were used in the present study. Seeds of rye were obtained from the Gene Bank of the Slovak Republic of the Plant Production

Research Center in Piešťany and Gene Bank of the Czech Republic of the Crop Research Institute in Prague. Fifteen genotypes of rye came from Czechoslovakia (CSK), another set of fifteen genotypes from Poland (PL), five from Czech Republik (CZ), another five from Hungary (HU) and last five genotypes from Union of Soviet Socialist Republics (SUN). All genotypes are of winter form.

Genomic DNA of rye cultivars was isolated from 100 mg freshly-collected leaf tissue according to GeneJET™ protocol (Fermentas, USA). The concentration and quality of DNA was checked up on 1.0% agarose gel coloured by ethidium bromide and detecting by comparing to λ-DNA with known concentration.

For analysis, six microsatellite primer pairs were chosen according to the literature **(Saal - Wricke, 1999)**. Used primers were localised on 6R, and 7R chromosomes (Table 1). PCR amplification was performed in 20 μL volume containing PCR water, 5 x Green GoTaq® Flexi Buffer, 100 μM dNTP Mix, 0.3 μM primers (Forward and Reverse primer), 1.5 mM $MgCl_2$, 0.4 U *GoTaq® polymerase* (Promega, USA). PCR reactions were performed in a thermocykler (Bio-Rad, USA). The PCR program consisted of these steps: an initial denaturation (1 cycle): 2 min. at 93 °C, (29 cycles) denaturation: 1 min. 93 °C, annealing 2 min. with different temperature for each primer pair and extension 2 min. at 72 °C.

The PCR amplicons (5μL) were resolved by electrophoresis on 6.0% denaturating polyacrylamide gel stained with silver according to **Bassam et al., (1991)**. Final PCR amplicons were scanned in UVP PhotoDoc-t® camera system. The size of alleles was determined by comparing with 10 bp standard lenght marker (Invitrogen: 100 – 330 bp). Each band was treated as a single allele.

Each reproducible band was visually scored for the presence (1) or absence (0) for all genotypes. For determination of the genetic relationships between rye genotypes a dendrogram was used. The dendrogram was constructed based on principle of hierarchical cluster analysis using UPGMA (Unweighted Pair Group Method using arithmetic Averages) algorithm on the basis of Jaccard's coefficient in statistical program SPSS version 17.

Frequencies of incidence of all polymorphic alleles were calculated and used for determination of statistical parameters: diversity index (DI) **(Weir, 1990)**, probability of identity (PI) **(Paetkau et al., 1995)** and polymorphic information content (PIC) **(Weber, 1990)**.

Diversity index (DI):

$$DI = 1 - \sum p_i^2$$

Probability of identity (PI):

$$PI = \sum p_i^4 + \sum_{i=1}^{i=n-1} \sum_{j=i+1}^{n} \left(2 p_i p_j\right)^2$$

Polymorphic information content (PIC):

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2 \right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 \cdot p_j^2$$

RESULTS AND DISCUSSION

The development of molecular markers has opened up numerous possibilities for their application in plant breeding. Suitable markers for detecting polymorphisms at individual and population levels are SSRs **(Shang et al., 2006; Bolibok et al., 2005, Akhavan et al., 2009)**.

Six rye specific microsatellite primer pairs produced 58 polymorphic alleles with an average of 9.7 alleles per locus. The most polymorphic locus was *SCM86* where 14 polymorphic amplification products were detected. On the other hand the lowest polymorphic locus was *SCM2* with 6 polymorphic alleles.

Jenabi et al., (2011) used fifteen wheat and rye derived microsatellite markers to evaluate genetic variation of the mountain rye *Secale strictum* in Iran and to examine the patterns of diversity related to the varieties and geography. They detected high levels of diversity, with an average number of 6.1 alleles per locus (ranging up to 11) and high level polymorphism with polymorphism rate averaging 0.624 (between populations) and 0.357 (within populations) were observed among 125 individuals from 19 populations collected from various regions of Iran. **Gailîte et al., (2013)** analyzed genetic polymorphism of a set of 9 genotypes originated from Latvia using 12 SSR markers. The number of alleles ranged from 1 to 6 with an average number of alleles per locus 3.4. The results from their study indicate that while the Latvian rye collection is small, the genetic and phenotypic diversity contained within and between the accessions is quite high. **Targońska et al., (2015)** studied genetic diversity among 367 Polish rye accessions using 22 previously published simple sequence repeat (SSR) markers.

Table 1 List and characterization of locus specific microsatellite primers used for SSR analysis.

SSR marker	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)	Chromosomal location	Anealing temperature
SCM 2	GATGACTATGACTACCAGGATGAA	GGAGTGAGAAGGCCGAGAAG	6R	55 °C
SCM 28	CTGGTCCTGGTCTGGTGGGTC	CGCATCGGGTGTGTCGCATAC	6R	60 °C
SCM 40	CGCATCGGGTGTGTCGCATAC	CACATCTTGGGCCTGACACC	7R	60 °C
SCM 86	CAGATAGATGGGTGTTGTGCG	CTCTTCTCGACATCCACACTCC	7R	60 °C
SCM 101	GCCAGCCGCCACCTTAATTG	AGCCCAACTCTTTCGTGCATG	6R	60 °C
SCM 180	GTTTCGTCCCCGTTGCCATC	ACGTGTCGCTTTCCATTGCCC	6R	60 °C

Table 2 Characteristics of used SSR markers in this study.

SSR marker	Number of alleles	DI	PIC	PI
SCM 2	6	0.547	0.484	0.144
SCM 28	11	0.771	0.764	0.027
SCM 40	13	0.867	0.865	0.004
SCM 86	14	0.887	0.885	0.002
SCM 101	9	0.857	0.851	0.004
SCM 180	5	0.740	0.708	0.022
average	**9.7**	**0.778**	**0.760**	**0.034**

Note: DI - diversity index; PI - probability of identity; PIC - polymorphic information content.

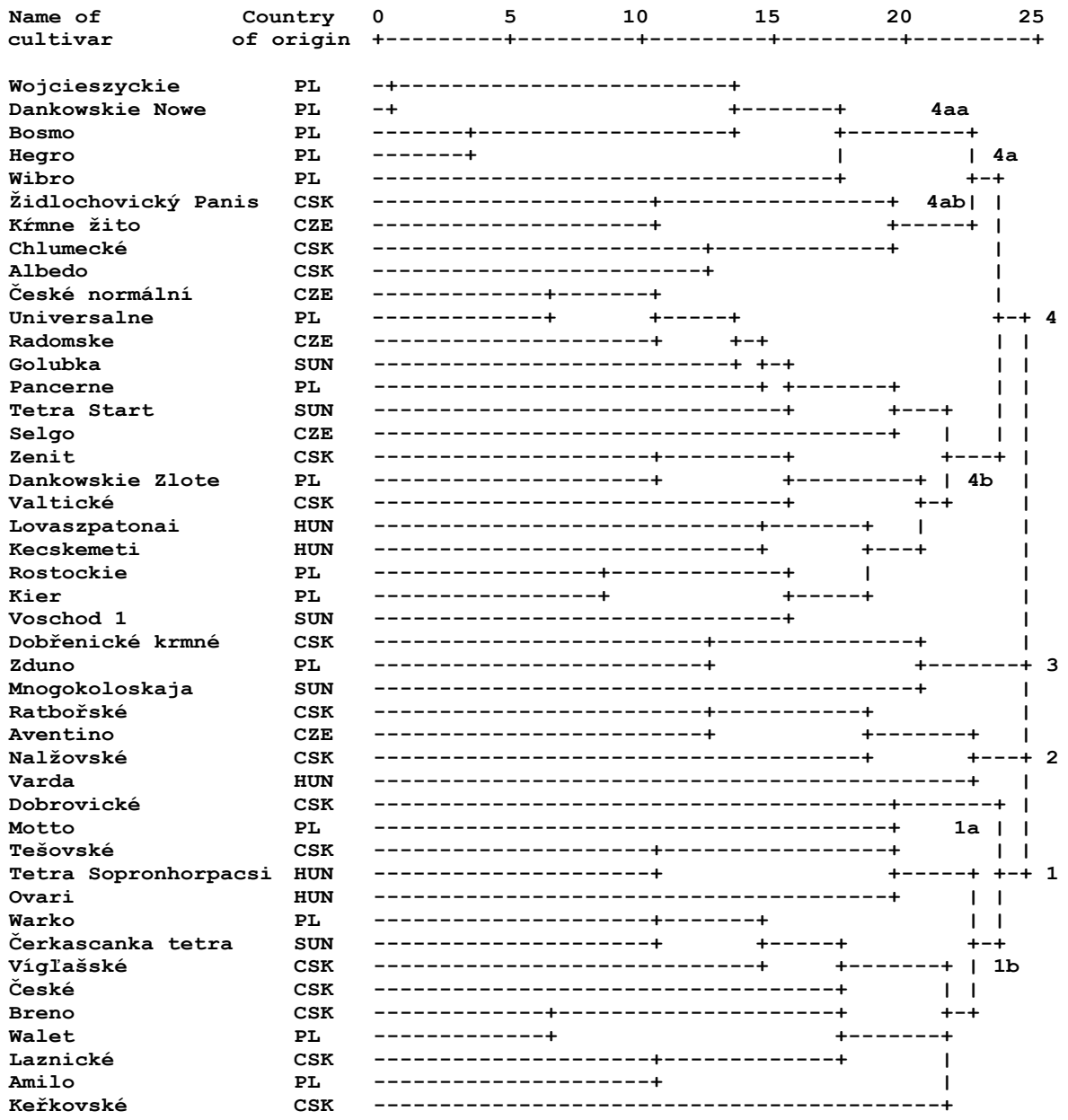

Figure 1 Dendrogram of 45 rye genotypes prepared based on 6 SSR markers.
CSK - Czechoslovakia, CZ - Czech Republic, HU - Hungary, PL - Poland, SUN - Union of Soviet Socialist Republics.

Resulting from the number and frequency of alleles, diversity index (DI), polymorphic information content (PIC) and probabilities of identity (PI) were calculated (Tab. 2). The diversity index (DI) of SSR markers ranged from 0.547 (*SCM2*) to 0.887 (*SCM86*) with an average of 0.778. The lowest value of polymorphic information content was recorded for *SCM2* (0.484) and the highest value for *SCM86* (0.885) of PIC was detected in *SCM86* with an average of 0.760. Only one marker (*SCM2*) reached considerably unfavourable results of DI, PIC, PI and number of alleles compared to average values of tested set. Probability of identity was low ranged from 0.002 (*SCM86*) to 0.144 *(SCM2*) with an average of 0.034 that indicates the possibility to differentiate genetically close genotypes.

Jenabi et al., (2011) found out lower polymorphism in their study. They calculated the within populations PIC value for all microsatellites which ranged from 0.246 to 0.451 with an average of 0.357. **Targońska et al., (2015)** detected the average PIC value for all markers used 0.57. The highest PIC value (0.93) was obtained for SCM152, and the lowest PIC (0.18) was determined for SCM050.

The dendrogram of genetic relationships among 45 rye cultivars based on SSR markers is presented in Figure 1. The hierarchical cluster analysis showed that the rye genotypes were divided into 4 main clusters. The first cluster was divided in two subclusters (1a and 1b). Subcluster 1a contains two genotypes of Czechoslovak and Polish origin. In the subgroup 1b were grouped 12 genotypes which were bred in Czechoslovakia (50%), Poland (25%), Hungary (16.7%) and one coming from Union of Soviet Socialist Republics. The second cluster contained four genotypes were three (75%) of them had Czech or Czechoslovak origin. In the third subcluster separated three rye genotypes of different origin. The rest of rye genotypes in the fourth cluster were divided into two subclusters (4a and 4b). Subcluster 4a was further subdivided into two subclusters, subcluster 4aa with 5 genotypes all coming from Poland and subcluster 4ab with four genotypes of Czech or Czechoslovak origin. Subcluster 4b of 15 genotypes included genotypes of Polish origin (33.3), SUN origin (20%), Czech origin (20%), Czechoslovak origin (13.3) and Hungarian origin (13.3). Two genotypes of 4aa subcluster (Wojcieszyckie and Dankowskie Nowe) from Poland were genetically the closest. We can assume that they have close genetic background (Figure 1).

Targońska et al., (2015) showed that the clustering of rye accessions studied was more weakly correlated with geographic origin than with the source of seeds. **Akhavan et al., (2010)** in the prepared SSR based dendrogram using UPGMA algorithm showed evident broad groupings related to the subspecies. The populations of subsp. *cereale* were mainly grouped but populations belonging to the subsp. *ancestrale* were divided in to two subgroups (groups I and III), indicating higher diversity of the latter subspecies.

CONCLUSION

The objective of this study was to determine the genetic variation among 45 rye varieties using SSR markers. Values of diversity index and polymorphic information content were higher than 0.7 in 83% of SSR markers that means high lever of polymorphism of used markers. WE can recommend them for further analyses. The dendrogram was prepared based on UPGMA algorithm using the Jaccard´s coefficient and divided in to four main clusters. All studied genotypes separated into four clusters. Clustering partially reflected geographic origin of studied rye genotypes. SSR are commonly and extensively used tools for assessment of variability in crops. These marker systems are efficient due to their locus specificity, reproducibility and reliability, for analysis of molecular differentiation and for resolving taxonomic problems in plants. Our result showed appreciably high genetic diversity among the rye genotypes studied. This survey showed the high genetic diversity within the European rye genepool as an important source for crop breeders, and indicated that there is value in sampling for useful genes for crops improvement.

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Contact address:

Želmíra Balážová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zelmira.balazova@uniag.sk.

Lenka Petrovičová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: petrovicovalenka22@gmail.com.

Zdenka Gálová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zdenka.galova@uniag.sk.

Martin Vivodík, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: martin.vivodik@uniag.sk.

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EVALUATION OF CAROTENOIDS, POLYPHENOLS CONTENT AND ANTIOXIDANT ACTIVITY IN THE SEA BUCKTHORN FRUIT JUICE

Andrea Mendelová, Ľubomír Mendel, Peter Czako, Ján Mareček

ABSTRACT

Due to the content of biologically active substances, sea buckthorn (*Hippophae rhamnoides* L.) has become the object of great interest of both, experts and the general public. It is appreciated particularly for the high content of vitamins and other biologically active substances, not only in berries but also in leaves and bark. The aim of the study was to evaluate the nutritional quality of sea buckthorn juice prepared from different varieties of sea buckthorn based on the content of total carotenoids, polyphenols and antioxidant activity. In this study we used varieties Hergo, Tytti, Vitaminaja, Raisa, Askola, Dorana, Slovan, Leikora, Bojan, Terhi and Masličnaja. Content of different components was quantified using spectrophotometry. The total carotenoids content expressed as β-carotene content in juice ranged from 50.63 mg.100 g^{-1} DM to 93.63 mg.100 g^{-1} DM, the highest content was in variety Askola and the lowest one in Terhi. Total polyphenols content determined by Folin-Ciocalteu method ranged from 13.03 mg GAE. dm^{-3} DM to 25.35 mg GAE. dm^{-3} DM. The highest content was identified in juice of variety Dorana and the lowest one in Raisa. The antioxidant activity quantified by the FOMO method ranges from 45.11 g AA. dm^{-3} DM to 108.77 g AA. dm^{-3} DM. The highest antioxidant activity was determined in juice of Dorana and the lowest in variety Bojan.

Keywords: sea buckthorn; sea buckthorn juice; carotenoids; polyphenols; antioxidant activity

INTRODUCTION

The first remarks about sea buckthorn are several centuries old. In traditional Chinese medicine it was used in the years 618-907 AD against cough, to improve blood circulation, to help with digestive problems, to relieve pain. Leaf extract was used in Mongolia to treat colitis **(Guliyev et al., 2004)**. Sea buckthorn caught considerable attention in Russia, where it has been seen as a very important plant for its healing and regenerative effects **(Bajer, 2014)**. Nowadays the public interest in sea buckthorn as a special dietary supplement is growing, mainly for its nutritional and health-related effects **(Yang and Kallio, 2002)**.

Sea buckthorn is native to Asia and very large Eurasian area at different altitudes. It is a unique plant that is currently domesticated in different countries, in particular China, Russia, Germany, Finland, Romania, France, Nepal, Pakistan and India **(Selvamuthukumaran et al., 2007)**. Sea buckthorn belongs to the less demanding timber species in terms of location requirements. The most suitable for its growth is light sandy clay soil but it thrives also in arid, semi-arid and fragile mountain areas. From the temperature point of view, it withstands high daytime temperatures in summer and severe winter frosts **(Letchamo et al., 2007)**.

Although almost all parts of the sea buckthorn plant are used, the fruit is the most valuable product, together with seeds. Sea buckthorn berries contain almost all water soluble vitamins and fat soluble vitamins and many other substances necessary for the human body. They contain pectin, essential oils, tannins, organic acids, oils, minerals and other substances. Significant sugars in sea buckthorn berries are glucose, fructose and xylose. From the organic acids there are mainly malic and quinic acids. Research has shown that Russian berries have relatively lower concentration of organic acids (2.1 to 3.2 g 100 mL^{-1} juice) than Finnish and Chinese genotypes holding the highest concentrations of organic acids (3.5 – 9.1 g 100 mL^{-1} juice) **(Bal et al., 2011)**. **Raffo et al., (2004)** state that the sour varieties contain predominantly malic acid (46.6 $mg.g^{-1}$) and quinic acid (28.2 $mg.g^{-1}$). **Bal et al., (2011)** reported that the fruits have a high proportion of aspartic acid and glutamic acid, but it is necessary to mention also the high content of essential amino acids, especially lysine, valine, threonine, methionine, leucine, izoleucine, tryptophan, phenylalanine.

Sea buckthorn is an excellent source of natural antioxidants **(Bal et al., 2011)**. The most important antioxidant in sea buckthorn juice is vitamin C **(Rosch et al., 2003)**. The authors report that one berry covers the recommended daily dose of vitamin C. The highest concentration of vitamin C is in Chinese subspecies *H. sinensis,* 2500 mg.100 g^{-1}. European subspecies of *H. rhamnoides* berries contain more than 360 mg.100 g^{-1} of vitamin C **(Bal et al., 2011)**. **Ercisli et al., (2007)** reported that there is lower content of ascorbic acid in varieties which are not cultivated. Vitamin E in sea buckthorn berries is found in the form α, β, γ,

δ - tocopherols **(Yang and Kallio, 2002)**. The content of vitamin E varies from 8.0 mg to 16 mg 100 g^{-1} of fruit. Is is mainly in pulp oil (100 to 160 mg.100 g^{-1} of oil) and seed oil (105 to 120 mg.100 g^{-1}) **(Bajer, 2014)**. According to **Rosch et al., (2003)**, the content of vitamin E in the seed oil corresponds to 61-113 mg.100 g^{-1} and the content in the juice oil varies from 162 to 255 mg.100 g^{-1}.

Carotenoids give sea buckthorn typical yellow to orange colour. Therefore, the oil from the pulp contains more carotenoids than seed oil. The most active representative of carotenoids is β-carotene. In addition to β-carotene in sea buckthorn, there are also lycopene, zeaxanthin, β-kryptoxanthin **(Yang and Kallio, 2002b)**.

In sea buckthorn berries there were identified 12 types of flavonoids: quercetin, kaempferol, hesperidin, rutin, and others. Myricetin, citrine, catechin and others are also present. Those are secondary plant metabolites that are beneficial for the body due to its antioxidant character **(Chen et al., 2013; Bal et al., 2011)**. **Arimboor et al., (2006)** reported that sea buckthorn berries and leaves contain 9 phenolic acids: gallic acid, protocatechuic, salicylic, p-hydroxybenzoic, vanillic, caffeic, cinnamic, p-coumaric and ferulic.

The aim of this study was to determine the content of total carotenoids, polyphenols and antioxidant activity of sea buckthorn juice from selected varieties.

MATERIAL AND METHODOLOGY

The study reviewed 11 varieties of sea buckthorn – Hergo, Tytti, Vitaminaja, Raisa, Askola, Dorana, Slovan, Leikora, Bojan, Terhi, Masličnaja, which were obtained from the Central Control and Testing Institute for Agriculture - State department of fruit variety testing in Veľké Ripňany. German varietis were represented by Hergo, Askola, Dorana, Leikora. Russian originating were varieties Vitaminaja and Masličnaja, Finnish varieties were represented by varieties Tytti, Terhi and Raisa and Slovak by Bojan and Slovan.

Harvesting of berries for the analysis was carried out in the second half of September, when the fruits had characteristic deep orange colour. Due to fine surface structure of berries and their vulnerability, the full branches with berries were collected. The shoots were then frozen at -18 °C and were stored in the freezer until the analysis was run. Analyses were performed within 48 hours after harvesting. Before the analysis, we separated berries from the shoots in frozen state by gently shaking them off. Frozen sea buckthorn berries of different varieties were partially thawed and then pressed to obtain sea buckthorn juice, which was then subject to analysis.

Content of total carotenoids expressed in β- carotene was analysed at a wavelength of 455 nm by spectrophotometer UV VIS Jenway model 6405 UV / VIS in accordance with the methodology STN 12136 Determination of total carotenoids content and individual carotenoid fractions. Samples were extracted in acetone followed by capturing carotenoids in the petroleum ether solution.

Polyphenolic substance content was determined by spectrophotometry at a wavelength of 700 nm by Folin – Ciocalteu method **(Singleton and Rossi, 1965)** and was measured as equivalent content of gallic acid. The method is based on reaction of the Folin - Ciocalteu reagent with polyphenols, with leads to formation of blue colour product. The intensity of the blue colour is proportional to the content of the polyphenols.

The antioxidant activity was determined by the FOMO method **(Prieto et al., 1999)**. The principle of the method is the reduction of Molybdenum (VI^{+}) to Molybdenum (V^{+}) by activity of the reducing component in the phosphorus presence. There is a green Phosphomolybdic complex which colour intensity is measured at a wavelength of 695 nm by spectrophotometer. The reductive ability of compounds can be expressed as ascorbic acid (AA) content, which is needed to achieve the same reduction effect.

The results were processed by the statistical package Statistica 8.0 (Statsoft, Inc., Tulsa, USA). The differences between the samples were followed by Tukey's HSD test and correlation dependence between evaluation indicators by using Pearson‘s correlation coefficient.

RESULTS AND DISCUSSION

Sea buckthorn fruits are rich in carotenoids, the most active representative is β-carotene, whose average content was mentioned by **Bajer (2014)** 1.8-3.9 g mg.100 g^{-1} fruit. In our samples the content of carotenoids in sea buckthorn juice ranged from 5.87 mg.100 g^{-1} in variety Terhi to 12.07 mg.100 g^{-1} in Askola (Table 1).

After the conversion of carotenoids to 100 % dry mater, the content of carotenoids ranged from 50.63 mg.100 g^{-1} dry matter in variety Terhi to 93.63 mg.100 g^{-1} dry matter in Askola. Carotenoid content in samples decreased in the following order Askola > Vitaminaja > Hergo > Leikora > Doran > Tytti > Slovan > Bojan > Raisa > Masličnaja > Terhi. Tukey's HSD test determined the lowest content of carotenoids in the juice of varieties Terhi and Masličnaja, among which there was no statistically significant difference. The highest content of carotenoids was in juice of German variety Askola, which was statistically significantly different from any other samples. High levels of carotenoids content over 70 mg.100 g^{-1} dry matter we also found in samples of juices from German varieties Dorana, Leikora, Hergo. From Russian varieties a high content of carotenoids in juice was evaluated variety Vitaminaja. Slovak varieties Slovan and Bojan were lower in content of carotenoids.

Yang and Kallio (2002) state the total content of carotenoids in sea buckthorn berries from 1.0 to 200.0 mg.100 g^{-1} and β-carotene content from 0.2 to 17.0 mg.100 g^{-1}. **Eccleston et al., (2002)** determined the total content of carotenoids in sea buckthorn juice at the amount of 7.3 ml mg.100 mL^{-1} and β-carotene formed 3.3 mg. 100 mL^{-1}. **Kuruczek et al., (2012)** analyzed nine Russian varieties of sea buckthorn and the maximum levels of carotenoids were found in varieties Aromatnaya (28.97 mg.100 g^{-1} fresh weight), Arumnyj (21.51 mg.100 g^{-1}) and Botanicheskaya (14.2 mg.100 g^{-1}). In the study of **Raffo et al., (2004)** in German varieties of sea buckthorn Askola, Hergo and Leikora a presence of major carotenoid zeaxanthin (3-15 mg.100 g^{-1}), β-carotene (0.3-5 mg.100 g^{-1}) and ß-cryptoxanthin (0.5-1.9 g mg.100 g^{-1}) were found.

Table 1 Rating of carotenoids content in sea buckthorn juice.

Varieties	Total carotenoids (mg.100 g^{-1})	Total carotenoids (mg.100 g^{-1} DM)
Terhi	5.87	50.63[a]
Masličnaja	6.23	53.23[b]
Raisa	9.89	64,20[b]
Bojan	8.29	64.27[b]
Slovan	8.56	67,92[c]
Tytti	8.98	69.64[cd]
Dorana	8.21	71.40[de]
Leikora	9.66	73.76[e]
Hergo	10.56	77.07[f]
Vitaminaja	11.99	87.55[g]
Askola	12.08	93.63[h]

NOTE: [a-h] means indicated by the same letter are insignificantly different at $P > 0.05$; DM – dry matter.

In these three varieties increased concentrations of carotenoids were observed while berry ripening. **Andersson (2009)** in his work examines the content of carotenoids in sea buckthorn berries. In his experiments he used varieties Ljubitelskaja, originating in Russia and BHi 72587, BHi 72588, BHi 727102 from the Swedish University of Agricultural Sciences Balsgard. The main carotenoids occurring in sea buckthorn berries include lutein, zeaxanthin, β cryptoxanthin, lycopene, γ-carotene, β-carotene. The author quantified that the total carotenoid content ranged from 1.5 to 18.5 g mg.100 g^{-1}, in variety Ljubitelskaja it was 5.9 mg.100 g^{-1}, in BHi 72587 15.1 mg.100 g^{-1}, in BHi 72588 13.8 mg.100 g^{-1}, in variety BHI 727102 9.4 mg.100 g^{-1}. **Mőrsel et al., (2014)** provided the total content of polyphenols and carotenoids in sea buckthorn and orange juices. The highest content of β-carotene (18.65 mg.100 mL^{-1}) and polyphenolic substances (156.65 mg.100 mL^{-1}) were found in sea buckthorn juice. In orange juice the content of β-carotene was 11.68 mg.100 mL^{-1} and polyphenols 140.73 mg.100 mL^{-1}.

While evaluating the polyphenols content we identified the lowest one expressed in mg GAE.dm^{-3} (gallic acid equivalent) in the juice of varieties Raisa (2.00 g GAE.dm^{-3}) and the highest in the juice of Dorana (2.92 g GAE.dm^{-3}) (Table 2). After conversion of polyphenols content into dry weight, monitored substances were found within the values from 13.3 g GAE.dm^{-3} dry matter in a sample of the varieties Raisa to 25.35 g GAE.dm^{-3} dry matter in the juice of Dorana. Polyphenols content in the studied samples of sea buckthorn juice declined in the following order of Dorana > Hergo > Leikora > Vitaminaja > Masličnaja > Tytti > Terhi > Slovan > Bojan > Askola > Raisa (Table 2).

Tukey's test proved statistically significant differences in polyphenols content in the evaluated juice samples. The difference was not statistically significant between the juices of Slovan and Terhi. Similarly like in the carotenoids evaluation, the high levels of polyphenols were found in juices of German varieties Dorana, Hergo,

Table 2 Rating of polyphenol content in sea buckthorn juice.

Varieties	Total polyphenols (g GAE.dm^{-3})	Total polyphenols (g GAE.dm^{-3} DM)
Raisa	2.00	13.03[a]
Askola	2.05	15.88[b]
Bojan	2.11	16.34[c]
Slovan	2.24	17.81[d]
Terhi	2.07	17.88[d]
Tytti	2.36	18.31[e]
Masličnaja	2.30	19.68[f]
Vitaminaja	2.72	19.89[g]
Leikora	2.64	20.13[h]
Hergo	2.84	20.76[i]
Dorana	2.92	25.35[j]

NOTE: [a-h] means indicated by the same letter are insignificantly different at $P > 0.05$, DM – dry matter.

Leikora. Variety Askola, reaching the highest levels of carotenoids did not belong to varieties with a high content of polyphenols. Correlation analysis proved that there is no correlation dependence between the polyphenol and carotenoiod contents in sea buckthorn juice.

Bončiková et al., (2012) followed the content of total polyphenols in apple varieties Topaz, Pinova, Jonagold and Idared. Analyzing the detected value of 496.7 mg. kg^{-1} in a variety Idared after 842.2 mg. kg^{-1} in a variety Topaz. While determining the antioxidants in sea buckthorn juice **Eccleston et al., (2002)** declare flavonoids content in the amount of 1.182 mg.100 mL^{-1}, isorhamnetin-rutinoside formed 355 mg. 100 mL^{-1}, isorhamnetin-glucoside 142 mg. 100 mL^{-1}, guercetin-glycoside and guercetin-rutinoside 35 mg.100 mL^{-1}. **Arimboor et al., (2006)** dealt with processing of the fresh sea buckthorn berries and their chemical evaluation and indicated that the pure sea buckthorn juice containing no oil is characteristic by a high content of vitamin C (168.3-184.0 mg.100 g^{-1}) and polyphenols (2392-2821 mg.100 g^{-1}). **Raffo et al., (2004)** demonstrated the presence of flavonoids isorhamnetine (350-660 g mg.100 g^{-1}, quercetin (30-100 mg.100 g^{-1}) and kaempferol (2-5 mg.100 g^{-1}) in the German varieties Askola, Hergo and Leikora. **Gutzeit et al., (2007)** in their study isolated certain flavonoids from the juice of sea buckthorn using highspeed countercurrent liquid chromatography. Isorhamnetin-3-O-β-D-glucoside (95 mg), isorhamnetin -3-O-β-rutinoside (10 mg), guercetin-3-O-β-D-glucoside (5 mg) were separated from 4.1 g of the crude ethyl acetate extract. **Rop et al., (2014)** investigated and determined in the fruit of sea buckthorn the total content of polyphenols, flavonoids and antioxidant activity. The following samples were analyzed – varieties of Czech origin – Botanicky, Buchlovicky, of German origin – Hergo, Leikora and Russian origin – Ljubitelna, Trofimovskij. Polyphenolic substances content were measured spectrophotometrically by Folin – Ciocalteu method. Total polyphenol content was detected in the range from 8.62 g GAE. kg^{-1} dry matter in variety Buchlovicky to 14.17 g GAE. kg^{-1} dry matter in variety Trofimovskij. In variety Hergo it was 9.65 g GAE. kg^{-1} dry matter and in variety Leikora 9.74 g GAE. kg^{-1} dry matter, which are the values lower to what we have found in our work.

In the samples of sea buckthorn juice antioxidant activity was assessed by using the FOMO method. The antioxidant activity of the samples was expressed in $mg.dm^{-3}$ equivalent of ascorbic acid (AA). The values of antioxidant activity of sea buckthorn juice samples ranged from 12.51 g $AA.dm^{-3}$ in varieties Dorana to 5.82 g $AA.dm^{-3}$ in Bojan (Table 3).

After conversion to dry weight we identified statistically the highest antioxidant activity in the juice of variety Dorana (107.88 g $AA.dm^{-3}$ dry matter). Evaluated varieties according to antioxidant activity formed six homogeneous groups. Relatively high levels of antioxidant activity in addition to variety Dorana were also found in juices of Slovan Masličnaja. Also varieties Leikora, Terhi and Vitaminaja achieved higher levels of antioxidant activity. Average values we found in the juice of varieties. Hergo and Askola, with no statistically significant difference, and variety Tytti. The lowest antioxidant activity we found in the juice of varieties Raisa and Bojan.

Kuruczek et al., (2012) devoted their study to analysis of antioxidant activity of crude extracts from sea buckthorn berries. Analyses were performed using spectrophotometric methods FRAP and DPPH and nine Russian sea buckthorn varieties grown in Poland were examined. The highest values of antioxidant activity by DPPH method the authors found in varieties Avgustinka (45.78%), Aromatnaya (45.37%) Arumnyj (44.08%), Prozachnaya (39.06%) and by the FRAP method it was in the varieties Botanicheskaya (1892 μmol.L^{-1}) Avgustinka (819 μmol.L^{-1}), Luchistaya (676 μmol.L^{-1}), Aromatnaya (648 μmol.L^{-1}). **Rop et al., (2014)** investigated the antioxidant activity of botanical varieties Botanicky, Buchlovicky, Hergo, Leikora, Ljubitelna, Trofimovskij. The highest antioxidant activity using DPPH method was found in the Russian variety Ljubitelna (18.11 g TEAC kg^{-1}), the lowest in the Czech variety Botanicky (11.26 g TEAC kg^{-1}). In the German variety Hergo authors found antioxidant activity 11.58 g TEAC kg^{-1} and in Leikora 11,50 g TEAC kg^{-1}.

Table 3 Evaluation of antioxidant activity of sea buckthorn juice.

Varieties	Total antioxidant activity (g $AA.dm^{-3}$)	Total antioxidant activity (g $AA.dm^{-3}$ DM)
Bojan	5.82	45.12^{a}
Raisa	7.01	45.53^{a}
Tytti	7.91	61.32^{b}
Hergo	9.04	65.98^{c}
Askola	7.80	68.21^{c}
Leikora	9.91	75.67^{d}
Terhi	8.84	76.22^{d}
Vitaminaja	10.51	77.95^{d}
Masličnaja	10.22	88.05^{e}
Slovan	11.16	89.06^{e}
Dorana	12.23	108.77^{f}

NOTE: $^{a-f}$ means indicated by the same letter are insignificantly different at $P > 0.05$, DM – dry matter.

Table 4 Correlation matrix between the monitored parameters, depending on the results of the Pearson's correlation coefficient.

	Polyphenols	Antioxidant activity
carotenoids (mg.100 g^{-1} DM)	0.08	-0.02
polyphenols (g.dm^{-3} DM)		**0.79****

NOTE: ** Correlations are significant at the level P <0.01; n= 33, DM – dry matter.

Yildiz et al. (2012) analyzed samples of seven genotypes of sea buckthorn from Turkey. They assessed their polyphenols content and antioxidant activity. The total polyphenols content was between 213 mg GAE.100 g^{-1} and 262 mg GAE.100 g^{-1}. Analyses of antioxidant activity by DPPH method showed the high value in the sea buckthorn genotypes, on average 94.2% inhibition of DPPH radical. **Ivanišová et al., (2015)** analyzed antioxidant activity in sea buckthorn and its products (fruit, sea buckthorn tea, oil and juice. The highest activity by DPPH method was observed in oil 8.75 mg TEAC. g^{-1}. Antioxidant activity by phosphomolybdenum method was in the range of 111.59 mg TEAC. g^{-1} to 196.4 mg TEAC. g^{-1}, with higher rates in sea buckhorn tea.

Using Pearson correlation coefficients, we watched a relationship between assessed parameters of sea buckthorn juice quality. We found statistically significant positive correlation between polyphenols content and antioxidant activity. Between the polyphenols and carotenoids contents and carotenoids content and antioxidant activity of sea buckthorn juice we did not find correlation dependence (Table 4).

CONCLUSION

Sea buckthorn (Hippophae rhamnoides L.) has become a product of interest thanks to its content of biologically active substances. It is appreciated especially for the high content of vitamins not only in fruit but also in its leaves or bark which are characterized by healing effects. Sea buckthorn fruits are unique due to the large amount of vitamins, vitamin C, tocopherol, minerals, ß-carotene, flavonoids and organic acids. Sea buckthorn juice is rich in vitamin C, indicating its antioxidant effects. The goal of this research was to assess the content of total carotenoids, polyphenols and antioxidant activity in juice of selected sea buckthorn varieties In this work juice samples of 11 sea buckthorn varieties were evaluated – Hergo, Tytti, Vitaminaja, Raisa and Asko Doran Slovan, Leikora, Bojan, Terhi, Masličnaja. The total carotenoids expressed in β-carotene content in the evaluated sample juices ranged from 50.63 mg.100 g^{-1} dry matter in variety Terhi to 93.63 mg.100 g^{-1} dry matter in Askola. The lowest content of polyphenols we found in the juice of the variety Raisa (13.03 g GAE.dm^{-3} dry matter) and the highest in the sample of Dorana (25.35 g GAE.dm^{-3} dry matter). The values of antioxidant activity of sea buckthorn juice samples ranged from 45.12 g AA.dm^{-3} dry matter of the Bojan sample to 108.77 g AA.dm^{-3} dry matter in the sample of Dorana. Among evaluated varieties Dorana juice was characterized by a high quality of content of nutritionally important substances. It clearly reached the highest content of polyphenolic compounds and high antioxidant activity.

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Contact address:

Ing. Andrea Mendelová, PhD., Slovak University of Agriculture in Nitra, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: andrea.mendelova@uniag.sk.

Ing. Ľubomír Mendel, PhD., Plant Production Research Center Piešťany, Bratislavská 122, 921 68 Piešťany, Slovak Republic, E-mail: mendel@vurv.sk.

Ing. Peter Czako, PhD., Slovak University of Agriculture in Nitra, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: peter.czako@uniag.

Doc. Ing. Ján Mareček, PhD., Slovak University of Agriculture in Nitra, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: jan.marecek@uniag.

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INCIDENCE OF BACTERIA AND ANTIBACTERIAL ACTIVITY OF SELECTED TYPES OF TEA

Jana Hutková, Attila Kántor, Margarita Terentjeva, Jana Petrová, Czeslaw Puchalski, Maciej Kluz, Rafal Kordiaka, Simona Kunová, Miroslava Kačániová

ABSTRACT

The purpose of this study was to determine *in vitro* antibacterial activity of selected teas (Assam: Indian black tea from *Camellia sinensis*, Pu-erh: darkpu-erh (shu) from *Camellia sinensis*, Sencha: Japanese green tea from *Camellia sinensis*) against five species of pathogenic microorganisms. In our study, we determined the total viable count (TVC), yeasts (Y) and *Enterobacteriaceae* counts (E). MALDI-TOF MS Biotyper was used for identification of colonies after cultivation. Evaluation of the antimicrobial activity was performed by disc diffusion method, well diffusion method and detection of minimum inhibitory concentration (MIC). For antibacterial activity against *Escherichia coli* CCM 2024, *Yersinia enterocolitica* CCM 5671, *Klebsiella pneumonie* CCM 2318, *Staphylococus aureus* CCM 2461 and *Bacillus thurigiensis* CCM19 were detected. The inhibition zones were measured in mm in disc diffusion method and well diffusion method. The MIC of the individual extracts was measured spectrophotometrically. The high number of total viable count was found in Pu-erh tea (2.1 log CFU.g^{-1}) and lowest number was found in Assam tea (0.7 log CFU.g^{-1}). The high number of *Enterobacteriacea* was found in Pu-erh tea (2.03 log CFU.g^{-1}) and lowest in Assam tea (0 log CFU.g^{-1}). The higher number of yeasts was found in Pu-erh tea (1.83 log CFU.g^{-1}) and lowest in Assam tea (0.3 log CFU.g^{-1}). Mass spectrometry revealed the presence of seven Gram positive bacteria *Bacillus cereus, B. mycoides, B. pumilus, Enterococcus durans, Staphylococcus epidermis, S. hominis, S. warneri*, four Gram negative bacteria *Acinetobacter junii, Hafnia alvei, Klebsiella pneumoniae, Sphingomonas* spp. and two yeast - *Candida glabrata, Cryptococcus albidus*. The results show that certain tea extracts are particularly active against various pathogenic bacteria. Tea extracts (Sencha, Rooibos, Mate, Assam) were found to have the strongest antibacterial activity against *Staphylococcus aureus* CCM 2461.

Keywords: bacteria; antibacterial activity; MALDI TOF MS Biotyper; tea

INTRODUCTION

Tea is a popular beverage due to its inherent liquor and flavour characteristics. Two major types of tea available in the market are green tea and black tea. Linnaeus first classified the tea plant as Thea sinensis and later named as (*Camellia sinensis* (L) O. Kuntze). The native place of tea plants is claimed as the area touching Nagaland, Manipur along Assam and Burma frontier in the west, even though China in the east touching southwardly through the hills of Burma, Thailand and Vietnam. India is the second largest producer of black CTC tea with a production of 1135.07 million kilograms in the year 2012. It supplies approximately 26% of global back tea demand. Moreover, Assam tea estates manufacture more than 50% of all Indian black tea production. Taste of the tea liquor and appearance of the made tea are two major characteristics quality parameters of black tea **(Dutta and Baruah, 2014)**.

Pu-erh black tea, which is subjected to a long-time of secondary oxidization and fermentation (post-fermented), is defined as a new type of tea in recent years **(Liang et al., 2005)**. Pu-erh black tea, originally produced in the Yunnan province of China, is used as a health beverage to prevent a variety of diseases. First parching crude green tea leaves (*Camellia sinensis* var. *assamica* (L.) obtain Pu-erh black tea O. Kuntze; *Theaceae*) and then undergoes secondary fermentation with microorganisms such as *Aspergillus niger* (postfermented). During the fermentation process, catechins are oxidized into quinone by polyphenol oxidase and then condensed to form bisflavanol, theaflavin, thearubigen, and other high molecular components **(Wang et al., 2010)**. These are regarded as the biologically important active components of Pu-erh black tea which may be responsible for its acclaimed health benefits. Examples of such health benefits include hypocholesterolemia, anti-obesity **(Fujita and Yamagami, 2008a, 2008b)**, anti-atherosclerosis **(Hou et al., 2009)** and anti-mutagenicity **(Wang et al., 2011a,b)**. It is generally believed that the popularity of Pu-erh black tea is linked to its long history of use, especially in Asia, and its health benefits.

Tea quality is important for its market value and is defined by colour, freshness, strength, and aroma. To date, approximately 600 volatiles have been described in black

tea, with fewer numbers in oolong and green tea, due to the lesser degree of fermentation when producing these teas, and thereby tea quality influences a certain market percentage. Fresh tea leaves of *Camellia sinensis* are steamed immediately after plucking to produce Japanese green tea (Sencha). Endogenous enzymes involved in aroma formation are inactivated by the steam treatment, producing low aroma contents. The commercial value of Sencha is mainly evaluated by umami (taste) and fresh green odor, whereas flowery and fruityodor is essential for black tea or semi-fermented tea (oolong tea). The volatile compounds in green tea, black tea and semifermented tea have been intensively analysed **(Katsuno et al., 2014)**. The potent odorants of Japanese and Chinese green teas and black tea have been investigated based on aroma extract dilution analysis (AEDA) **(Kumazawa and Masuda, 2002; Kumazawa et al., 2006)**. To enrich green tea with more aroma attributes, a selection of raw materials (tea cultivars) can be employed. Amongst several types of Sencha aroma-rich green teas, made from *C. sinensis* cultivars, Kohshun and Shizu 7132 have a sweet odor **(Yang et al., 2009)**, and are becoming popular in Japan.

Despite the significant role of herbal teas in improving nutrition and health, there have been reports of microbial contamination and adverse effects resulting from their consumption. These include neurological, cardiovascular and hematological hazards **(Palmer et al., 2003)**. Toxin-producing microbial contaminants are often the cause of these adverse effects. Therefore, it is important to identify the microbial contaminants of herbal tea products as indicators of safety and quality **(Schweiggert et al., 2005)**. A few reports demonstrating microbial contamination of medicinal herbs from various parts of the world exist in the literature. **Rizzo et al., (2004)** indicated that medicinal plants in Argentina harbored toxigenic fungi such as *A. flavus*, *A. parasiticus* and several members of the Genus *Fusarium*.

In recent years, much attention has been focused on the role of tea flavonoids in the promotion of health, especially of catechins **(Ivanišová et al. 2013; Ivanišová et al., 2015 a,b)**. In plants, these metabolites are involved in their protection against several pathogens including insects, bacteria, fungi, and viruses. In the human organism, these polyphenols may exert health promoting properties, mainly antioxidant, anticancer, anti-inflammatory, antidiabetic and antimicrobial activities **(Dias et al., 2013; Sharangi, 2009; Silva, 2012; Wheeler, 2004)**.

The purpose of this study was to determine *in vitro* the antibacterial activity of selected teas (Assam: Indian black tea from*Camellia sinensis*, Pu-erh: dark pu-erh (shu) from*Camellia sinensis*, Sencha: Japanese green tea from *Camellia sinensis*) against five species of pathogenic microorganisms. In our study, we determined the total viable count, number of yeasts and number of*Enterobacteriaceae* genera.

MATERIAL AND METHODOLOGY

For microbial analysis and antimicrobial activity threeselected teas (Assam: Indian black tea from*Camellia sinensis*, Pu-erh: dark pu-erh (shu) from*Camellia sinensis*, Sencha: Japanese green tea from *Camellia sinensis*) were used.

Microbiological analysis

Five grams of the tea was sampled using sterile scalpels and forceps, immediately transferred into a sterile stomacher bag, containing 45 mL of 0.1% peptone water (pH 7.0), and homogenized for 60 s in a Stomacher at room temperature. Microbiological analyses were conducted by using standard microbiological methods. Total viable count (TVC) were determined using Plate Count Agar (PCA, Oxoid, UK) after incubation for 2 days at 35°C. For *Enterobacteriaceae*, Violet red bile glucose agar (VRBL, Oxoid, UK) were inoculated with sample suspension and incubated at 37°C for 24 h. Number of yeasts (Y) were determined using Tryptic Glucose Yeast agar (TGYA, Oxoid, UK). Inoculated plates were incubated for 5 days at 25°C. All plates were examined for typical colony types and morphology characteristics associated after the incubation.

We used MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany) for identification of bacteria and yeasts isolated from tea samples. After incubation of yeasts at 25°C for 5 days, isolated colonies were picked and suspended in 300 µL of sterile distilled water and mixed thoroughly. 900 µL of absolute ethanol was added. The mixture was centrifuged at 13 000 × g for 2 min. After the supernatant was discarded, the pellet was centrifuged again. Residual ethanol was completely removed by pipetting and the pellet was allowed to dry at room temperature. Subsequently 10 µL of formic acid (70%) was added and mixed with the pellet with a sterile toothpick. Next, 10 µL of acetonitrile (100%) was added and mixed thoroughly. The solution was centrifuged at maximum speed for 2 minutes again, and 1 µL of the supernatant was spotted on a polished MALDI target plate (Bruker Daltonics, Germany). Immediately after drying 1 µL of the matrix solution was added to each spot and allowed to air dry. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, Germany) dissolved in 50% acetonitrile with 0.025% trifluoroacetic acid (TFA). The matrix solution preparation (2.5 mg of HCCA) contains 500 µL of acetonitrile, 475 µL of ultra-pure water and 25 µL of trifluoroacetic acid. Next added 250 µL of this solution to the 2.5 mg of HCCA. Samples were then processed in the MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics, Germany) with flex Control software and results obtained with Real-time Classification software (RTC) by used database "Taxonomy" (Bruker Daltonics, Germany).

Antimicrobial activity

The dry materials were crushed, weighed out to 10 g and soaked separately in 100 mL of ethanol p.a. (96%, Sigma, Germany) during two weeks at room temperature in the dark. Exposure to sunlight was avoided to prevent the degradation of active components. Then, ethanolic tea extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol (Stuart RE300DB rotary evaporator, Bibby scientific limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany).

For antibacterial activity, bacteria *Escherichia coli* CCM 2024, *Yersinia enterocolitica* CCM 5671, *Klebsiella pneumonie* CCM 2318, *Staphylococus aureus* CCM 2461

and *Bacillus thurigiensis* CCM19 were used. Bacteria were collected from Czech Collection of Microorganisms. The bacterial cultures were cultivated in Muller Hinton broth (Imuna, Slovakia) at 37 °C.

Antimicrobial activity of tea extract was determined using a disc diffusion method and well diffusion method. The MIC of the individual extracts was measured spectrophotometrically. Briefly, a 100 μL of the test bacteria were grown in 10 ml of fresh media until they reached a count of approximately of 10^5 cells.mL^{-1}. An amount of 100 μL of the microbial suspension was spread onto Mueller Hinton agars. Each antimicrobial assay was performed in at least triplicate.

RESULTS AND DISCUSSION

Green tea is produced from tea leaves that have not undergone the process of fermentation. Until recently, the world trade in tea focused almost exclusively on black tea **(Ošťádalová et al., 2014)**.

The number of microorganisms identified in tea is shown in Figure 1. The highest number of total viable count was found in Pu-erh tea (2.1 log CFU.g^{-1}) and the lowest in Assam tea (0.7 log CFU.g^{-1}). The high number of *Enterobacteriacea* was found in Pu-erh tea (2.03 log CFU.g^{-1}) and the lowest in Assam tea (0 log CFU.g^{-1}). The higher number of yeasts was found in Pu-erh tea (1.83 log CFU.g^{-1}), while the in Assam tea (0.3 log CFU.g^{-1}). Mass spectrometry revealed the presence of seven Gram positive bacteria *Bacillus cereus, B. mycoides, B. pumilus, Enterococcus durans, Staphylococcus epidermis, S. hominis, S. warneri*, four Gram negative bacteria *Acinetobacter junii, Hafnia alvei, Klebsiella pneumoniae, Sphingomonas* spp. and two yeast *Candida glabrata, Cryptococcus albidus*.

Microflora of tea was studied before. The microorganisms identified in tea wer *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Pseudomonas flourescens, Pseudomonas aeruginosa, Klebsiella pneumoniae, Serratia marcencens, Salmonella typhimurium* and *Escherichia coli*, respectively. Fungal isolates were identified as *Aspergillus niger, Aspergillus flavus, Penicillium expansum, Rhizopus stolonifer*, and *Fusarium solanii*, respectively **(Omogbal and Ikenebomeh, 2013)**. This finding indicates that tea could be contaminated with large numbers of different microorganisms.

Mbata et al. 2005 reported that daily consumption of green tea can kill gram positive *Staphylococcus aureus* and other harmful bacteria. Also it has been reported that the green tea contain catechin and polyphenols. These compounds have been found to possess antibacterial and antiviral action as well as anticarcinogenic and antimutagenic properties. This suggests that these compounds could be responsible for the inhibitory of *L. monocytogenes*.

The crude ethanolic extract of white tea exhibited moderate antimicrobial activity against *Sigella sonnei* (11.0 mm), *Pseudomonas aeroginosa* (10.0 mm), *Escherichia coli* (9.0 mm) and *Bacillus cereus* (8.0 mm) at 500 μg.disc^{-1} **(Ur Rashid et al., 2013)**.

The antibacterial activity detected with disc diffusion method is shown in Figure 2. The best antibacterial activity against the tested bacteria with disc diffusion method was found against *Stapylococcus aureus* for Sencha tea (5 mm). The highest antibacterial activity against *Escherichia coli* with disc diffusion method was found for Assam tea (1 mm). The highest antibacterial activity against *Yersinia enterocolitica* was found for Assam and Pu-erh tea (2 mm). The higher antibacterial activity against *Klebsiela pneumonie* was found for Assam and Sencha tea (1 mm). The higher antibacterial activity against *Bacillus thurigiensis* was found for Pu-erh tea (2 mm).

The results of the study showed that the tea extract of *Camellia sinensis* indicates the presence of potent antibacterial activity, which confirms its use against the pathogenic microorganisms. The assessment of antimicrobial activity was based on measurement of inhibition zones formed around the discs. Disk diffusion method did not produce recordable results for all the three type of tea leaves against the pathogens. Among these the methanolic extract of fresh green tea exhibited greater antimicrobial activity. The methanol extracts of the test plant produced larger zones of inhibition against the bacteria. These observations may be attributed to green tea catechin compounds and polyphenols. These compounds have been found to possess antibacterial action **(Saikia et al., 2006)**.

The microorganisms which were found to be sensitive to fresh green tea extracts were *E. coli, Enterococcus faecalis, Staphylococcus aureus, Candida albicans* and *Pseudomonas aeruginosa* **(Archana and Abraham, 2011)**.

It has been documented that green tea contains catechin and polyphenols which are highly sensitive to the oxidation process. The catechin and polyphenols have been found to possess antibacterial and antiviral action as well as anticarcinogenic and antimutagenic properties. These compounds could be responsible for the inhibition of pathogens. The antibacterial effects of tea polyphenols (TPP) extracted from Korean green tea (*Camellia sinensis*) against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated in the previous study. The earlier works by **Mabe et al. (1999)** showed that tea catechins have an antibacterial effect against *H. pylori* and may have a therapeutic effect against gastric mucosal injury induced by this organism.

The antibacterial activity with well diffusion method is shown in Figure 3. The best antibacterial activity against tested bacteria with well diffusion method was found against *Bacillus thurigiensis* for Sencha tea.

The agar-well diffusion method was used for the concentrations of 100, 200, 300, and 400 mg.mL^{-1}, respectively. Results showed that the minimum inhibitory concentration of tea alcohol extract was 400 mg.mL^{-1} with inhibition zone of 20 mm. The extract decreased the bacterial viable count since it showed a visible decrease to $<5\times10^6$ CFU.mL^{-1} after 24 hours of incubation. Black tea extract also had the ability to completely inhibit *Pseudomonas* growth on blood agar and inhibited protease activity and adhesion **(Flayyih et al. 2013)**.

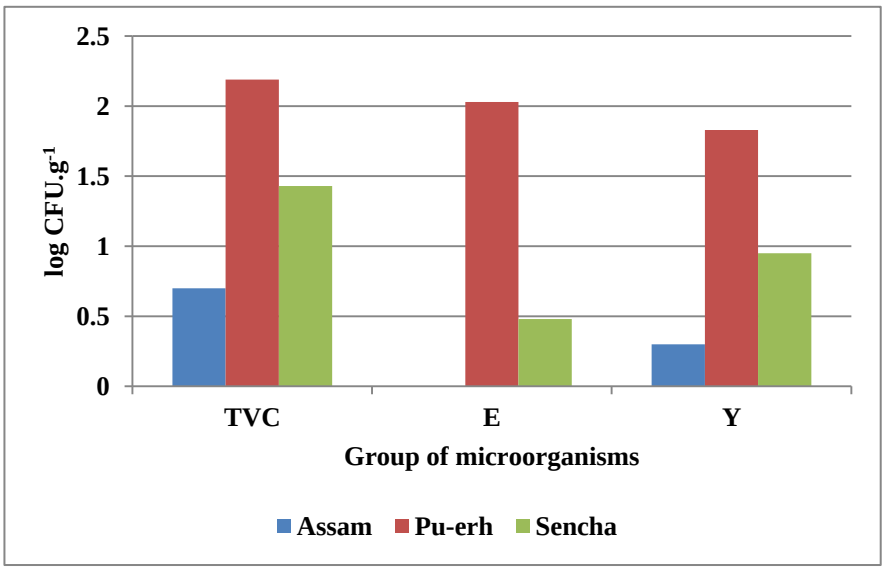

Figure 1 The number of microorganisms in log CFU.g^{-1} in tea samples.

NOTE: TVC - total viable count, E - *Enterocacteriaceae*,Y - yeasts.

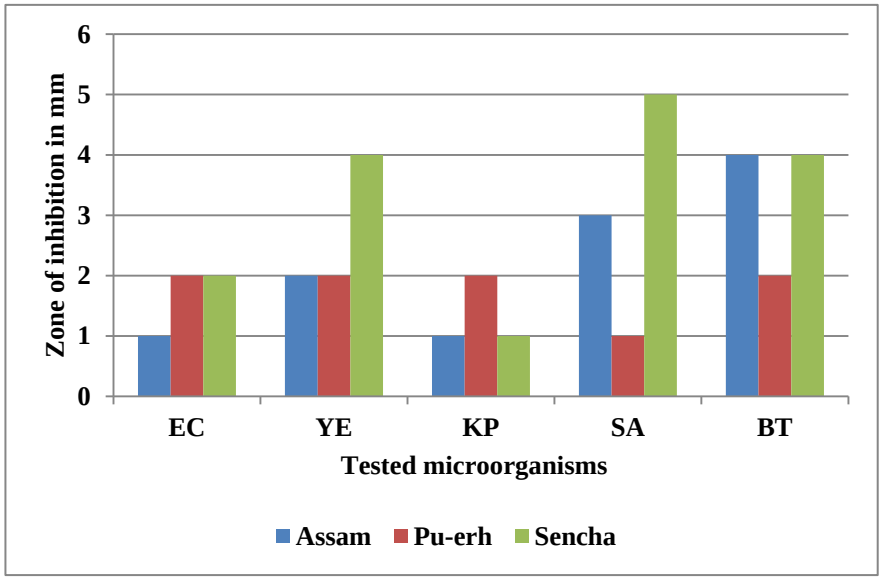

Figure 2 Antimicrobial activity of selected tea against bacteria by disc diffusion method.

NOTE: EC - *Escherichia coli*, YE - *Yersinia enterocolitica*, KP – *Klebsiella pneumonie*, SA – *Staphylococus aureus*, BT - *Bacillus thurigiensis.*

In the study of **Radji et al. 2013** the MIC of green tea extract against MRSA was 400 µg.mL^{-1}, while the MIC for MDR– *P. Aeruginosa* was 800 µg.mL^{-1}. The anti-bacterial activity of green tea extract is comparable to standard antibiotic. The activity of 16 µg of green tea extract against the laboratory strain of *S. Aureus* ATCC 25923 was comparable to that of commercially available oxacillin (1 µg), whereas the activity of 16 µg green tea extract was comparable to that of commercially available gentamicin (10 µg) against the laboratory strain *P. Aeruginosa* ATCC 27853, even though green tea extract was slightly less effective. Green tea extract showed good antimicrobial activity against MRSA and MDR – *P. aeruginosa*, although both of these bacteria have been resistant to multiple classes of antibiotics.

The polyphenol contents of green tea have been reported to inhibit the varieties of pathogenic bacterial growth such as *Helicobacter pylori*, methicillin-resistant *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Salmonella typhi*, *Shigella dysenteriae*, *Shigella flexneri* and *Vibrio cholera*. Polyphenols in green tea were also found to be effective against human immunodeficiency virus, hepatitis, and influenza viruses. Dental caries and periodontal diseases are two the most prevalent plaques associated with oral infectious diseases produced by endogenous oral flora. *S. mutans* and *S. sobrinus* are known as the main etiological

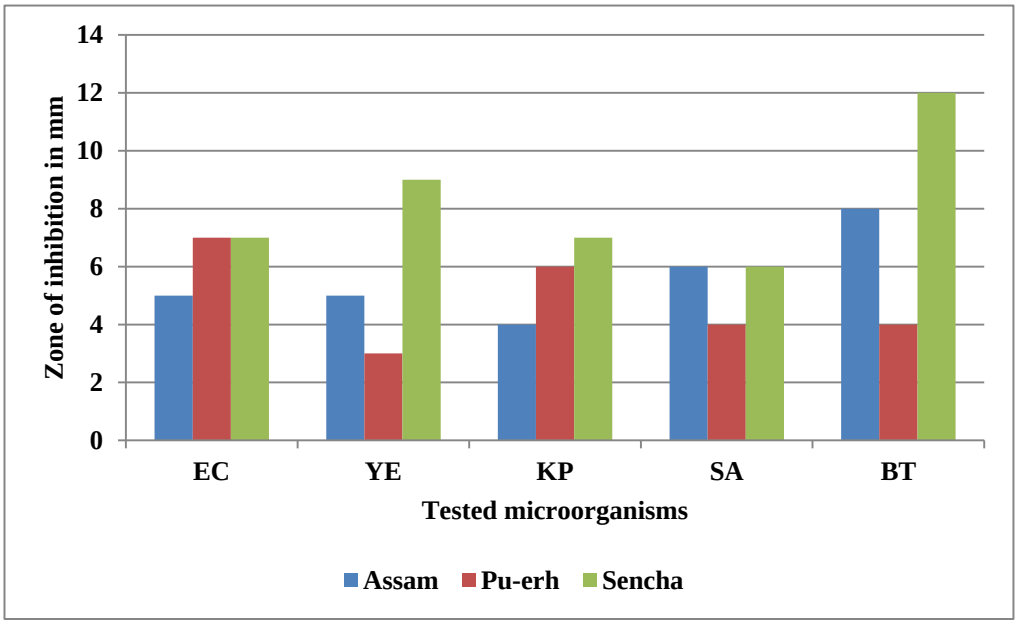

Figure 3 Antimicrobial activity of selected tea against bacteria in mm.

NOTE: *EC - Escherichia coli, YE - Yersinia enterocolitica, KP - Klebsiella pneumonie, SA - Staphylococcus aureus, BT - Bacillus thurigiensis.*

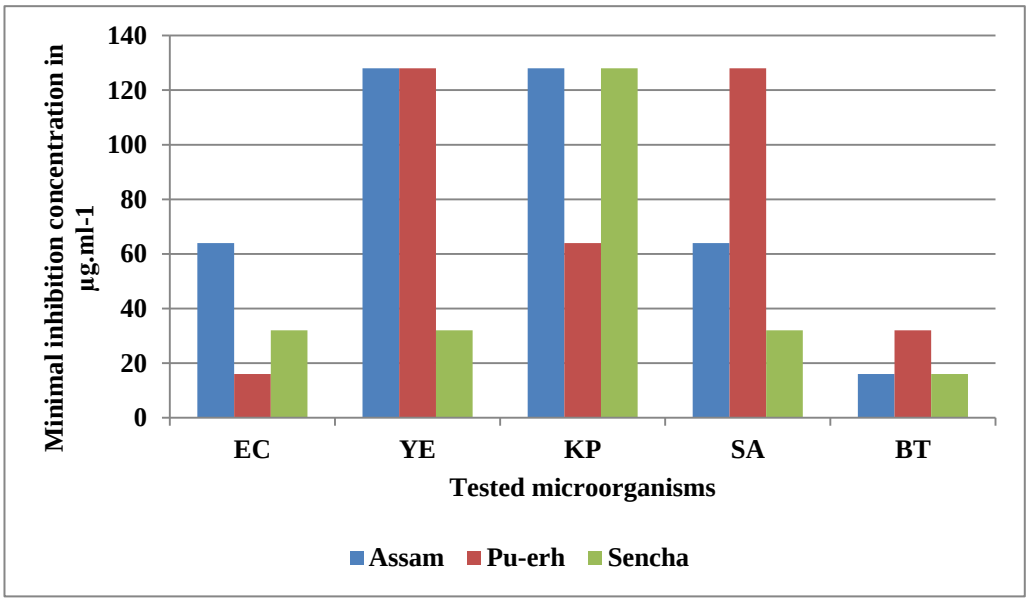

Figure 4 Minimal inhibition concentration of selected tea against bacteria in µg.mL^{-1}

NOTE: *EC - Escherichia coli, YE - Yersinia enterocolitica, KP - Klebsiella pneumonie, SA - Staphylococcus aureus, BT - Bacillus thurigiensis.*

agents of dental caries. These cariogenic bacteria adhere to the tooth surface and produce a sticky glycocalyx film composed of glucan resulting from the action of glucosyltransferase on dietary sucrose. Accumulation of bacteria causes dental plaque formation within which there is continuing acid production by the bacterial plaque **(Araghizadehet al., 2013)**. Tea extract exhibited the inhibitory effect also to those microorganisms.

The minimum inhibitory concentration (MIC) of the ethanol extract is shown in Figure 4. The lowest antibacterial activity was typical for Pu-erh against *Escherichia coli*.

CONCLUSION

The mass spectrometry for identification of tea microflora was used and altogether the presence of seven Gram-positive and four Gram-negative bacteria species were revealed. Tea extracts exhibited the antimicrobial activity, thus have a potential antimicrobial activity against microorganisms even agains the pathogens.

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Contact address:

Jana Hutková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: rapcanova.pk@gmail.com.

Attila Kántor, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: attila.kantor@uniag.sk.

Margarita Terentjeva, Latvia University of Agriculture in Nitra, Faculty of Veterinary Medicine Institute of Food and Environmental Hygiene, K. Helmaņa iela 8, LV-3004, Jelgava, Latvia, E-mail: margarita.terentjeva@llu.lv.

Jana Petrová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.petrova@uniag.sk.

Czeslaw Puchalski, Faculty of biology and agriculture, University of Rzeszow, Department of bioenergy Technologies, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: cpuchal@univ.rzeszow.pl.

Maciej Kluz, Faculty of biology and agriculture, University of Rzeszow, Department of Biotechnology and Microbiology, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: kluczyk82@op.pl.

Rafal Kordiaka, Faculty of biology and agriculture, University of Rzeszow, Department of Biotechnology and Microbiology, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: rafal.kordiaka@wp.pl

Simona Kunová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety and, Tr. A. Hlinku 2, 949 76, Nitra, Slovakia, E-mail: simona.kunova@uniag.sk.

Miroslava Kačániová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@uniag.sk.

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THE CONTENT OF TOTAL POLYPHENOLS IN DIFFERENT VARIETIES OF *SOLANUM TUBEROSUM* GROW IN SPIŠ AREA

Beáta Volnová, Janette Musilová, Miriam Kopernická, Petra Kavalcová, Ľuboš Harangozo, Michal Medvecký

ABSTRACT
Potatoes can be classified into groups of foods that are consumed regularly and in relatively large quantities, they are an essential source of polyphenol compounds. Phenolic compounds are the predominant antioxidants in nutrition and their study is currently being paid much attention. These antioxidants act synergistically; polyphenol compounds protect vitamin C and β-carotene, which in turn helps to increase the effect of vitamin E. Potatoes are very popular vegetables in Slovakia, not only in terms that they are easy to prepare, but also by the fact that they combine the wholesomeness of cereals and delicacy and characteristic chemical composition of vegetables. It is important that they find their place in our diet. Nutritional value of potatoes is determined by the content of nutrients such as protein, starch, fat, minerals, and absence of toxins, as well as by a significant content of bioactive components from the group of polyphenols. The study was performed in order to analyse 7 Slovak potato varieties from Spiš area, according to biologically active compounds: such as polyphenols. The content of total polyphenols was determined by the method of Lachman et al., (2003). The lowest determined content of total polyphenol (mg.kg^{-1} dry matter) in locality Spišský Štvrtok was measured in a variety Victoria (795.05 mg.kg^{-1} dry matter) and the highest content of total polyphenols in locality Spišský Štvrtok was measured in variety Laura (1238.42 mg.kg^{-1} dry matter). In the locality Odorín was determine the lowest content of total polyphenols in variety Red Anna (974.09 mg.kg^{-1} dry matter) and the highest content of total polyphenols was determined in variety Laura (978.95 mg.kg^{-1} dry matter). Between all varieties in locality Spišský Štvrtok was confirmed the statistically significant difference in the influence of the variety in the contents of total polyphenols (mg.kg^{-1} DM). This varietal dependence was not appear in samples taken in the locality Odorín. The total polyphenols content of the potatoes can be influenced by other factors, for example locality. In this case, there were statistically significant differences in the content of total polyphenols in variety Laura obtained from two different localities.

Keywords: potato; variety; total polyphenol; compound; Spiš area

INTRODUCTION

Eatable potatoes have an important role in the production of agricultural crops and also of produced food raw materials. The potatoes are frequently consumed in Europe but sometimes overlooked nutritional quality of this staple crop. *Solanum tuberosum* follows only rice and wheat in world importance as a food crop for human consumption. Cultivated potatoes have spread from the Andes of South America where they originated to 160 countries around the world. Consumption of fresh potatoes has declined while processed products have increased in popularity.

According to **Frančáková, et al., (2001)**, potato tuber is growing for its rhizone tubers, which is known as potatoes. Potatoes filled in human nutrition mainly for volume function, than eating function and protective function. Potatoes are an important food, industrial raw materials, feed and a major agricultural crops with high yield potentially useful biomass. As the potato becomes a staple in the diets of an increasing number of humans, small differences in potato nutritional composition will have major impacts on population health (**Camire et al., 2009)**.

According to **Lisińska (2006)** the nutritive value of potato is relatively high, because of protein content and composition (high percentage of essential amino acids: lysine, leucine, phenyloalanine, threonine and valine). Potato is also characterised by high amounts of starch, and lower content of sugars, minerals (K, Mg, Fe, Cu, J, P) and vitamins of group B, folic acid, fat-soluble vitamins E, K, and carotenoids, which may be converted into vitamin A **(Wroniak, 2006)**. The content of vitamins in tubers is not high, however 200 g of potatoes covers much of the daily requirement for these compounds, especially vitamin B6 (20 – 26%), vitamin B1 (12 – 20%), niacin (10 – 20%), folic acid (4 – 12%), and pantothenic acid (10%) **(Lisińska and Leszczyński, 1989)**. According to **Astley (2003)** *Solanum tuberosum* is an excellent source of vitamin C and other biologically active substances, such as polyphenols and flavonoids, which are commonly described as antioxidants. These substances have beneficial influence on human organism, as they protect against cardiovascular disease, and cancer, as well as reduce blood cholesterol level. The potato is a carbohydrate-rich, energy-providing food with little fat. Potato protein content is fairly low but has an excellent biological value of 90 – 100. Potatoes are particularly high in vitamin C and are a good source of several B vitamins and potassium. The skins provide

Table 1 Characterictics of soil and nutrient content.

Point of delivery	pH (KCl)	C_{ox}	mold	P	K	Ca	Mg
		%		(mg.kg^{-1})			
Matejovce	5.75	1.56	2.69	36.27	191.03	2780	193.50
Spišský Štvrtok	5.22	2.74	3.21	30.23	178.38	1710	180.0
Odorín	5.19	2.22	2.83	82.71	179.75	1590	161.0

substantial dietary fiber. Many compounds in potatoes contribute to antioxidant activity and interest in cultivars with pigmented flesh is growing. Potato tubers present in human nutrition an important source of antioxidants. According to **Musilová et al., (2010)** in the potatoes are the greatest extent represented polyphenols (1226 – 4405 mg.kg^{-1}) and ascorbic acid (170 to 990 mg.kg^{-1}), carotenoids (4 – 4.5 mg.kg^{-1}), α-tocopherol (0.5 to 2.8 mg.kg^{-1}), in small amounts of selenium (0.1 mg.kg^{-1}) and α-lipoic acid. Polyphenols are important sources in potatoes. They are divided into two main groups: phenolic acids and flavonoids, which create from 1/3 to 2/3 of all antioxidants **(Tapiero et al., 2002**; **Musilová et al., 2013)**.

Polyphenols are a group of plant secondary metabolites that are markedly represents in the diet of humans and animals. The main factor responsible for the delayed research on polyphenols is the variety and the complexity of their chemical structure **(D'Archivio et al., 2007**; **Hegedűsová et al., 2015)**.

Lachman et al., (2013) present that phenolic compounds are the most commonly used group of antioxidants, most of which is represented by the chlorogenic acid isomers, and caffeic acid. Polyphenolic substances contained in foods of plant origin are at present pursued plant components. According to **Suli et al., (2014)** polyphenols are found in normal foods of plant origin in varying amounts. The content of phenolic compounds in natural materials is quite variable, depending on each type of crop, but also of their varieties. According to **Mareček et al., (2013)** in the varietal composition, we have to take more account of varietal differences, especially in the carbohydrates content and in the options of processing to different products. Polyphenol content is conditioned by genetically influenced and agronomic soil and weather or environmental conditions. **André et al., (2009)** classed the polyphenols into a group of natural antioxidants. About the effect of polyphenolic substances on human health is constantly debated in professional and general level, with views on the action of these agents are not completely uniform **(Lachman et al., 2013**; **Volnová et al., 2015)**. Content of total polyphenolic compounds and anthocyanins is dissimilar at different stages of tuber maturity; it is affected by different environmental conditions, e.g. longer days and lower temperatures **(Reyes et al., 2004)** or ecological way of cultivation **(Hamouz et al., 2005)**.

The aim of this research was to evaluate a set of seven potato varieties and watched the content of total polyphenols in different varieties of *Solanum tuberosum* grow in Spiš area.

MATERIAL AND METHODOLOGY

Material: For analyses we used seven potato varieties from Spiš area: Victoria, Laura (Spišský Štvrtok), Belana, Laura (Odorín), Red Anna, Marabel, Malvína (Matejovce), which were analysed for the content of biologically active compounds: total polyphenols and potato varieties were collected from Spiš area. Each variety was removed from four places of our area of interest.

Methods: analysis of potatoes: Total polyphenols were determined by the method of **Lachman et al., (2003)** and expressed in mg eq. gallic acid per kg dry matter. 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 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.

Analysis of soil: In each locality we determined exchange soil reaction (pH/KCl) – was determined oxidimetry %, with using translation method of Ťurin, Cox carbon content (%) and mold (%) – were determined oxidimetry %, with using translation method of Ťurin, and content of macroelements (mg.kg^{-1}) – we set by Mehlich II method, analytical method for the determination of output was atomic absorption spectrophotometer (AAS Varian AA Spectr DUO 240FS/240Z/UltrAA). We evaluated the indicators based on the Code of Good Agricultural Practice **(Bielek, 1996, Decree no. 338/2005 Coll.)** Results were statistically evaluated by the Analysis of Variance (ANOVA – Multiple Range Tests, Method: 95.0 percent LSD) using statistical software STATGRAPHICS (Centurion XVI.I, USA) and the regression and correlation analysis (Microsoft Excel) was used.

RESULTS AND DISCUSSION

The results of the analysis of different locations are referred to Table 1. Soil from Matejovce area have been weakly alkaline, with middle content of mold, very low content of phosphorus, middle content of potassium and good content of magnesium (193.50 mg.kg^{-1}). The soil from locality Odorín is alkaline, with middle content of mold, high content of P, middle content of K and good content of Mg and soil from Spišský Štvrtok area is alkaline too, with middle content of mold, with good content of phosphorus, middle content of K and good content of magnesium. Nowadays, the great emphasis is placed on research of polyphenols from plant extracts, as well as their biological activity **(Arnal et al., 2012; Duchnowicz et al., 2012**; **Stojadinovic et al., 2013**; **Zhang et al., 2013)**. The polyphenols are the most abundant antioxidants in the

Table 2 Multiple Range Tests for the locality effect on the total polyphenols content (mg.kg^{-1} DM) in potato tubers (Spišský Štvrtok locality).

Variety	Count	Mean	Homogenous groups
Victoria	16	795.05 ±108.92	X
Belana	16	956.75 ±129.54	X
Laura	16	1238.42 ±23.31	X

Method: 95,0 percent LSD.

Table 3 ANOVA Table for total polyphenol content by variety.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*P*-Value
Between groups	1.61096E6	2	805480.0	82.79	0.0000
Within groups	437810.0	45	9729.1		
Total (Corr.)	2.04877E6	47			

human diet **(Bystrická et al., 2010)**. They are known to exhibit stronger antioxidant activity than monophenols **(Troszynska et al., 2002)**. In our work, we watched the locality effect on the total polyphenols content in different varieties of potatoes. The results of analyzes of individual samples have statistically processed (Table 2 – 7)

As we can see in Table 2, determined values of total polyphenol content in three different varieties were in range from 795.05 mg.kg^{-1} DM (locality Spišský Štvrtok, Victoria variety) to 1238.42 mg.kg^{-1} DM (Spišský Štvrtok area, Laura variety). Minimal measured values in variety Victoria was 614.72 mg.kg^{-1} DM, in variety Belana was 722.72 mg.kg^{-1} DM and in variety Laura was 1202.72 mg.kg^{-1} DM. Maximal measured value in variety Victoria was 993.28 mg.kg^{-1} DM, in variety Belana was 1112.12 mg.kg^{-1} DM and in variety Laura was 1268.88 mg.kg^{-1} DM. Statistically significant differences in total polyphenols content between individual variety is confirmed. The differences were between varieties Victoria – Belana and Laura, between varieties Belana – Victoria and Laura and between variety Laura – Belana and Victoria.

Between the content of total polyphenols in varieties of locality Odorín were only minimal differences, while the lowest and highest TPC we have established in variety Laura (minimum: 883.12 mg.kg^{-1} DM and maximum 1037.76 mg.kg^{-1} DM, which is almost 18% difference). The difference between the lowest and highest average value of TPC is only 0.5%. These contents of total polyphenols in testing varieties were determined only with minimal difference. There were not statistically significant differences between different varieties. The content of total polyphenols was not different in average

Based on the results of the statistical evaluation it can be stated that there are significant differences in potatoes of the same variety Laura from different areas (Odorín and Spišský Štvrtok). The TPC in variety Laura from locality Spišský Štrvtok was nearly about 27% higher as from Odorín area. Minimal measured value was in variety Laura 883.12 mg.kg^{-1} DM from locality Odorín, and maximum was in variety Laura 1268.88 mg.kg^{-1} DM from locality Spišský Štvrtok.

The average TPC of all samples middle early varieties was in the variety Victoria 795.05 mg.kg^{-1} DM and standard deviation was 108.92, in variety Belana was average value of TPC 956.75 mg.kg^{-1} DM and standard deviation was about 129.54 and in the last variety Laura from Spišský Štvrtok was average value TPC 1238.42 mg.kg^{-1} DM and standard deviation was 23.31. Based on these results it can be assumed correlation between the location and the total polyphenol content in potatoes, which is confirmed by the results of many authors who deal with the issue **Reddivari et al., (2007)**; **Hamouz et al., (2007)**. **Burgos et al., (2013)** as one of the key factors indicate variety and the conditions in their processing, too. **Lachman et al., (2008)** also confirm the significant effect of locality, which have a high content of TPC and higher

Table 4 Multiple Range Tests for the effect of variety on the total polyphenols content (mg.kg^{-1} DM) in potato tubers (locality Odorín).

Variety	Count	Mean	Homogenous groups
Red Anna	16	974.09 ±42.44	X
Marabel	12	977.79 ±31.77	X
Laura	16	978.95 ±54.16	X

Method: 95,0 percent LSD

Table 5 ANOVA Table for total polyphenol content by variety.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*P*-Value
Between groups	203.218	2	101.609	0.05	0.9506
Within groups	82124.7	41	2003.04		
Total (Corr.)	82327.9	43			

Table 6 Multiple Range Tests for the effect of variety Laura on the total polyphenols content (mg.kg^{-1} DM) in potato tubers (Odorín and Spišský Štvrtok localities).

C.locality	Count	Mean	Homogenous groups
Odorín	16	978.95	X
Sp. Štvrtok	16	1238.42	X

Method: 95,0 percent LSD

Table 7 ANOVA Table for total polyphenols content by variety Laura.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*P*-Value
Between groups	538597.0	1	538597.0	309.82	0.0000
Within groups	52152.3	30	1738.41		
Total (Corr.)	590750.0	31			

yields of potatoes in the area, which had the lowest average annual temperature and minimum daily temperatures. Further the author notes, that the content of total polyphenols can be influenced by variety. This fact is confirmed by many other authors. According to **Navarre et al., (2011)** a difference in the content of total polyphenols may be caused, for example, genotype or varietal affiliation. The influence of variety as an important factor influence the polyphenol content in potato tubers is confirmed by our results.

Pawelzik et al., (1999) and **Friedman (1997)** determined a significant effect of variety on TP content, which has already been confirmed by our results.

CONCLUSION

Polyphenols are secondary metabolites of plants with antioxidant properties.

The potato is one of the richest sources of antioxidants in the human diet. The main antioxidants are polyphenols (123 – 441 mg 100 g^{-1}), ascorbic acid (8 – 54 mg 100 g^{-1}), carotenoids (up to 0.4 mg 100 g^{-1}) and tocopherols (up to 0.3 mg 100 g^{-1}). L-Tyrosine, caffeic acid, chlorogenic acid and ferulic acid are amongst the main polyphenols, which have about twice the level in the skin compared with the flesh of the potato. In terms of chemical structure, it is a diverse group of chemically related substances, which are divided into several classes and subclasses. Technological processes used in the food production, storage and the meals treatment lead to changes in polyphenol content in foods. These factors together effect the representation of polyphenols in foods and also their utility.

Content of polyphenols is especially affected by variety, year of cultivation, stress factors (mechanical damage of tubers, attack of pathogens or action of light on tubers) and by cooking treatment. In a lesser extent the effect of locality, potassium fertilization, storage temperature, γ-irradiation and other factors could be involved, but there is only a little demonstrable empirical evidence in the literature references.

Polyphenolic exceed biological activity in the human body, among others they can take active part in the removal of free radicals, metal ion chelatation as well as affect enzyme activity and protein availability. Although their health beneficial properties, polyphenolic compounds are prevalent, between others, coronary heart disease, cancer, inflammatory diseases.

Nutritional value of potatoes is influenced by the content of nutrients, absence of toxic substances and presence of biologically active polyphenols, which are responsible for antioxidant activity of this vegetable. Potato is easy to prepare, widespread and versa, as it combines energy value of cereals and chemical composition typical for vegetables. It is therefore very important to include it in our everyday diet.

In our work we deal with the research of changes to the total polyphenols content in different varieties of potatoes. In conclusion we can say that the lowest content of total polyphenols we found in a variety Victoria of locality Spišský Štvrtok and the highest content of total polyphenols we have established in a Laura variety of locality Spišský Štvrtok. Total polyphenols content was statistically significant in the area Spišský Štvrtok and statistically not significant in the area Odorín. So the effect of locality on the content of total polyphenols in potato tubers in variety Laura was statistically significant.

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Contact address:

Ing. Beáta Volnová, Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, Tel.: +421 376 414 375, E-mail: bejatkavolnova@pobox.sk.

Ing. Miriama Kopernická, Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, Tel.: +421 376 414 376, E-mail: m.kopernicka@gmail.com.

doc. Ing. Janette Musilová, PhD., Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia; Tel.: +421 37 641 4606, E-mail: janette.musilova@uniag.sk.

Ing. Petra Kavalcová, Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra,

Slovakia, Tel.: +421 37 641 4375, E-mail: kavalcova.petra@gmail.com

Ing. Ľuboš Harangozo, PhD., Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, Tel.: +421 37 641 4497, E-mail: harangozolubos@gmail.com.

Ing. Michal Medvecký, Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, Tel.: +421 37 641 4497, E-mail: medvecký.michaling@gmail.com.

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CHARACTERIZATION OF PROTEIN FRACTIONS AND ANTIOXIDANT ACTIVITY OF CHIA SEEDS (*Salvia Hispanica* L.)

Kvetoslava Kačmárová, Blažena Lavová, Peter Socha, Dana Urminská

ABSTRACT

Chia seed (*Salvia hispanica* L.) is an annual herbaceous plant categorized under *Lamiaceae* family. Chia seeds were investigated as a source of proteins and natural antioxidants. It is a potential alternative source of high quality protein, fats, carbohydrates, high dietary fibre, vitamins and mineral elements. The objective of this study was to evaluate chia seed from protein content and antioxidant acivity and highlight the quality of this pseudocereal. A crude protein, moisture content, content of protein fractions, total antioxidant capacity (TAC) and superoxide dismutase activity of chia seeds and food products containing chia seeds were determined. The protein content of chia seeds ranged from 2.9% to 4.6% dry matter from that albumins and globulins ranged from 54.6% to 62.8%. Chia is poor in a prolamines (<15%). Various chia seeds showed differences in their SOD activity and exhibited the high antiradical activity against 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The highest antioxidant capacity was found in sample chia seeds from Bolivia (1.46 mM TEAC.g^{-1} in the dry matter) and the lowest values of antioxidant activity was estimated in sample chia seeds from Argentina (1.05 mM TEAC.g^{-1} in the dry matter). The highest SOD activity was determined in sample chia from Argentina (2191.8 U.g^{-1} in the dry matter). The lowest SOD activity was found in sample chia-bio from Argentina (754.0 U.g^{-1} in the dry matter.). It makes them potentially suitable for use in the gluten-free diet of coeliac people and it can be used as a potential ingredient in health food because of its high antioxidant activity.

Keywords: chia seed; protein; protein fraction; antioxidant activity

INTRODUCTION

In recent years, demand for food with multiple health benefits has increased. There is an interest to introduce a new food to prevent various disorders **(Mohd Ali et al., 2012)**.

During recent decades, it has been demonstrated worldwide increase in allergies and intolerances to certain foods, which is associated with nutrition, lifestyle, economic growth and urbanization **(Gilissen et al., 2014)**. For example, the most common food-induced enteropathy, caused by intolerance to cereal proteins (gluten), is coeliac disease, also termed coeliac sprue. Large number of coeliac patients is also lactose-intolerant. It leads to mineral, vitamin and protein deficiencies in the diet suitable for them **(Arendt et al., 2011)**.

Chia seeds (*Salvia hispanica* L.) are one of the potential alternative sources of high quality protein, fats, carbohydrates, high dietary fibre, vitamins and mineral elements. They also contain a high amout of antioxidants, and therefore are reintroduced to diets to provide health benefits for patients and healthy persons **(Segura-Campos et al., 2014)**.

Salvia hispanica L. is a plant of the *Lamiaceae* or *Labiatae* family native of central and southern America, and grows in arid climates. It can grow up to 1 meter tall, has opposite arranged leaves and small flowers (Figure 1). It produces a small white and dark seeds **(Mohd Ali et al., 2012)**, which are considered a pseudocereals and an oilseeds (Figure 2) **(Sandoval-Oliveros and Paredes-Lopéz, 2012)**.

Due to the composition of seeds, they present a good alternative source of proteins for humans. Chia seeds contain a higher amount of proteins (19-23%) than other traditionally used grains, such as wheat (14%), barley (9.2%), oats (15.3%), corn (14%) and rice (8.5%) **(Monroy-Torres et al., 2008; Sandoval-Oliveros and Paredes-Lopéz, 2012)**.

Determinant of quality proteins is digestibility. It is the amount of protein absorbed into the body relative to the amount that was consumed. Protein digestibility of c hia flour is 79.8%, according to **Monroy-Torres et al., (2008)**, as well as cereals processed for direct consumption (corn, wheat, oats, etc.).

Chia seeds are also rich in natural antioxidants, especially phenolic compounds such as chlorogenic acids, caffeic acids, kaempferol and quercetin. All of the mentioned characteristics may reduce cardiovascular diseases, regulate an intestinal transit or prevent of some diseases such as type II diabetes and some types of cancer **(Sandoval-Oliveros and Paredes-Lopéz, 2012)**.

The main objective of the present study is to characterize and evaluate a content of proteins, protein fractions and antioxidant activity in Slovakia commercially available chia seeds.

MATERIAL AND METHODOLOGY

Chia seeds (*Salvia hispanica* L.) were obtained from local markets in Nitra, Slovak Republic. Three types of chia seeds from different producers were used for analysis. The first sample of chia seeds is originating in Bolivia, the

Figure 1 *Salvia hispanica* L. plant, **(Mohd Ali et al., 2012)**.

Figure 2 Chia seed, **(Mohd Ali et al., 2012)**.

second sample of chia was originating in Argentina and as produced by ecological farming (bio). The last sample of chia was harvested also in Argentina (conventional farming). For analysis, we used a bio-raw apricot flapjack (containing 4% of chia seeds) and chia spelled biscuits (containing 3% of chia seeds). Flours and food products for analyses were prepared by milling (BOSCH, MKM 6000).

Moisture content was determined according to the ICC Standard Method No. 110/1 for cereals and cereal products. Approximately 8 g of each sample of seeds were weighed into special aluminium dishes and dried until constant weight, using a moisture analyzer KERN DBS 60-3.

For determination of crude protein content was used 500 mg of each sample of milled chia flours and products with chia seeds. Nitrogen content was measured by the Kjeldahl method according to the **ICC Standard Method No. 105/2 (1994)**. The samples were digested in a Kjeldahl Digestion Unit type DK6 (Velp Scietifica), using cupric sulfate and potassium sulfate as catalysts. The digested samples were than destilled using UDK 127 Destilation Unit (Velp Scientifica) and the destilates were titrated with H_2SO_4 (c = 0,1 M). The protein content was calculated as nitrogen x conversion factor f (N x 6.25).

For extraction of protein fractions was used 2500 mg of each sample of chia flours and milled food products. Fractionation of proteins (albumin, globulins, prolamins and glutelins) was carried out according to the Golenkov, using modification of the method reported by **Michalik (2002)**. The protein content of the isolated fractions was assessed by Kjeldahl method.

In this study the QUENCHER procedure was used to measure the total antioxidant capacity (TAC) using $ABTS^+$ assay **(Serpen et al., 2012)**. All three samples of chia seeds needed to be diluted at 1:1 (w/w) with cellulose. ABTS was dissolved in deionized water to a concentration of 7 mM. The radical cation of ABTS was obtained by reaction with 2.45 mM potassium persulfate and allowing the stock solution to stand in the dark at room temperature for at least 12 hours **(Re et al., 1999)**. The working solution of $ABTS^+$ was prepared by diluting 10 mL of $ABTS^+$ stock solution with approximately 800 mL of a water/ethanol (50:50, v/v) mixture. The working solution absorbance was 0.750 – 0.800 at 734 nm **(Sargi et al., 2013)**. Ten (±1.0) mg of powdered sample was weighed into a centrifuge tube having 15 mL capacity. The reaction was started by adding 10 mL of $ABTS^+$ working solution. The tube was shaken rigorously for 1 minute and placed on shaker in the dark. The mixture was shaken at 350 rpm at room temperature on the shaker (ThermoMixer C, Eppendorf) to facilitate the surface reaction between the solid samples and $ABTS^+$ solution. After exactly 30 minutes for ABTS probe from the first introduction of radical/oxidant solution onto solid samples, centrifugation (Avanti J-25, Beckman Coulter) was performed at 9,200 x g for 2 minutes. Optically clear supernatants were transferred into spectrophotometric cuvette and the absorbance values were measured at 734 nm for ABTS assay (6705 UV/VIS spectrophotometer, JENWAY). The TAC of samples determined with ABTS assay were calculated in mmol of Trolox equivalent antioxidant capacity (TEAC) per g of sample using the calibration curves **(Serpen et al., 2012)**.

In this study the diagnostic Ransod set (RANDOX, Great Britain) was used for the determination of superoxide dismutase activity. The principle of the method was based on the xanthine and xanthine oxidase that produce superoxide radicals reacting with tetrazolium salt to red formasan. SOD activity is determined as a degree of inhibition of this reaction which occurs at 37 °C. Following preparation was identical both for the prepared yeast samples and standards from which the calibration curve was constructed **(Březinová Belcredi et al., 2010)**.

The chia seeds were homogenized in chilled 0.1 M sodium phosphate buffer (pH 7.4) to prepare a 10% homogenate. The homogenate was centrifuged at 10,000 x g at 4 °C for 10 minutes and the supernatant was used for assays **(Sangeetha, 2010)**. The sample (0.05 mL) and the substrate (1.7 mL) were added into a cuvette and the mixture was carefully blended. Reaction was started by addition of xanthine oxidase (0.25 mL). The cuvette was placed into the spectrophotometer and an absorbance of 505 nm was measured. The first absorbance was measured after 30 seconds (A_1) and the second after 3 minutes (A_2). The result was converting to SOD units/g of sample.

RESULTS AND DISCUSSION

The moisture content, the total protein content and the proportion of the protein fractions of chia seeds and products with chia are summarized in the Table 1.

The moisture content of chia samples ranged from 5.8 to 6.72%. These results are within the range of 4.5% to 6.8% reported by numerous authors **(Monroy-Torres et al. 2008; Coorey et al. 2012; Sandoval-Oliveros and Paredes-Lopéz 2012; Segura-Campos et al. 2014)**.

Chia seeds are characterized by a high protein content. Studies according to **Monroy-Torres et al., (2008)**, **Sandoval-Oliveros and Paredes-Lopéz (2012)** and **Segura-Campos et al., (2014)**, describe the value of protein content of 15 – 23%. In the present study, protein content of all samples of chia flour was very low and ranged from 2.9 to 4.6%. It could be caused by using unmodified chia flours. **Sandoval-Oliveros and Paredes-Lopéz (2012)** used defatted and dried flours of mucilage-free chia seeds for the same analysis, with the result 23% of proteins in dry solids.

After protein extaction and fractionation by solubility, all fractions were quantified by Kjeldahl method. The proportion obtained from chia 1 (Bolivia) was 55.8% of crude albumins and globulins, 13.8% of prolamins, 9.5% of glutelins, whereas 20.9% of the protein wasn´t recovered. The proportion obtained from chia 2 (bio-chia, Argentina) was 62.8% of crude albumins and globulins, 14.2% of prolamins, 15.1% of glutelins, whereas 7.9% of insoluble residue. In the chia 3 (Argentina), the content of albumins was 54.6%, 12.5% were prolamins, 15.2% were glutelins and the content of insoluble residue was 17.7%. Albumins and globulins were the most abundant (from 55.8% to 62.8%) followed by glutelins (9.5% – 15.2%), and prolamins (12.5 – 14.2%). The values are similar to those reported by **Sandoval-Oliveros and Paredes-López (2012)**, excluding the values of the insoluble residues (7.9 – 20.9%), which were higher.

Palenčárová and Gálová (2009) investigated the proportion of each protein fraction in selected cereals. Compared to their results, chia seeds contained double amount of nutritionally valuable fraction of storage proteins, albumins and globulins, compared to the common used cereals (wheat 25.4%, barley 27.12%, rye 41.34% and oats 20.22%). The content of the celiac active prolamin fraction was twice lower, compared to wheat (36.7%), barley (32.57%) and rye (28.75%). The prolamin content in chia was also lower than that of oats (16.65%). The glutelins content was determined lower, and content of insoluble residues was detected higher in chia seeds to that of above-mentioned cereals. The values of mentioned protein fractions are also consistent with those reported by **Gálová et al., (2011)**.

The dominance of albumin and globulin fractions was proved in pseudocereals such as amaranth **(Hricová et al., 2011)** and buckwheat **(Guo and Yao, 2006)**. The only difference found between amaranth (*Amaranthus cruentus*) and chia was a higher proportion of glutelins and lower proportion of prolamins in chia seeds. On the other hand, the protein fractions of chia and buckwheat (*Fagopyrum tataricum*) were very similar, except insoluble residues that were not mentioned by authors **Guo and Yao (2006)**.

From the results shown in Table 1 it follows that a content of crude protein detected in chia biscuits and apricot chia flapjack was 1.3% and 1.5%, respectively. Both types of chia meals presented a various composition of protein fractions. The main protein fraction corresponded to glutelins (41.9%) and fractions of albumins and globulins (39.3%) in spelt chia biscuits and apricot flapjack, respectively. The food products contain low level of prolamins, 11.9% in spelt biscuits and 17.3% in apricot flapjack. The differences were caused by various compositions of these meals, which represent a complex food composed of several different nutritionally valuable constituents. Spelt and oat, the two main components of the products, were also different in the nutritional value, thus in the composition of protein fractions **(Socha et al., 2010)**.

The values of a crude protein in products were also different than those found for chia seed samples. From the nutrition point of view, chia seeds are better source of valuable proteins, compared to food products containing insignificant amount of chia seeds.

In the present study was used TAC measurement by QUENCHER method. The QUENCHER procedure eliminates time-consuming extraction steps, which assists to build a unique database and ease of comparison for the TAC of different food types. The solvent composition of probe radical solution had a significant influence on TAC measured by direct QUENCHER. In this procedure, the solvent not only acted as a reactant carrier but also a food matrix solubilizer **(Serpen et al., 2012)**.

Based on the results of trolox standard curve (Figure 3) it was possible to calculate the trolox equivalent antioxidant capacity for chia seeds.

Table 1 The total protein content and the proportion of the protein fractions of chia seeds and products containing chia.

Sample (Coutry of Origin)	Content of Chia seeds (%)	Crude Protein (%)	Albumins and Globulins (%)	Glutelins (%)	Insoluble Residue (%)
Chia (Bolivia)	100	2.9	55.8	9.5	20.9
Chia, bio (Argentina)	100	3.6	62.8	15.1	7.9
Chia (Argentina)	100	4.6	54.6	15.2	17.7
Apricot flapjack (United Kingdom)	4	1.5	39.3	26.2	17.2
Chia spelled biscuits (Slovakia)	3	1.3	26.9	41.9	19.3
n*	-	3	3	3	3

Figure 3 Percentage quenching of absorbance at 734 nm as a function of Trolox concentrations built by using $ABTS^{+}$ working solution.

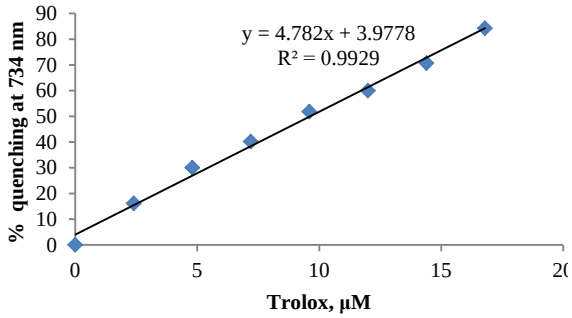

Figure 4 Calibration curve using for calculation of SOD activity.

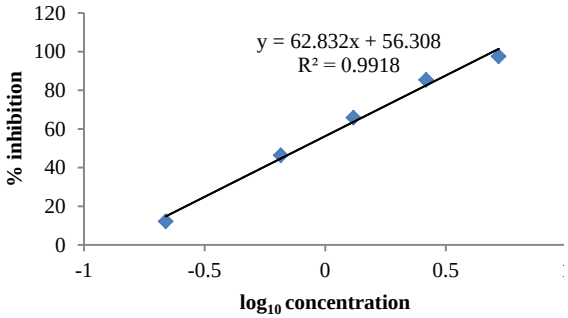

Table 2 Antioxidant capacity ($ABT^{\cdot+}$) and superoxide dismutase activity of chia seeds in the dry matter.

Samples (Country of Origin)	ATBS (mM TEAC.g^{-1} d.w.)	SOD (U.g^{-1} d.w.)	Moisture (%)
Chia (Bolivia)	1.46	1231.4	5.80
Chia, bio (Argentina)	1.13	754.0	6.72
Chia (Argentina)	1.05	2191.8	6.62
n*	3	3	3

* The number of repetitions, TEAC = trolox Equivalent Antioxidant Capacity, d.w. = dry weight.

Based on results (Table 2) it can be concluded that the highest antioxidant capacity was found in sample chia seeds from Bolivia (1.46 mM TEAC.g^{-1} in the dry matter) and the lowest values of antioxidant activity was estimated in sample chia seeds from Argentina (1.05 mM TEAC.g^{-1} in the dry matter). As seen from the Table 2, obtained data showed that there was a difference between TAC of tested samples which can be caused by a different variety or growing conditions.

Serpen et al., (2012) used the QUENCHER procedure for the $ABTS^{+\cdot}$ assay and they determined the antioxidant capacity of some seeds such as wheat (17.0 mM TEAC.kg^{-1} in the dry matter.), rice (14.9 mM TEAC.kg^{-1} in the dry matter) and rye (32.7 mM TEAC.kg^{-1} in the dry matter). These results are lower than those found for all seeds in present study for the $ABTS^{+\cdot}$ assay.

Sargi et al., (2013) determined the antioxidant capacity of chia seeds 2.56 mM TEAC.g^{-1} in the dry matter. These results are higher than those found for seeds in present study. **Vázques-Ovando et al., (2009)** found that antioxidant activity in the fiber-rich fraction of chia was 488 µM TEAC.g^{-1} in the dry mater, **Marineli et al., (2014)** reported for Chilean chia seeds 436 µM TEAC.g^{-1} and for Argentina chia meals **Capitani et al., (2012)** reported 557.2 µM TEAC.g^{-1} in the dry matter.

Chia is considered a seed with high antioxidant capacity, because is loaded with high amount of phenolic compounds **(Martínez-Cruz and Peredes-López, 2014)**. In this study was measured activity of superoxide dismutase, which protects the organism against the oxidatie damage caused by active oxygen forms **(Piterková et al., 2005)**. In this study was used a standard curve for determintion of SOD activity (Figure 4).

The highest SOD activity was determined in sample chia from Argentina (2191.8 U.g^{-1} in the dry matter). The lowest SOD activity was found in sample chia-bio from Argentina (754.0 U.g^{-1} in the dry matter.). There is no exact information about SOD activity in chia seed, so **Kolahi-Ahari (2006)** determined SOD activity in different species of kiwifruit on the level about 40 U.g^{-1} fresh weight. **Březinová-Belcredi et al., (2010)** detected superoxide dismutase activity in grain samples of 12 varieties and lines of spring barley in the interval 62 – 147 U.g^{-1} in the dry matter. In comparisom with these results it can be concluded that SOD activity in chia seeds is very high. According to the results in Table 2 it is evident that SOD activity in chia tested samples was very hight. Detection of hight antioxidant activity and SOD activity in chia seeds indicate the chia seeds to be a potential ingredient in health food products such as nutrition bars or cookies.

CONCLUSION

Based on the current research findings, chia seed is a good source of valuable protein fractions (albumins and globulins) and antioxidant compounds. The content of prolamins is low (<15%) what makes chia seeds potentially useful in the preparation of gluten-free products suitable for celiacs. The isolation and preparation of selected compounds from chia seeds could be used to produce potent natural antioxidants or ingredients with commercial applications in pharmacy, food industry or as a dietary suplements.

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Contact address:

Kvetoslava Kačmárová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra Slovakia, E-mail: xkacmarova@is.uniag.sk.

Blažena Lavová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra Slovakia, E-mail: blazena.lavova@uniag.sk.

Peter Socha, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology and Center of AgroBioTech, Tr. A. Hlinku 2, 949 76 Nitra Slovakia, E-mail: peter.socha@uniag.sk.

Dana Urminská, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra Slovakia and Institute of Chemistry, Slovak Academy of Sciences, Center for White-Green Biotechnology, Tr. A. Hlinku 2, SK-949 76 Nitra, E-mail: dana.urminska@uniag.sk.

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ANTIFUNGAL ACTIVITY OF LEMON, EUCALYPTUS, THYME, OREGANO, SAGE AND LAVENDER ESSENTIAL OILS AGAINST *ASPERGILLUS NIGER* AND *ASPERGILLUS TUBINGENSIS* ISOLATED FROM GRAPES

Miroslava Císarová, Dana Tančinová, Juraj Medo

ABSTRACT

Today, it is very important to find out the protection of products of natural origin as an alternative to synthetic fungicides. The promising alternative is the use of the essential oils (EOs). Essential oils from plants have great potential as a new source of fungicide to control the pathogenic fungi.The main objective of this study was evaluation of the antifungal activity of lemon (*Citrus lemon* L.), eucalyptus (*Eucalyptus globulus* LABILL.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) sage (*Salvia officinalis* L.) and lavender (*Lavandula angustifolia* MILLER.) EOs against *Aspergillus niger* and *Aspergillus tubingensis* isolated from grapes and their ability to affect the growth. It was tested by using the vapor contact with them. At first both tested isolates were identified by using PCR method. Sequence data of 18S rRNA supported the assignment of these isolates to the genus *Aspergillus* and species *A. niger* (ITS region: KT824061; RPB2: KT824060) and *A. tubingensis* (ITS region: KT824062; RPB2: KT824059). Second, EO antifungal activity was evaluated. The effect of the EO volatile phase was confirmed to inhibit growth of *A. niger and A tubingensis*. EOs were diluted in DMSO (dimethyl sulfoxide) final volume of 100 µL. Only 50 µL this solution was distributed on a round sterile filter paper (1 x 1 cm) by micropipette, and the paper was placed in the center of the lid of Petri dishes. Dishes were kept in an inverted position. The essential oils with the most significant activity were determined by method of graded concentration of oils - minimum inhibitory doses (MIDs). The most effective tested EOs were oregano and thyme oils, which totally inhibited growth of tested isolates for all days of incubation at 0.625 $\mu L.cm^{-3}$ (in air) with MFDs 0.125 $\mu L.cm^{-3}$ (in air). Lavender EO was less active aginst tested strains (MIDs 0.313 $\mu L.cm^{-3}$). The results showed that the tested EOs had antifungal activity, except lemon and eucalyptus. Sage EO was the only one which decelerated the radial growth of colony of both tested strains after all days of cultivation in comparison with a control sets. Our study provides the support that essential oils can be used to control plant pathogens such as *A. niger* and *A. tubingensis*.

Keywords: *Aspergillus*; essential oils; antifungal activity; vapor

INTRODUCTION

Fruit deterioration is a key postharvest problem because fungal spoilage can cause great economic losses. Grape, as a perishable fruit, is susceptible to fungal infection, especially from *Aspergillus niger* which causes a disease called black mold, one of the major causes of rapid and extensive deterioration of table grapes during the harvest and the major obstacle for storage **(dos Santos et al., 2012; de Sousa et al., 2013)**. *Aspergillus niger*, the most important member of *Aspergillus* subgenus *Circumdati* section *Nigri*, is primarily a plant pathogenic fungi responsible for deterioration of stored food material, as well as *Aspergillus tubingensis*, which includes species that morphologically resemble *Aspergillus niger* **(Samson et al., 2000)**. In addition, the genus *Aspergillus* and its species are producers of several mycotoxins. *A. flavus* and *A. parasiticus* are the main aflatoxins-producing species, while production of ochratoxin A is mainly associated with *Aspergillus carbonarius* and *A. niger* or *Nigri* section species, which has also been reported to produce fumunosin, sterigmatocystin, cyclopiazonic acid and patulin **(Plascencia-Jatomea et al., 2014)**. Spoilage and poisoning of food by fungi are the major problem for food industry and consumers. Decay may increase post harvest losses up to 50% without fungicide treatment. However, the use of synthetic fungicides is becoming more restrictive and thus alternative treatments need to be developed to reduce environmental risk and satisfy the demands of consumer groups **(Phillips et al., 2012)**. This negative consumer perception of chemical preservatives drives attention towards natural alternatives **(Sharma and Tripathi, 2008)**. Due to an increasing risk of chemical contamination upon the application of synthetic fungicides to preserve fresh fruits and vegetables, essential oils are gaining increasing attention **(Farzaneh et al., 2015)**.

Essential oils are aromatic and volatile liquids extracted from plants. The chemicals in essential oils are secondary metabolites, which play an important role in plant defense as they often possess antimicrobial properties **(Hyldgaard et al., 2012)**. Some of EOs have been reported to be active *in vitro* against *A. niger* such as lemongrass **(Tzortzakis and Economakis, 2007)** and *Matricaria chamomilla* flower **(Tolouee et al., 2010)**. A number of EO components have been registered by the European Commission for use as flavourings in food stuffs **(Commission Decision of 23 January, 2002)**. Some EO formulations are currently used

as food preservatives and are kept in the category "GRAS" in view of their favourable safety profile. Being volatile in nature, such EOs may be used as plant-based fumigants for the stored food commodities. Hence, EOs may play a significant role in overcoming storage losses and in enhancing food shelf life **(Prakash et al., 2015)**.

The objective of this study was evaluation of the antifungal activity of 6 EOs by using vapor contact against the fungal species of the genus *Aspergillus* section *Nigri* isolated from grapes in Slovakia.

MATERIAL AND METHODOLOGY

Fungal isolation and identification

Two isolates of black aspergilly, *Aspergillus niger KMi-116-LR* and *Aspergillus tubingensis KMi-144-LR* isolated from grapes, were used. These isolates belong to the collection of microorganisms at the Department of Microbiology of the Slovak Agricultural University in Nitra. They were inoculated on Czapek Yeast Autolysate Agar (CYA) **(Samson et al., 2002)** dishes.

Culturing conditions and DNA extraction

Single spore fungal isolates grown on SDA (Pancreatic Digest of Casein 5 $g.L^{-1}$, Peptic Digest of Meat 5 $g.L^{-1}$, Glucose 40 $g.L^{-1}$, Agar 15 $g.L^{-1}$, BioLife, Italy, Srl) plates (2 weeks, 26 °C, 16/8 light regime) were used for DNA extraction. DNA was extracted using a ZR fungal/bacterial DNA extraction kit (Zymo Research Corp. USA, CA). Identification of isolates was based on 18S rDNA-ITS1-5.8S rDNA-ITS2-28S rDNA region (ITS). We used also partial sequences of second largest subunit of DNA dependent RNA polymerase II (RBP2) because ITS region has low discrimination power among species in *Aspergillus* sect. *nigri*. Amplification reactions were carried out in 25 μL volumes containing: 200 mM dNTPs, 1x dreamTaq buffer, 0.5 unit DreamTaq DNA polymerase (Life technologies, USA), 0.5 mM of corresponding primer, and 0.5 μL DNA. Conditions of PCR reactions were following: initial denaturation at 95 °C for 3 min, 35 cycles were performed consisting of denaturation at 95 °C for 30 s, annealing at corresponding temperature for each primer set for 45 s, and extension at 72 °C for 90 s, final step was 10 min incubation at 72 °C. PCR reactions were carried out in a Biorad MJ mini thermal cycler (BioRad Corp., USA, CA). Primers used for PCR and sequencing of ITS region were ITS1 and ITS4 **(White et al., 1990)**. Primer pair for PCR amplifdication was partial RPB2 where RPB2-5F and RPB2-7cR and sequencing primer was RPB-6F **(Liu et al.,**

Table 1 The major constituents of essential oils analyzed by Calendula company a.s.

Essential oils	Compound	Amount (%)
Lemon	β-pinene	7.0 – 17
	sabinene	1.0 – 3.0
	limonene	56 – 78
	γ-terpinene	6.0 – 12
	β-caryophyllene	max. 0.5
	neral	0.3 – 1.5
	α-terpineol	max. 0.6
	neryl acetate	0.2 – 0.9
	geranial	0.5 – 2.3
	geranyl acetate	0.1 – 0.8
Oregano	carvacrol	min. 50
Lavender	limonene	<1.0
	cineole	<2.5
	3-octanone	0.1 – 2.5
	camphor	<1.2
	linalool	20 – 45
	linalyl acetate	25 – 46
	terpinen-4-ol	0.1 – 6.0
	lavandulyl acetate	>0.2
	lavandulol	>0.1
	α-terpineol	<2.0
Thyme	ρ-cimene	40 ±3
	thymol	32 ±2
Eucalyptus	α-pinene	9.0
	β-pinene	max. 1.5
	sabinene	max 0.3
	α-phellandrene	max. 1.5
	limonene	12
	1,8-cineole	min. 70
	camphor	max. 0.1
Sage	1,8-cineole	min. 5.0
	thujone	min. 15.0
	borneole	min. 5.0

Note: max. (maximum), min. (minimum).

1999). PCR products were cleaned-up by ExoI/FastAP (Life technologies, USA) and sent to Macrogen (Korea) for Sanger sequencing. Acquired sequences were assembled and processed using the Seaview software **(Gouy et al., 2010)**. Isolates were identified by comparison with records in genbank database using genbank BlastN tool. (http://blast.ncbi.nlm.nih.gov/). Sequences of both used isolates was deposited in genbank database under folowing accesion numbers: *A. niger* isolate KMi-116-LR, ITS: KT824061; RPB2: KT824060. *A. tubigensis* isolate KMi-144-LR, ITS: KT824062; RPB2: KT824059.

Essential plant oils

The essential oils used in this study were extracts of lemon (*Citrus lemon* L.), eucalyptus (*Eucalyptus globulus* LABILL.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) sage (*Salvia officinalis* L.) and lavender (*Lavandula angustifolia* MILLER.), they all were supplied by Calendula company a.s. (Nová Ľubovňa, 238 A, Slovakia). The gas chromatography analysis of the main components of each essential oils were determined by Calendula company a.s. (Table 1). Essential oils were extracted by hydro distillation and its quality and stability were certified by suppliers.

Antifungal activity of essential oils

The antifungal activity of selected EOs was investigated by microatmosphere method. The test was performed in sterile Petri dishes (Ø 90 mm) containing 15 mL of CYA. Evaluation by filter paper was made by the method adapted from **Guynot et al., (2003)**. First, all EOs were tested in highest concentration (0,625 µL.cm^{-3} of air). EOs were diluted in DMSO (dimethyl sulfoxide) final volume of 100 µL. Only 50 µL of this solution was distributed on a round sterile filter paper (1 x 1 cm) by micropipette, and the paper was placed in the center of the lid of Petri dishes. Dishes were kept in inverted position. Filter paper discs impregnated with dimethyl sulfoxide (DMSO) were only used as a control to confirm no solvent effect of bioactivity. Each fungus was inoculated in the center of Petri dishes with needle – inoculated. Dishes were tightly sealed with parafilm and incubated for fourteen days at 25 ±1 °C (three replicates were used for each treatment). Diameters (Ø mm) of the growing colonies were measured at the 3rd, 7th, 11th and 14th day with a ruler. Essential oils able to inhibit each fungus (visible inhibition- non growth of fungus) were used in the following test.

Minimum inhibitory doses (MIDs)

After incubation, the minimum inhibitory doses (MIDs) of EOs with the most significant activity were recorded by the method adapted from **Kloucek et al., (2012)**. The essential oils with the most significant activity were determined by method of graded concentration of oils. EOs dissolved in DMSO were prepared at different concentrations (0.500, 0.313, 0.188, 0.125, 0.063 µL.cm^{-3} of air). Cultivation was carried out at the 25 ±1 °C and measured after 14 days. The MID (expressed as microlitres of EOs per volume unit of atmosphere above the organism growing on the agar surface) was defined as the lowest concentration of the oil which did not permit any visible growth after 14 days in comparison with control sets.

Statistical analysis

All analyses were performed in triplicate and the results were expressed as the mean of the data obtained in each replicate. Statistical analyses were performed with descriptive statistics (mean and standard deviation) and inferential tests (ANOVA followed by 95.0% Tukey HSD test) to determine statistically significant differences ($p < 0.05$) between treatments.

RESULTS AND DISCUSSION

Contamination of grapes and grape products by *Aspergillus* section *Nigri* is known to occur widely. The fungal species *Aspergillus niger*, *Aspergillus tubingensis*, and *Aspergillus carbonarius* are included within this section and during their growth are able to produce mycotoxins **(Somma et al., 2012)**.

The objective of this study was to find the activity of the volatile phase of lemon, oregano, lavender, eucalyptus, thyme and sage essential oils against the fungal growth of *Aspergillus niger* and *Aspergillus tubingensis*. First, all EOs were tested at the higherst concentration (0,625 µL.cm^{-3}). Both tested strains, *Aspergillus niger* (Figure 1) and *A. tubingensis* (Figure 2) were sensitive in treatment with oregano, lavender and thyme EOs, which completely inhibited their growth after all days of cultivation (14 days). Strain *A. niger* was not sensitive in treatment with lemon EO, as same as *A. tubingensis*. Eucalyptus EO had very similar antifungal activity against both tested strains. *A. niger* showed the most significant sensibility to the sage EO at the highest concentration (0,625 µL.cm^{-3}) after 7 days of cultivation. *A. tubingensis* seems to be more resistant in treatment with sage EO. It was inhibited by sage EO only after 3 days of cultivation in a comparison with control sets and *A. niger* strain.

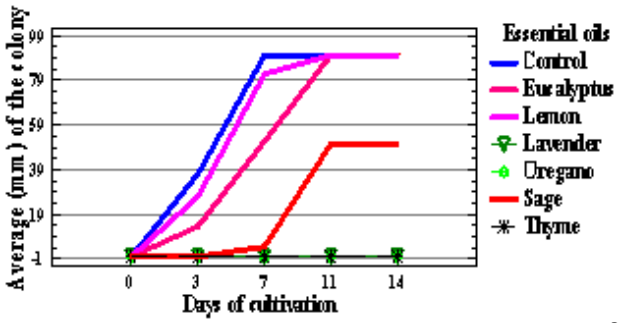

Figure 1 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *Aspergillus niger* KMi-116-LR.

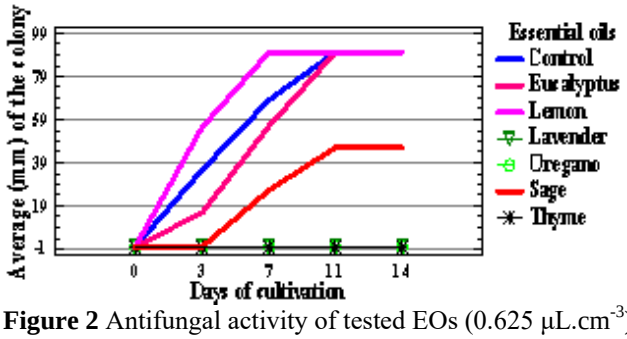

Figure 2 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *Aspergillus tubingensis* KMi-144-LR.

Table 2 Effect of different concentrations of lavender, oregano and thyme essential oils on radial growth inhibition (after 14 days) of *A. niger* and *A. tubingensis*.

Conc. µL.cm^{-3}	***Aspergillus niger*** KMi-116-LR			***Aspergillus tubingensis*** KMi-144-LR		
Essential oils	**Lavender**	**Oregano**	**Thyme**	**Lavender**	**Oregano**	**Thyme**
0.500	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0
0.313	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0
0.188	24.50^{b} ±2.29	0^{a} ±0	0^{a} ±0	7.67^{b} ±2.08	0^{a} ±0	0^{a} ±0
0.125	34.67^{c} ±8.39	0^{a} ±0	0^{a} ±0	22.67^{c} ±6.43	0^{a} ±0	0^{a} ±0
0.063	66.67^{e} ±20.82	7.33^{a} ±0.58	45.67^{d} ±16.01	36.00^{d} ±8.54	6.50^{ab} ±1.80	44.67^{e} ±0.76
Control	90^{f} ±0	90^{f} ±0	90^{f} ±0	90^{f} ±0	90^{f} ±0	90^{f} ±0

* Data in the column followed by different letters are significantly different in 95% Tukey HSD test.

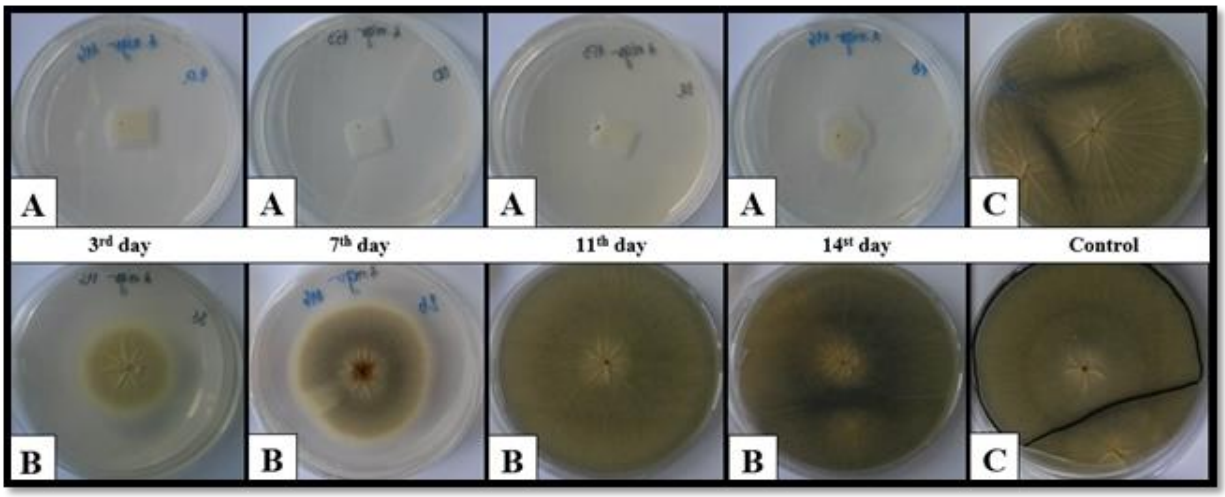

Figure 3 Antifungal activity of oregano (A) and lemon essential oils (B) against *Aspergillus niger*; (C) control.

Pinto et al., (2007) in their study also demonstrated similar results of antifungal activity of sage EO against fungi, but different results were found by **Suhr and Nielsen (2003)** where sage EO showed very poor inhibitor effects. Our results showed that all tested EOs have antifungal activity, except lemon and eucalyptus EOs, and demonstrated significant differences between each other (p <0.001). **Velázquez-Nuñez et al., (2013)** studied antifungal activity of citrus essential oils. They reported the minimum inhibitory concentration for the growth of *A. flavus* by direct addition 16.000 mg.L^{-1}, while for the vapor contact 8000 mg of EO mg.L^{-1} in air. For the both studied methods, growth of *A. flavus* decreased with increasing EO concentration. Further, studies have also documented that eucalyptus and lemon essential oils are effective even against fungal strains in vapor contact, e.g.: *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum and P. verrucosum* **(Viuda-Martos et al., 2008)**, *A. clavatus*, *A. niger*, etc. **(Su et al., 2006)**. Regarding to previous studies, this study demonstrated that lemon and eucalyptus EOs were not effective against tested strains in comparison with other tested EOs (sage, oregano, lavender and thyme). Also **Vilela et al., (2009)** reported that eucalyptus EOs and its major compound 1,8-cineole demonstrated very poor fungicidal activity against *A. flavus* and *A. parasiticus* in both contact and headspace volatile exposure assays.

In this study the most effective EOs were able to inhibit growth of tested strains all days of cultivation at the highest concentration (0.625 µL.cm^{-3}) and were used for determination of MIDs. Among all oils tested, thyme, oregano and lavender oils proved to be the best inhibitor of the black aspergilly. Results are showed in Table 2. The best results (MIDs 0.125 µL.cm^{-3}) (p <0.05) for both, *A. niger* and *A. tubingensis* showed oregano and thyme EOs.

In study of **Combrinck et al., (2011)** thyme EO proved to be the most effective inhibitor, totally inhibiting all of the pathogens tested at concentrations of 1000 µL.L^{-1} and lower, with the exception of a resistant *Penicillium* strain. Several researchers **(Stević et al., 2014; Kocić-Tanackov et al., 2012; Zabka et al., 2014)** found hight inhibitory effect of oregano EOs against fungi, too.

In our study, *A. niger* showed visible growth after 14 days only in treatment with lavender EO with a higher MIDs value 0.313 µL.cm^{-3}, as same as *A. tubingensis* (MIDs 0.313 µL.cm^{-3}) (p <0.05). **Soylu et al., (2010)** tested

rosemary and lavender EOs against *Botrytis cinerea*, and they also found that rosemary and lavender EOs were inhibitory at relatively higher concentrations (25.6 $\mu g.mL^{-1}$). Also **Daferera et al., (2003)** demonstrated that lavender, rosemary, sage, and pennyroyal essential oils have less inhibitory activity against tested fungal species. Although the concentrations of oils tested in this work were not the same. But antifungal activity of tested EOs depends on concentration of EOs, cultivation time and used method. In a previous study conducted by **Goñi et al., (2009)** behavior of clove EO was not the same in direct contact and vapor phase. **Bluma et al., (2009)** demonstrated that the vapor generated by 5000 $\mu L.L^{-1}$ of poleo oil significantly reduced growth of *Aspergillus* section *Flavi* in the order of 78.0%, whereas the dose of 3000 $\mu L.L^{-1}$ completely inhibited fungal development in the direct contact assay **(Bluma and Etcheverry, 2008)**. In study of **Velázquez-Nuñez et al., (2013)** direct addition of orange peel EO had a rapid effect on *A. flavus* growth, but exposure to orange peel EO vapors was more effective, requiring lower concentrations of EO to inhibit mold growth. They concluded that vapor contact is an alternative when essential oils (EO's) and microorganisms are placed separately in some sealed environment.

CONCLUSION

As a conclusion, volatile substances from oregano, thyme (MIDs 0.125 $\mu L.cm^{-3}$) and lavender (MID 0.313 $\mu L.cm^{-3}$) essential oils had a potential antifungal activity against tested strains of black aspergilly. Results showed that the tested EOs had antifungal activity, except lemon and eucalyptus EOs in comparison with control sets. In spite of the fact that sage EO showed only weak antifungal activity, and was able only to delayed growth of *A. niger* (after 7 days of cultivation) and *A. tubingensis* (after 3 days) could be used in food preservation, but further research is needed. Our study gives support that essential oils can be used to control plant pathogens such as *A. niger* and *A. tubingensis*.

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Contact address:

Miroslava Císarová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.cisarova@gmail.com.

Dana Tančinová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: name@domainname.sk.

Juraj Medo, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: juraj.medo.spu@gmail.com.

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ANTIOXIDANT ACTIVITY, PHENOLIC CONTENT AND COLOUR OF THE SLOVAK CABERNET SAUVIGNON WINES

Daniel Bajčan, Alena Vollmannová, Vladimír Šimanský, Judita Bystrická, Pavol Trebichalský, Július Árvay, Peter Czako

ABSTRACT

Antioxidants are specific substances that oxidize themselves and in this way they protect other sensitive bioactive food components against destruction. At the same time, they restrict the activity of free radicals and change them to less active forms. Grapes and wine are a significant source of antioxidants in human nutrition. One of the most important group occuring in grapes and wines are polyphenols. Many of phenolic compounds have been reported to have multiple biological activities, including cardioprotective, anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties attributed mainly to their antioxidant and antiradical activity. Therefore, it is important to know the content of polyphenols and their antioxidant effects in foods and beverages. Twenty-eight Cabernet Sauvignon wine samples, originated from different Slovak vineyard regions, were analyzed using spectrophotometry for the content of total polyphenols, content of total anthocyanins, antioxidant activity and wine colour density. Determined values of antioxidant activity in observed wines were within the interval 69.0 – 84.2% inhibition of DPPH (average value was 78.8% inhibition of DPPH) and total polyphenol content ranged from 1,218 to 3,444 mg gallic acid per liter (average content was 2,424 mg gallic acid.L^{-1}). Determined total anthocyanin contents were from 68.6 to 430.7 mg.L^{-1} (average content was 220.6 mg.L^{-1}) and values of wine colour density ranged from 0.756 to 2.782 (average value was 1.399). The statistical evaluation of the obtained results did not confirm any linear correlations between total polyphenol content, resp. total anthocyanin content and antioxidant activity. The correlations between total polyphenol content and total anthocyanin content, resp. the content of total anthocyanins and wine colour density were strong. The results confirmed very strong correlations between wine colour density and total polyphenol content, resp. antioxidant activity.

Keywords: polyphenol; antioxidant activity; anthocyanin; red wine; Cabernet Sauvignon

INTRODUCTION

Phenolic compounds are the most abundant secondary metabolites present in the plant kingdom. They possess a common structure comprising an aromatic benzene ring with one or more hydroxyl substituents. They represent a large and diverse group of molecules including two main families: the flavonoids based on common C6-C3-C6 skeleton and the non-flavonoids. In plant, they play a role in growth, fertility and reproduction and in various defence reactions to protect against abiotic stress like UV-light or biotic stresses such as predator and pathogen attacks. They also constitute basic components of pigments, essences and flavors **(Weisshaar and Jenkins 1998; Winkel-Shirley, 2002)**. Recent interest, however, in food phenolics has increased greatly because of the antioxidant and free radical-scavenging abilities associated with some phenolics and their potential effects on human health **(Bravo, 1998)**. Many of phenolic compounds (resveratrol, quercetin, rutin, catechin, proanthocyanidins) have been reported to have multiple biological activities, including cardioprotective, anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties attributed mainly to their antioxidant and antiradical activity **(Lorrain et al., 2013)**.

Grapes and grape products (mainly wines and juices) are a rich source of phenolic compounds. From the clue of "French paradox", polyphenolics from grapes and red wines attracted the attention of scientists to define their chemical composition and quantity **(Urpi-Sarda et al., 2009)**. Globally, red wines contain more phenolic compounds than white wines. It is caused by the technology of winemaking, when making white wines the grapes' skin is removed before fermentation **(Beer et al., 2006)**. The total polyphenols in wine besides variety of grapes, locality of growing, climatic conditions, are affected also by procedure of winemaking: length of contact of stum with grapes's skin, mixing, temperature, content of SO_2, pH value, content of alcohol etc. **(Villano et al., 2006; Lachman and Šulc 2006)**.

Cabernet Sauvignon (CS) is perhaps best known, most popular and one of most cultivated blue grapevine varieties in the world. This variety gives a lower harvest, wines are full-bodied, higher acids and polyphenols content (tannins and dyes) and excellent aging potential. Variety has traditionally mixing with other blue sort to achieve overall softer feel and a more balanced wine taste. Colder climate of Central Europe often makes the aroma of Slovak Cabernets with flavour of green pepper and grass

denouncing the lack of ripeness of the grapes. Cabernet Sauvignon is grown mainly in southwestern France, where this variety spread around the world (northern Italy, USA, South Africa, Australia, South America). In Slovakia, CS grown at about 13% of the areas planted with blue grapevine varieties and CS is the third most cultivated blue variety after Blaufränkisch and St. Laurent **(Ďurčová, 2011**; **Šajbidorová, 2012)**.

The purpose of this study was to determine and evaluate chosen antioxidant and sensory properties (the content of total polyphenols, content of total anthocyans, antioxidant activity and wine colour density) and their mutual correlations in red wine samples – Cabernet Sauvignon, originated from different Slovak vineyard areas.

MATERIAL AND METHODOLOGY

Chemicals and instruments

All analysed parameters – total polyphenol content, total anthocyanin content, antioxidant activity and wine colour density in wines were analyzed using UV/VIS spectrophotometry (spectrophotometer Shimadzu UV/VIS – 1240, *Shimadzu, Japan*). The chemicals used for all analysis were: Folin-Ciocalteau reagent, monohydrate of gallic acid p.a., anhydrous natrium carbonate p.a., citric acid p.a., dodecahydrate of disodium hydrogen phosphate, 35% hydrochloric acid p.a., ethanol p.a., methanol p.a., 1,1-diphenyl-1-picrylhydrazyl (DPPH) radical p.a.

Samples

Analysed, bottled, red, especially quality and dry wines

Table 1 Characteristics of analysed Cabernet Sauvignon wine samples.

Sample	Producer	Vineyard area	Vintage	Quality
LC-1	Vitis Pezinok / Hubert J.E. Sereď	Little Carpathian	2008	quality
LC-2	Bočko Víno, Šenkvice	Little Carpathian	2008	quality
LC-3	VPS, Pezinok	Little Carpathian	2010	quality
LC-4	Víno Jano, Limbach	Little Carpathian	2009	quality
LC-5	Villa Víno Rača, Bratislava	Little Carpathian	2013	quality
SS-1	Vitis Pezinok / Hubert J.E. Sereď	South Slovak	2007	quality
SS-2	Villa Víno Rača, Bratislava	South Slovak	2008	quality
SS-3	Víno Matyšák, s.r.o., Pezinok	South Slovak	2010	quality
SS-4	VINIDI, s.r.o., Bratislava	South Slovak	2008	late harvest
SS-5	Vinárske závody Topoľčianky	South Slovak	2010	quality
SS-6	Hubert J.E., Sereď	South Slovak	2007	quality
SS-7	Malokarpatská vinohrad. spol., Pezinok	South Slovak	2009	quality
N-1	Víno Nitra, Nitra	Nitra	2009	quality
N-2	Chateau Modra, Modra	Nitra	2009	late harvest
N-3	Vinárske závody Topoľčianky	Nitra	2006	quality
N-4	Vinárske závody Topoľčianky	Nitra	2009	quality
N-5	Víno Nitra, Nitra	Nitra	2009	quality
N-6	Mrva a Stanko, Trnava	Nitra	2011	grapes selection
ES-1	J&J Ostrožovič, Veľká Tŕňa	East Slovak	2009	quality
ES-2	PD Vinohrady, Choňkovce	East Slovak	2008	late harvest
ES-3	PD Vinohrady, Choňkovce	East Slovak	2007	grapes selection
ES-4	Pivnica Tibava, Tibava	East Slovak	2008	quality
ES-5	Pivnica Tibava, Tibava	East Slovak	2009	quality
CS-1	Agro Movino, Veľký Krtíš	Central Slovak	2009	quality
CS-2	Agro Movino, Veľký Krtíš	Central Slovak	2010	quality
CS-3	Agro Movino, Veľký Krtíš	Central Slovak	2011	grapes selection
CS-4	Agro Movino, Veľký Krtíš	Central Slovak	2011	quality
CS-5	L. Korcsog, Korvinum, Rykynčice	Central Slovak	2011	late harvest

Cabernet Sauvignon (CS) and their characteristics are mentioned in Table 1. Wine samples with origin in various Slovak vineyard areas (VA) were purchased in retail network, to provide that analysed samples of wine would have the same properties as wines that are consumed by common consumers (properties of wine affected by various factors, such as period and conditions of storage or distribution of wine).

Antioxidant activity determination

Antioxidant activity (AA) was assessed by method of **Brand-Williams et al., (1995)** using of DPPH (1,1-diphenyl-1-picrylhydrazyl) radical. Absorbance was read at 515.6 nm and antioxidant effectiveness was expressed as % inhibition of DPPH (quantitative ability of tested compound to remove in certain period a part of DPPH radical).

Determination of total polyphenol content

Total polyphenol content (TPC) was determined by modified method of **Singleton and Rossi (1965)**. 0.1 mL of wine sample was pipetted into 50 mL volumetric flask and diluted with 5 mL of distilled water. To diluted mixture 2.5 mL Folin-Ciocalteau reagent was added and after 3 minutes 7.5 mL of 20% solution of Na_2CO_3 was added. Then the sample was filled with distilled water to volume 50 mL and after mixing left at the laboratory temperature for 2 hours. By the same procedure the blank and calibration solutions of gallic acid were prepared. Absorbance of samples solutions was measured against blank at 765 nm. The content of total polyphenols (TP) in wines was calculated as amount of gallic acid equivalent (GAE) in mg per 1 litre of wine.

Determination of total anthocyanin content

Total anthocyanin content (TAC) was assessed by modified pH differential method of **Lapornik et al., (2005)**. The principle of this method is reduction of the pH of wine samples with hydrochloric acid to values 0.5 – 0.8 associated with the transformation of all anthocyans to red colored flavilium cation. The content of total anthocyanins (TA) was calculated from the difference absorbance values of both solutions (origin and acidified) and expressed as the amount of anthocyans in mg per 1 liter of wine.

Determination of wine colour density

Wine colour density (WCD) was assessed by method of **Sudrand (1958)** as the sum of the absorbance at 420 nm and 520 nm. The absorbance of the wine samples was measured in 0.2 cm path lenth glass cells.

All analyses were performed as four parallels.

Statistical analysis

Statistical analysis was performed using the software Statistica 6.0 (StatSoft, Czech Republic) and the results were evaluated by analysis of variance ANOVA.

RESULTS AND DISCUSSION

All studied parameters – the content of total polyphenols, the content of total anthocyanins, antioxidant activity and wine colour density of the Slovak wines Cabernet Sauvignon are described in Table 2.

Antioxidant activity in analysed wine samples was in range 69.0 – 84.2% inhibition of DPPH. Average value of AA was 78.8% inhibition of DPPH. The average value of AA in Cabernet Sauvignon wines is a slightly lower than we found out in the other two major Slovak red wines Blaufränkisch – 83.3% and St. Laurent – 81.2% inhibition of DDPH **(Bajčan et al., 2012)**, but slightly higher compared to Slovak Alibernet wine samples – 74.0% inhibition of DPPH **(Bajčan et al., 2015)**. Similar results of AA reported **Slezák (2007)** and **Špakovská et al., (2012)**, who found out AA in Slovak wines – Cabernet Sauvignon in range from 71.6 to 90.9% inhibition of DPPH. On the basis of value of AA an order could be as following: wines from Little Carpathian VA > wines from East Slovak VA > wines from Central Slovak VA > wines from Nitra VA > wines from South Slovak VA. Gained results did not exert statistically significant differences (at significance level $p = 0.05$) between values of antioxidant activity in wines made in various vineyard areas in Slovakia.

Total polyphenol content in analysed wine samples was in the range from 1,218 to 3,444 mg GAE.L^{-1}. Average content of TP was 2,424 mg GAE.L^{-1}. The average content of total polyphenols in wines - Cabernet Sauvignon is a little higher than we found out in the other two major Slovak varietal red wines Blaufränkisch – 2,003 mg GAE.L^{-1} and St. Laurent – 2,297 mg GAE.L^{-1} **(Bajčan et al., 2012)**. On the other hand, average content of TP in Slovak Cabernet Sauvignon wines was much lower than we determined in Alibernet wines – 3,057 mg GAE.L^{-1} **(Bajčan et al., 2015)**. The results are similar to results reported by **Slezák (2007)** and **Špakovská et al., (2012)**, who found out the content of TP in Slovak wines – Cabernet Sauvignon in range from 2,150 to 3,102 mg GAE.L^{-1}. Other (foreign) scientists **(Kondrashov et al., 2009; Burin et al., 2010**; **Yoo et al., 2011)** analyzing TPC in CS wines reported also very similar results (1,453 – 3,589 mg GAE.L^{-1}). **Cliff et al., (2007)** reported much lower average value of TPC (1,055 mg GAE.L^{-1}) in CS wines originated in British Columbia, Canada what is probably due to cold weather and lack of mature grapes. According to the average value of TPC an order for wines could be as following: wines from Central Slovak VA > wines from South Slovak VA > wines from Nitra VA > wines from Little Carpathian VA > wines from East Slovak VA. Gained results exerted statistically significant differences (at significance level $p = 0.05$) between TPC in wines made in East Slovak VA and TPC in wines made in Central Slovak VA, resp. South Slovak VA.

Total anthocyanin content in analysed wine samples was in the range from 68.6 to 430.7 mg.L^{-1}. Average content of TA was 220.6 mg.L^{-1}. The average TAC in wines Cabernet Sauvignon is significantly lower than we found out in the other three Slovak varietal red wines Blaufränkisch – 266.1 mg.L^{-1}, St. Laurent – 264 mg.L^{-1} and Alibernet – 403 mg.L^{-1} (**Bajčan et al., 2015; Tóth et al., 2011)**. According to the average value of TAC an order for wines could be as following: wines from Central Slovak VA > wines from Nitra VA > wines from Little Carpathian VA > wines from South Slovak VA > wines from East Slovak VA. Gained results exerted statistically significant differences between TAC in wines made in

East Slovak VA and TAC in wines made in Central Slovak VA, resp. Nitra VA.

Wine colour density in analysed wine samples was in range from 0.756 to 2.782. Average value of WCD was 1.399. The average value of WCD in wines Cabernet Sauvignon is a little higher than we found out in the other two major Slovak varietal red wines Blaufränkisch – 1.110 and St. Laurent – 1.224 **(Tóth et al., 2011)**. But on the other hand, average value of WCD in Slovak Cabernet Sauvignon wines was much lower than we determined in Alibernet wines – 2.317 **(Bajčan et al., 2015)**. This is the first study monitoring WCD in Slovak wines Cabernet Sauvignon, so we can´t compare our data with other scientists. The results are little higher to results reported by **Poiana et al., (2007)**, who found out WCD in Romanian wines - Cabernet Sauvignon in range from 0.708 to 1.474 (average value – 1.206).

According to the average value of WCD an order for

Table 2 The content of total polyphenols (TPC), content of total anthocyanins (TAC), antioxidant activity (AA) and wine colour density (WCD) in analysed wines.

Sample	TPC mg GAE.L^{-1}	TAC Mg.L^{-1}	AA %	WCD
LC-1	2,206 ±22	82.5 ±2.7	82.9 ±2.7	1.059 ±0.006
LC-2	1,926 ±23	246.3 ±3.7	79.1 ±3.3	1.182 ±0.004
LC-3	2,667 ±46	246.9 ±4.2	82.1 ±3.8	0.896 ±0.011
LC-4	2,237 ±117	151.1 ±5.3	80.1 ±2.5	1.177 ±0.015
LC-5	2,642 ±30	282.4 ±2.8	79.8 ±0.8	1.449 ±0.012
Average LCVA	***2,336 ±308^{a}***	***201.8* ±85.8^{a}**	***80.8 ±1.6^{a}***	***1.153* ±0.237^{a}**
SS-1	2,215 ±46	68.6 ±3.1	79.5 ±2.6	1.137 ±0.021
SS-2	2,267 ±46	208.2 ±1.6	81.8 ±1.4	1.064 ±0.009
SS-3	2,966 ±46	292.7 ±2.8	77.1 ±2.6	1.385 ±0.012
SS-4	2,634 ±22	206.4 ±7.4	75.9 ±1.7	1.608 ±0.008
SS-5	2,886 ±22	330.8 ±7.7	76.6 ±2.0	1.861 ±0.008
SS-6	3,365 ±22	111.8 ±7.4	73.5 ±3.8	1.927 ±0.019
SS-7	2,118 ±44	152.3 ±2.5	80.5 ±2.5	1.053 ±0.015
Average SSVA	***2,636 ±461^{b}***	***195.8* ±97.0^{b}**	***77.8 ±3.1^{b}***	***1.434* ±0.323^{b}**
N-1	1,632 ±69	103.9 ±0.7	81.1 ±1.7	1.096 ±0.006
N-2	2,747 ±44	330.0 ±2.1	76.2 ±2.0	1.801 ±0.014
N-3	2,513 ±46	272.1 ±5.6	80.0 ±3.9	1.426 ±0.018
N-4	2,885 ±68	293.5 ±3.5	69.0 ±1.8	2.782 ±0.023
N-5	2,628 ±23	162.6 ±6.7	84.2 ±4.0	1.076 ±0.005
N-6	2,798 ±43	363.3 ±8.4	77.4 ±1.0	1.968 ±0.021
Average NVA	***2,534 ±495^{c}***	***254.2* ±102.5^{c}**	***78.0 ±3.2^{c}***	***1.691* ±0.674^{c}**
ES-1	1,270 ±23	147.5 ±3.8	71.2 ±4.8	1.066 ±0.011
ES-2	2,206 ±22	84.9 ±3.2	81.9 ±1.2	1.159 ±0.010
ES-3	2,268 ±44	77.3 ±2.1	79.9 ±1.3	1.105 ±0.017
ES-4	2,230 ±22	120.6 ±5.3	83.8 ±2.0	0.888 ±0.009
ES-5	1,218 ±23	111.8 ±2.5	78.7 ±2.7	0.756 ±0.008
Average ESVA	***1,838 ±451^{bd}***	***108.4* ±30.2^{cd}**	***79.1 ±5.4^{d}***	***0.995* ±0.173^{bd}**
CS-1	2,409 ±23	236.8 ±3.1	79.9 ±1.7	1.175 ±0.004
CS-2	2,359 ±46	421.2 ±23.9	82.4 ±2.9	1.509 ±0.016
CS-3	3,444 ±91	430.7 ±9.5	74.4 ±3.1	2.095 ±0.022
CS-4	2,873 ±46	341.1 ±5.6	78.8 ±3.7	1.805 ±0.023
CS-5	2,275 ±31	299.8 ±4.9	78.6 ±1.1	1.667 ±0.017
Average CSVA	***2,672 ±502^{d}***	***345.9* ±83.3a^{d}**	***78.8 ±3.4^{e}***	***1.650* ±0.397^{d}**
Total average	**2,424 ±537**	**220.6 ±106.4**	**78.8 ±3.7**	**1.399 ±0.483**

NOTE: Values of TPC, TAC, AA and WCD are expressed as arithmetic average ±standard deviation.
[a-e] Values with the same letters denote significant differences (p <0.05) among vineyard areas.
LCVA – Little Carpathian vineyard area, SSVA – South Slovak vineyard area, NVA – Nitra vineyard area, ESVA – East Slovak vineyard area, CSVA – Central Slovak vineyard area.

wines could be as following: wines from Nitra VA > wines from Central Slovak VA > wines from South Slovak VA > wines from Little Carpathian VA > wines from East Slovak VA. Gained results exerted statistically significant differences (at significance level $p = 0.05$) between WCD in wines made in East Slovak VA and WCD in wines made in Central Slovak VA, and South Slovak VA.

In order to investigate the mutual relations between analyzed parameters, the linear regressions were obtained. The statistical evaluation of the obtained results did not confirm any linear correlations between TPC and AA, resp. TAC and AA (r = -0.255, resp. r = -0.279) at significance level $p < 0.1$. This is not in the agreement with the study of **Burin et al., (2010)**, **Kondrashov et al., (2009)** and **Balík et al., (2008)** who found out very strong linear correlations between TPC, resp. TAC and AA in wines and grape juices. Explanation lies in the differences in the methodology of AA determination. The correlations between TPC and TAC (r = 0.542), resp. TAC and WCD (r = 0.600) were highly significant at significance level $p < 0.01$. **Cioroi and Musat (2007)** reported stronger correlation between TPC and TAC (r = 0.739 and 0.771) in red wines. The statistical evaluation of the obtained results confirmed very highly significant correlations at significance level $p < 0.001$ between WCD and TPC, resp. WCD and AA (r = 0.697, resp. r = - 0.714).

CONCLUSION

Slovak red wines – Cabernet Sauvignon have high antioxidant activity (average value 78.8% inhibition of DPPH), high content of healthy useful phenolic compounds (average value of TPC 2,424 mg GAE.L^{-1}), moderate value of TAC (average value 220.6 mg.L^{-1}) and good colour (average value of WCD 1.399). The results showed statistically significant differences for 3 studied parameters (TPC, TAC and WCD) in wines made in some vineyard areas in Slovakia. On the basis of statistical evaluation of our results, statistically significant correlations were demonstrated between wine colour density and other 3 parameters (TPC, TAC and AA), resp. between TPC and TAC.

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Contact address:

Daniel Bajčan, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia, E-mail: bajcan@gmail.com.

Alena Vollmannová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia, E-mail: alena.vollmannova@uniag.sk.

Vladimír Šimanský, Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of pedology and geology, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia, E-mail: vladimir.sinamsky@uniag.sk.

Judita Bystrická, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia, E-mail: judita.bystricka@uniag.sk.

Pavol Trebichalský, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia, E-mail: pavol.trebichalsky@uniag.sk.

Július Árvay, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia, E-mail: julius.arvay@uniag.sk.

Peter Czako, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storing and Processing of Plant Products, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.czako@uniag.sk.

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DETERMINATION OF HEAVY METALS CONCENTRATION IN RAW SHEEP MILK FROM MERCURY POLLUTED AREA

Radovan Stanovič, Július Árvay, Martin Hauptvogl, Ján Tomáš, Anton Kováčik, Zuzana Záhorcová, Marek Slávik

ABSTRACT

The paper focuses on determining the content of monitored contaminants (Cd, Cu, Hg, Pb and Zn) in 53 samples of raw sheep milk collected in 2013 and 2014 on the sites Poráč and Matejovce nad Hornádom (middle Spiš). The area is characterized by historical mining and metalworking activity (mining and processing of polymetallic ores rich in Hg, Cd and Pb). Currently, the area is one of the most mercury contaminated areas in Central Europe. All statistical analyses were carried out using the statistical software Statistica 10.0 *(*Statsoft, USA). Descriptive data analysis included minimum value, maximum value, arithmetic mean and standard deviation. The results of the studied contaminant content show that the limit value for cadmium (10 $\mu g.kg^{-1}$) was exceeded in 25 samples. In the case of lead, the limit value of 20 $\mu g.kg^{-1}$ was exceeded in 16 cases. The limit value for copper (0.4 $mg.kg^{-1}$) was exceeded in one case. The limit value for zinc is not defined by a legislative standard. The risk level of the studied contaminants in the samples of raw sheep milk decreases as follows: Cd > Pb > Hg > Cu > Zn. It can be concluded that frequent and long-term consumption of the raw sheep milk originating from the studied sites poses a health risk. The content of the contaminants in the milk and their eventual transition into dairy products should be monitored over a longer term in more detail.

Keywords: former mercury mining area; health hazard; heavy metal; raw sheep milk; Slovakia

INTRODUCTION

Heavy metals and/or trace elements are ubiquitous components of the environment that may be of natural origin: volcanic activity, fires, geogenic origin **(Rutter et al., 2008)**, or anthropogenic origin: metal industry, mining, heavy industry, transportation **(Cui et al., 2005; Navarro et al., 2008; Singh et al., 2005)**. Increasing level of environmental contamination is directly correlated with the level of industrialization **(Tubaro and Hungerford, 2007)**. Metalworking industry and mining of minerals that contain hazardous heavy metals represent a major risk of the environmental contamination, especially of local nature.

Consuming local food poses the greatest risk of intoxication of the consumers by heavy metals that consequently affects their health. **Loutfy et al., (2006)** reported that consumers receive 90% of the total amount of heavy metals by consumption of food from contaminated areas. As a result, human exposure to toxic metals has become a major health risk. Chronic intake of heavy metals above their safe threshold by humans and animals has damaging effects and can cause non-carcinogenic hazards, such as neurologic involvement, headache and liver disease **(John and Andrew, 2011; Lai et al., 2010)**.

Children are particularly sensitive to increased concentrations of heavy metals (especially Hg, Cd and Pb) and arsenic because their tissues and organs accumulate high concentrations of contaminants reflecting in their health during the process of their development and growth. Central nervous system is especially sensitive due to its progressive development and even small amounts of heavy metals can cause irreversible processes resulting in mental retardation and behavioral disorders **(Ataro et al., 2008)**.

Milk and dairy products contain many essential nutrients and their regular consumption is recommended, especialy for young children **(Maas et al., 2011)**. Sheep raw milk has a higher content of essential vitamins and minerals than cow's milk and could be used to cater to consumers' appetite for healthy and safer products **(Bogdanovičová et al., 2015)**. Ovine milk is the most completed natural fluid, one of the most important basic and healthiest raw materials, which plays important role in the dairy nutrition of all population **(Lačanin et al., 2015)**. However, milk and dairy products may contain varying amounts of different toxic contaminants, especially heavy metals **(Ataro et al., 2008)**. In recent years, several reports have indicated the presence of heavy metals in milk and other dairy products **(Kazi et al., 2009; Soylak et al., 2005; Tuzen et al., 2008)**. Due to the fact that milk and milk products are very common food, it is necessary to make great efforts to control the content of the monitored contaminants and at the same time to monitor the quality of individual environmental components that are the main sources of heavy metals in the human food chain **(Caggiano et al., 2005)**.

The paper focuses on the evaluation of the contamination level of raw sheep milk by heavy metals (Cd, Cu, Hg, Pb

and Zn). The studied area was formerly characterized by important mining and metal processing activities **(Angelovičová and Fazekašová, 2014)**. Currently, the area is considered as one of the most contaminated sites by mercury, cadmium and lead in Slovakia, but also in Central Europe, which is significantly reflected in the quality of the grown vegetables **(Slávik et al., 2014)**, edible wild mushrooms **(Árvay et al., 2015; Svoboda et al., 2006)**. Thus it is assumed that it will be reflected in the quality and contamination level of the monitored raw sheep milk, the production of which belongs to the major and characteristic agricultural activities in the area.

MATERIAL AND METHODOLOGY

Samples collection

Samples of fresh sheep milk (N = 53) were obtained during 2013 and 2014 from identical individuals in two locations: Poráč (N = 20) and Matejovce nad Hornádom (N = 33). Immediately after the milking, the samples were temporarily stored in PE centrifuge bottles (50 cm^3) and frozen. Just before the analysis, the milk samples were defrosted at room temperature, filtered, homogenized and subsequently analytically processed.

Pre-analytical and analytical procedure

Frozen samples of the sheep milk were defrosted at room temperature just before the analysis. Subsequently, the samples were homogenized by shaking and 2 g were weighed and poured into mineralization tubes. The homogenized sheep milk samples were mineralized in a closed system of microwave digestion using Mars X-Press 5 (CEM Corp., Matthews, NC, USA) in a mixture of 5 cm^3 of HNO_3 (SupraPUR, Merck, Darmstadt, Germany) and 5 cm^3 of deionized water (0.054 $\mu S.cm^{-1}$) from Simplicity185 (Millipore SAS, Molsheim, France). Digestion conditions for the applied microwave system comprised of the heat, which ran up to 150 °C for 10 minutes and was kept at the constant temperature for 10 minutes. A blank sample was carried out in the same way. The sample was subsequently filtered through a quantitative filter paper Filtrak 390 (Munktell & Filtrak GmbH, Bärenstein, Germany) and filled up with deionized water to a volume of 50 cm^3 **(Árvay et al, 2014)**.

The contents of the studied contaminants were determined by flame atomic absorption spectrometry: F-AAS (Cu and Zn) on the SpectrAA 240 FS (Varian Inc., Mulgrave, VIC, Australia), electrothermal atomic absorption spectrometry: GF-AAS (Cd and Pb) with Zeemann background correction on the SpectrAA 240 Z (Varian Inc., Mulgrave, VIC, Australia). Total mercury content (THg) was determined directly in the liquid milk samples (200 µL) by a selective mercury analyzer AMA-254 (Altec, Praque, Czech Republic) based on CV–AAS. Detection limit for F-AAS was 2.0, 0.6 $\mu g.kg^{-1}$ for Cu and Zn, respectively. Detection limit for GF-AAS was 10.0 $ng.kg^{-1}$ for both Cd and Pb. Detection limit for mercury was 1.5 $ng.kg^{-1}$. Certipur® (Merck, Darmstadt, Germany) calibration solution was used for the calibration of all instruments.

Statistical analysis and risks assessment

All statistical analyses were carried out using the statistical software Statistica 10.0 (Statsoft, USA). Descriptive data analysis included minimum value, maximum value, arithmetic mean and standard deviation. The limit of the statistical significance was set up at $p < 0.05$ for all descriptive statistical analyses. To evaluate a health risk resulting from the consumption of raw sheep milk, the obtained data on the content of the studied contaminants were compared with limit values defined by the Codex Alimentarius of the Slovak Republic **(PK SR, 2006)** and EC Regulation 1881/2006 **(EC, 2006)**.

Table 1 Heavy metals in sheep milk samples with descriptive statistics.

Year of collection	Number of samples	Heavy metals in sheep milk samples Median ±SD (range)				
		Hg $\mu g.kg^{-1}$	**Cd** $\mu g.kg^{-1}$	**Pb** $\mu g.kg^{-1}$	**Cu** $mg.kg^{-1}$	**Zn** $mg.kg^{-1}$
		Matejovce nad Hornádom				
2013	19	0.138 ±0.587 (0.079 – 0.286)	1.66 ±1.86 (0.71 – 9.07)	17.3 ±46.5 (ND – 193)	0.14 ±0.06 (0.08 – 0.26)	3.41 ±1.63 (1.02 – 8.04)
2014	14	0.220 ±0.121 (0.063 – 0.450)	8.35 ±9.31 (2.95 – 30.5)	13.6 ±28.3 (ND – 93.6)	0.16 ±0.08 (0.10 – 0.36)	5.51 ±1.07 (4.43 – 7.93)
		Poráč				
2013	10	0.061 ±0.016 (0.036 – 0.079)	12.9 ±4.33 (9.19 – 22.2)	7.85 ±33.9 (6.05 – 113)	0.07 ±0.44 (0.02 – 1.47)	5.53 ±0.99 (3.53 – 6.01)
2014	10	0.068 ±0.025 (0.025 – 0.103)	22.2 ±13.3 (10.1 – 52.9)	12.5 ±9.83 (5.73 – 39.5)	0.12 ±0.14 (0.02 – 0.51)	5.64 ±1.28 (4.10 – 7.97)
Maximum Allowable Levels		**50**[a]	**10**[a]	**1000**[a] **20**[b]	**0.4**[a]	--

NOTE: ND – not detected, SD – standard deviation.

[a] Maximum allowable levels of monitored heavy metals - Codex allimentarius of Slovakia **(PKSR, 2006)**.

[b] Maximum allowable levels of monitored heavy metals - Commission regulation (EC) 1881/2006 **(EC, 2006)**.

RESULTS AND DISCUSSION

The contents of the studied heavy metals together with the basic statistical indicators are shown in Table 1. Due to the fact that the sites of interest were characterized in the past by intensive extraction and processing of mercury **(Árvay et al., 2014)**, mercury is considered to be the main heavy metal in terms of quality assessment of the raw sheep milk in this paper. Its content varied in relatively wide intervals within the years, as well as the sites. The highest concentration in terms of the site was recorded in Matejovce nad Hornádom where the mean value of Hg was 0.138 $\mu g.kg^{-1}$ of the raw sheep milk in 2013 and 0.220 $\mu g.kg^{-1}$ in 2014. The mean value of the Hg content in the milk from Poráč was about one order of magnitude lower: 0.061 $\mu g.kg^{-1}$ in 2013 and 0.068 $\mu g.kg^{-1}$ in 2014. The data are balanced also within the set, as evidenced by the lower standard deviation (Table 1) in comparison with the variability of the Hg values obtained from Matejovce. Such significant differences in the content of the studied contaminant from the sites that are about 2 km apart are due to a significant difference in the atmospheric distribution of emissions from the sources. This is confirmed by other studies **(Angelovičová and Fazekašová, 2014; Svoboda et al., 2000)**. The mercury content in the milk samples from the both sites did not exceed the maximum level of 50 $\mu g.kg^{-1}$ set by the Codex Alimentarius SR **(PKSR, 2006)**.

The content of cadmium, which is an accompanying element in polymetallic ores mined in the area of interest ranged in a much wider intervals. It is evidenced by the extremely high standard deviations (Table 1). The mean values of Cd content were 9.12 $\mu g.kg^{-1}$ (2013) and 22.2 $\mu g.kg^{-1}$ (2014) in the Poráč area and 1.66 $\mu g.kg^{-1}$ (2013) and 8.35 $\mu g.kg^{-1}$ (2014) in the Matejovce area. The highest concentration of cadmium was recorded in the Poráč area in 2014 (52.9 $\mu g.kg^{-1}$). Large differences in the cadmium content in the sheep milk samples can be caused by several factors such as: seasonality, climatic conditions and variability of feed ration **(Rahimi, 2013)**, since the samples were obtained during outdoor breeding and pasturing. Hygiene standard defined by the Codex Alimentarius sets the contaminant content at 10 $\mu g.kg^{-1}$. The obtained results show that the limit value was exceeded in 6 out of 53 samples taken in Matejovce in 2014. In the Poráč, 9 samples exceeded the limit value in 2013 and 10 in 2014. It can be stated that the cadmium content exceeded the limit value in almost 50% of all samples of the raw sheep milk.

The lead content in the milk samples varied at different intervals, depending on the site. The mean Pb content was 7.85 $\mu g.kg^{-1}$ (2013) and 12.5 $\mu g.kg^{-1}$ (2014) in the Poráč area. Similarly to the cadmium, the lead content varied widely, which was reflected in the standard deviations (Table 1). In comparison with Matejovce, where the Pb content varied in a higher concentration: 3.17 $\mu g.kg^{-1}$ (2013) and 13.6 $\mu g.kg^{-1}$ (2014), the Poráč area seems to be less risky. However, the results show that both sites pose a potential risk resulting from the sheep milk consumption, since the limit value (20 $\mu g.kg^{-1}$) set by the EC Regulation 1881/2006 **(EC, 2006)** was exceeded in 9 (2013) and 3 samples (2014) taken from Matejovce and in 2 samples (2013 and 2014) taken from Poráč. Codex Alimentarius SR does not state a maximum allowed content of lead.

Copper and zinc are considered essential micronutrients and their content in food is desirable in an optimal amount **(Maas et al., 2011)**. The copper content in the milk samples varied in a relatively low concentrations compared with zinc. The mean value of the copper content was 0.07 $mg.kg^{-1}$ (2013) and 0.12 $mg.kg^{-1}$ (2014) in the samples from the Poráč site and 0.14 $mg.kg^{-1}$ (2013) and 0.16 $mg.kg^{-1}$ (2014) in the samples from the Matejovce site. Codex Alimentarius SR set the maximum level of copper to 0.40 $mg.kg^{-1}$. The limit value was not exeeded in any samples on the mean level. However, in the case of individual samples, the limit was exceeded in one sample from the Poráč site taken in 2013 (1.47 $mg.kg^{-1}$). It can be concluded that in terms of the copper content, consumption of the sheep milk does not pose a health risk. The mean values of the zinc content varied at higher levels. In the samples taken from the Poráč site, the Zn content was 5.53 $mg.kg^{-1}$ (2013) and 5.64 $mg.kg^{-1}$ (2014). The Zn content recorded in the samples taken from the Matejovce site was 3.41 $mg.kg^{-1}$ (2013) and 5.51 $mg.kg^{-1}$ (2014). The content of Zn was relatively balanced as evidenced by the relatively small standard deviations (Table 1). Due to the fact that no legislative standard defines limit values for Zn in milk, it is not possible to make conclusion on the hygienic quality of the sheep milk in terms of zinc content.

CONCLUSION

Evaluation of the contamination level of agricultural products and food ingredients is important in terms of maintaining an adequate health safety of human food chain components, especially in areas that are significantly contaminated by risk elements such as heavy metals. The contents of the studied contaminants in the raw sheep milk samples taken from two areas: Poráč and Matejovce nad Hornádom, ranged in various levels posing different degrees of health risk resulting from consumption of the milk. Mercury was assumed to pose the highest health risk, however, the hygiene standards for this element were not exceeded. The most hazardous contaminant was cadmium. The maximum allowed level of Cd (10 $\mu g.kg^{-1}$) was exceeded in 25 out of 53 samples. The limit value of lead (20 $\mu g.kg^{-1}$) was exceeded in 16 cases. The copper content exceeded the limit value (0.4 $mg.kg^{-1}$) in one case. The limit value for zinc is not defined by any legislation. It can be concluded that regular consumption of the sheep milk, in connection with intake of the studied contaminants from other sources, may pose a health risk in the long term. Therefore, it is necessary to monitor the contaminants in the milk, as well as milk products in long-term and more detail.

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Contact address:
Radovan Stanovič, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: radovan.stanovic@uniag.sk.
Július Árvay, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: julius.arvay@gmail.com.
Martin Hauptvogl, Slovak University of Agriculture in Nitra, Faculty of European Studies and Regional Development, Department of Sustainable Development, Tr. A. Hlinku 2, 949 76 Nitra, E-mail: martin.hauptvogl@uniag.sk.
Ján Tomáš, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jan.tomas@uniag.sk.
Anton Kováčik, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: anton.kovacik@uniag.sk.
Zuzana Záhorcová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zuzana.zahorcova@uniag.sk.
Marek Slávik, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marek.slavik@uniag.sk.

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BIOACCUMULATION OF CADMIUM BY SPRING BARLEY (*HORDEUM VULGARE* L.) AND ITS EFFECT ON SELECTED PHYSIOLOGICAL AND MORPHOLOGICAL PARAMETERS

Miriama Kopernická, Ján Tomáš, Tomáš Tóth, Ľuboš Harangozo, Beáta Volnová

ABSTRACT

Heavy metals and other toxic elements in the environment, mainly located in soil and groundwater, have a significant effect on plant and its productivity that has a huge attention in recent years. Accumulation of heavy metals in soil cause toxicity to plants, and contaminate the food chain. The industrial areas, as well as developing countries have been contaminated with high concentration of heavy metals. Main sources of contamination are mining and other industrial processes, as well as military and or lanfills, sludge dumps or waste disposal sites. The heavy metals are very dangerous to environment and pose serious danger to public health by entering throught the food chain or into drinking water. Phytoextraction is one way how to remove the contaminants from soil by plants. Phytoextraction of heavy metals is a technology that has been studied for several years. It is more ecological and cheaper way how to clean our environment.Several plant species are known becauce they hyperaccumulate a high contents of metals from the soil. The accumulators are mainly herbaceous species, crops and nowadays angiosperm trees with a high growth such as poplars or willows. We have focused on the determination of some morphological (lenght and weight of roots and biomass) and physiological (contents of dry mass and number of lief stomata) characteristics and the determination of the bioaccumulation factor and the translocation factor of cadmium by spring barley (*Hordeum vulgare* L.). Imprints of leaves were evaluated using an optical microscope Axiostar Plus, Carl Zeiss, lens CP Achromat 40x/0.65, eyepiece PI 10x / 18, Canon Utilities Software Zoom Browser EX 4.6 and hardware Acer Travel Mate 4600, Canon Power Shot A95. The density of stomata was evaluated on an area of 1 mm^2. Samples of the dried plants (leaves and roots) were mineralized by acid digestion using microwave digestion device MARS X - press 5. The end of determination to obtain the cadmium content was performed by atomic absorption spectrometer Varian 240 Z with GTA120 graphite furnace. The effect of contamination by cadmium to germination, length of leaves and number of stomata on abaxial side of leaf was confirmed. The contaminated soil by cadmium does not pose a risk of heavy metal entry into the feed and food chain by spring barley (*Hordeum vulgare* L.).

Keywords: stomata; barley; phytoextraction; cadmium; heavy metals;

INTRODUCTION

Trace metals in the aquatic environment can be traced to both natural and anthropogenic sources. Trace metals are classified as being light or heavy with densities less or greater than 5 $g.cm^{-3}$. Natural and anthropogenic activities usually result in gaseous emissions and wastewater discharges into the environment. When these substances in the emissions and effluent discharged into the environment are in very minute amounts or in low concentrations and are toxic to plants and animals and have short residence time in the environment, they are described as contaminants **(Tyokumbur and Okorie, 2014)**.

Heavy metals are extremely persistent in the environment because they are not biodegradable and may not be broken down by chemical oxidation or through thermal processes. Some metals are essential for plant growth. Very high or low contents of some heavy metals may be inhibitory to plant growth **(Ochonogor and Atagana, 2014)**.

Heavy metals are inorganic chemical hazards. The most contaminated sites are by lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni). Soils are the major sink for heavy metals released into the environment by. Their total concentration in soils persists for a long time after their introduction. Changes in their chemical forms (speciation) and bioavailability are possible **(Maslin and Maier, 2000)**.

Heavy metal contamination of soil may pose risk human's health and the ecosystem through soil, the food chain, drinking ground water, reduction in food quality (safety and marketability) via phytotoxicity, reduction in land usability for agricultural production causing food insecurity, and land tenure problems. The adequate protection and restoration of soil ecosystems contaminated by heavy metals require their characterization and remediation **(Wuana and Okieimen, 2011)**.

Cadmium (Cd^{2+}) is a highly toxic trace element whose presence in the environment is cxaused by human

activities. It is taken up by roots via essential metal transporters **(Cohen et al., 1998; Lasat et al., 2000; Pence et al., 2000)**.

After longer exposure to heavy metal decreases growth rate by affecting various aspects of plant physiology, as well as decreases carbon assimilation that can lead to wilting **(Perfus - Barbeoch et al., 2002)**.

Plants throughout their life cycle experience various types of environmental stresses (such as drought, salinity, high temperature, cold, heavy metal and other similar stresses) due to their sessile nature. Among these stresses, salinity stress has become the limiting factor for the productivity of agricultural crops by affecting germination, plant vigor and finally crop yield **(Munns and Tester, 2008; Zhang et al., 2011; Arif Shafi Wani, 2013)**.

Cadmium is toxic to many plant species even at very low concentration. It is mainly generated from smelting industries, abrasion of automobile tires, burning of diesel and heating oils and from phosphate fertilizers originated by aerobically digested sewage sludge. Concerning to its effects on plants, Cd is accumulated in them and interacts with several physiological processes such as photosynthetic, respiratoryand nitrogen metabolism, resulting in poor growth and low biomass. Furthermore, Cd is associated with oxidative stress and it can result in the production of free radicals and active oxygen species **(Puertas-Mejı´a et al., 2010)**.

The role of oxidative stress in metal toxicity has been assessed by measuring alterations in the redox metabolic components of stressed plants. Over the past few years major progress has been achieved, particularly by comparing metal tolerant and/or metal hyperaccumulator genotypes with their non-tolerant relatives and by using transgenic plants that overexpress or lack specific redox elements. These approaches provided novel insight into the relationship between metal sensitivity and cellular redox imbalance **(Sharma, 2008)**. Metal ions may directly interfere with the metabolic activities by altering the conformation of proteins, for example enzymes, transporters or regulator proteins, owing to their strong affinities as ligands to sulfhydryl and carboxylic groups. This is taken to be a major cause for metal imposed toxic effects **(Sharma, 2004)**. Stress factors generally applied at higher levels may cause irreversible changes in physiological processes as stomatal closure or slowing down the biochemical processes. Low levels of toxic metals such as cadmium also slow growth and affect biochemical processes. Strength and duration of stress exposure can also cause permanent changes. In addition to toxic metals, changes in the membranes of plant cells are mostly affected by water stress, changes in temperature and by frost. Toxicity of the metals (such as cadmium), can cause an accumulation in tissues, which consequently affects the metabolism of plants, particularly the photosynthetic apparatus **(Lachman et al., 2015)**.

The mechanisms of cadmium (Cd) uptake and tolerance in plants have been studied extensively, but a clear understanding of what controls the translocation of Cd to aboveground tissues is lacking. One approach to better understanding the factors that control Cd accumulation and distribution is to determine where Cd is bound as it travels from the root surface to aboveground parts **(Akhter et al., 2014)**.

Accumulation and translocation of the environmental pollutants as cadmium was evaluated in different parts of plants. Although roots comprise usually only a little part of whole plant biomass, they consistently contain 70 – 100% of the whole plant metal burden **(Lachman et al., 2004)**.

The effect of Cd on transpiration of water from leaves has been studied extensively. At low concentrations, Cd increased the permeability of the leaf cuticle and increased transpiration in sugar beet. At high concentrations, Cd induced stomatal closure and decreased leaf transpiration in mustard (*Brassica juncea* L.), barley (*Hordeum vulgare* L.), and lettuce (*Lactuca sativa* L.). However, the mechanism of Cd-induced stomatal closure is still poorly understood. Some studies reported increased production of abscisic acid (ABA) with increased Cd-exposure and suggested that ABA might regulate stomata closure in Cd-stressed conditions, however, in ABA-insensitive mutants of Arabidopsis thaliana L. Cd^{2+} affected guard cell regulation in an ABA-independent manner by entering the cytosol via Ca^{2+} channels **(Akhter and Macfie, 2012)**.

Environmental contamination by Cd in human food typically comes from crops and contaminated water. The effects of Cd range from shortness of breath, effects on respiratory system, vomiting and diarrhoea, kidney damage and renal failure, bone damage, Itai-Itai disease (osteomalacia), to low birth weight and increase in abortions **(Stanbrough et al., 2013)**.

Crops grown in contaminated soil may accumulate Cd in different plant parts, such as root, leaf, grain etc., and consumers may develop a number of Cd-related chronic diseases. It is recommended to keep Cd concentrations below regulatory guidelines in vegetables, fruits, grains and other agricultural products to avoid metal toxicity. Because the concentration of Cd in edible plant tissues is not always directly proportional to the concentration of Cd in the soil, understanding the mechanisms of Cd accumulation and translocation in plants is important to ensuring food safety **(Akhter and Macfie, 2012)**.

On the other hand cereals are main foods in many countries, as human foods or as animal feeds. Epidemiological studies indicate that the consumption of whole - grain and whole - grain products is related to reduction in total mortality, coronary heart disease mortality, diabetes and cancer incidense. These beneficial effects are attributed to the bioactive factors in cereal grain such as non digestible carbohydrates and phytochemicals **(Ivanišová et al., 2010)**. Cereals and pseudocereals have a significant role in human nutrition. They are source of specific carbohydrates, proteins, lipids, fibre and wide spectrum of vitamins and minerals. Cereals and pseudocereals may also contain some antinutritional factors, such as phytic acid, polyphenols, trypsin inhibitors and inhibitors of α-amylase. These are responsible for reducing of protein and carbohydrate digestibility and decreasing accessibility of minerals due to complex formation **(Kocková and Valík, 2011)**.

The legislation should respect environmental protection and public health, at national and international level **(Kabata - Pendias and Pendias, 2001).**

MATERIAL AND METHODOLOGY

The aim of our work is the evaluation of selected morphological (lenght and weight of roots and biomass) and physiological (contents of dry mass and number of lief stomata) characteristics and the determination of the bioaccumulation factor and the translocation factor of cadmium by spring barley (*Hordeum vulgare* L.).

The seeds were germinated in Petri dishes on a filter paper for 48 hours in the dark with temperature 25 °C and 80% air humidity. After 2 days 100 germinated seeds were transferred into each container filled with 950 g of washed silica sand. The containers were watered firstly by Hoagland's solution **(Hoagland and Arnon, 1950)** and after that on alternate days as needed with destilled water. The water – soluble $CdCl_2$. 2.5 H_2O was added to containers to obtain the application of 1, 5 and 25 mg.kg^{-1}. The control treatments (0 mg.kg^{-1}) had no added heavy metal. The plants were grown with supplementary lihting 16/8 hours photoperiod and controlled temperature 20 – 25 °C. The plants were harvested after four weeks of cultivation. They were cleaned, washed with deionized water and separated into roots and aerial parts.

Imprints of leaves have been transferred to a glass slide and preparations were made for further analysis. Microreliefs we collected in the central part of the leaf on adaxial (upper) and abaxial (lower) side. Preparations were evaluated using an optical microscope Axiostar Plus, Carl Zeiss, lens CP Achromat 40x/0.65, eyepiece PI 10x / 18, Canon Utilities Software Zoom Browser EX 4.6 and hardware Acer Travel Mate 4600, Canon Power Shot A95. The density of stomata was evaluated on an area of 1 mm^2.

Cadmium concentrations were obtained by treating the samples by 10 cm^3 of aqua regia (2.5 cm^3 HNO_3 and 7.5 cm^3 HCl) using microwave digestion unit Mars X-press 5 (CEM Corp., USA). The mineralization was carried out in teflon vessels. The concentrations were measured by atomic absorption spectrometry (AAS) in a Varian AA 240 Z (Varian, Australia) with GTA120 graphite furnace.

The significance of selected parametres was verified by LSD test. We used Pearson correlation coefficients at significance level of p <0.05 (weak statistical significance) and p <0.01 (very strong statistical significance) by STATGRAPHICS Plus 5.1.

RESULTS AND DISCUSSION

In the experiment, the barley plants showed visual symptoms of the external toxic effect of metal, such as leaf discoloration and dehydration.

The changes in dry matter content of roots and leaves, and the length of the leaves indicate that the plant react to changing of environmental conditions **(Piršelová et al., 2010)**.

Strong statistical significance was confirmed between contamination by cadmium with (Table 1):

- germination → low negative correlation,
- length of the leaves→ high negative correlation,

Weak statistical significance was confirmed between contaminanation by cadmium with (Table 2):

- weight of biomass→ low negative correlation.

The negative impacts of heavy metals on plants are decreasing of seed germination, lipid content, enzyme activity and plant growth, the inhibition of photosynthesis or reduction of chlorophyll production **(Gardea-Torresdey et al., 2005; Akpor et al., 2014)**.

The adaxial (Figure 1) and abaxial (Figure 2) side preparations of the barley leaves were evaluated using an optical microscope. Very strong statistical significance was between contaminanation by cadmium with (Table 3) number of leaf stomata on abaxial side → high positive correlation. After microwave digestion of the harvested biomass (roots and leaves) of spring barley (*Hordeum vulgare* L.) was measured the content of Cd and the obtained results are shown in Figure 3 and Figure 4.

The cadmium content in roots in first variant (application 1 mg.kg^{-1} of $CdCl_2$. 2.5 H_2O) was 219.39 ±68.65 mg.kg^{-1}, in second variant (application 5 mg.kg^{-1} of $CdCl_2$. 2.5 H_2O) was 489.38 ±140.41 mg.kg^{-1} and in third variant (application 25 mg.kg^{-1} of $CdCl_2$. 2.5 H_2O) was 2064.36 ±108.32 mg.kg^{-1} of dry mass.

Table 1 Cadmium effect on the germination and length of the leaves of barley.

Contamination by Cd (mg.kg^{-1})	Germination (%)	Length of the leaves (cm)
0	94	29.40
1	91	28.60
5	88	24.90
25	84	23.90

Table 2 Cadmium effect on the dry weight of the roots and leaves of barley.

Contamination by Cd (mg.kg^{-1})	Dry weight of the roots (g)	Dry weight of the leaves (g)	Total biomass (g)
0	0.8902	1.4850	2.3752
1	0.7232	1.4848	2.2080
5	0.4792	1.2613	1.7405
25	0.6400	1.2850	1.9250

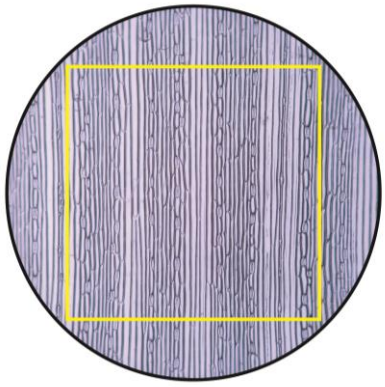

Figure 1 Detail of the adaxial (upper) side of spring barley (*Hordeum vulgare* L.) leaf on an area of 1 mm^2.

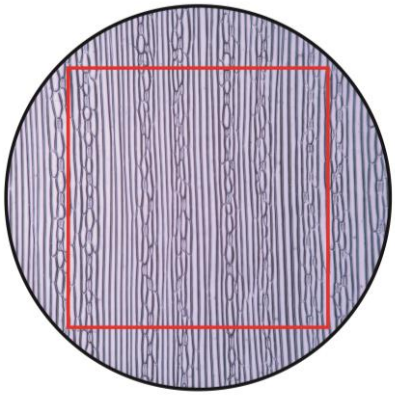

Figure 2 Detail of the abaxial (lower) side of spring barley (*Hordeum vulgare* L.) leaf on an area of 1 mm^2.

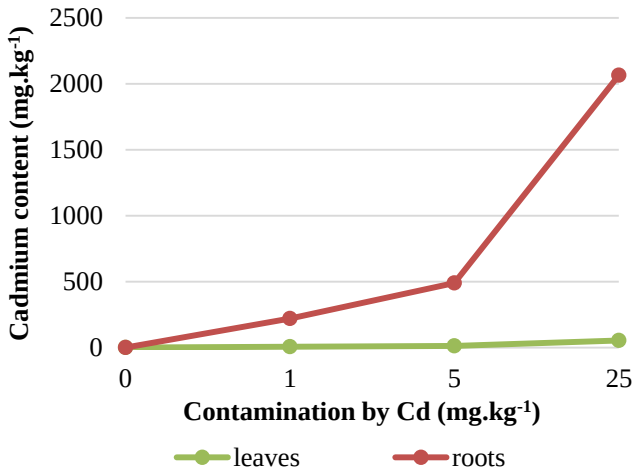

Figure 3 The evaluation of cadmium content in the roots and leaves of barley.

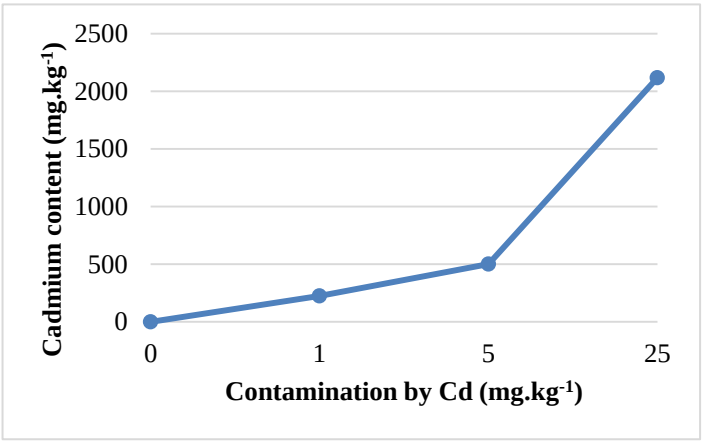

Figure 4 The evaluation of cadmium content in the barley.

The content of cadmium in leaves varied over a value in first variant 5.57 ±0.29 mg.kg^{-1}, in second variant 11.68 ±2.14 mg.kg^{-1} and in third variant 52.93 ±6.73 mg.kg^{-1} of dry mass.

The cadmium content in different parts of the plant increases proportionally with an increasing application of heavy metal. The cadmium content in the root system was up to 40 times higher than the aboveground part of barley.

The bioaccumulation factor has been used as an effective way to show the potencial of the plants for phytoremediation. It is the indicator of the ability of metal accumulation by plants. A good accumulator plant should

Table 3 Cadmium effect on the number of leaf stomata in the central part of the barley leaf on adaxial (upper) and abaxial (lower) side.

Contamination by Cd ($mg.kg^{-1}$)	Number of lief stomata per $1mm^2$ (adaxial side)	Number of lief stomata per $1mm^2$ (abaxial side)
0	59	51
1	53	58
5	59	63
25	60	67

Table 4 Bioaccumulation factor and the translocation factor of barley according to contamination by cadmium.

Contamination by Cd ($mg.kg^{-1}$)	BF of the roots (%)	BF of the leaves (%)	TF of the barley x 100
0	-	-	-
1	219.39	5.57	2.54
5	97.88	2.34	2.39
25	82.57	2.12	2.56

have a bioaccumulation factor lower than 100%. The translocation factor discribes in which part of plant body is the highest accumulation of contaminant **(Kherbani et al., 2015).**

The bioaccumulation factor was calculated as:

BF = Cd content in the plant/ Cd content in the soil.

The translocation factor was described as:

TF = Cd content in the leaves/ Cd content in the roots.

In our case, the maximum bioaccumulation factor of roots was obtained with the value 97.88% to a 5 $mg.kg^{-1}$ for Cd.

The cadmium content in the leaves of barley was much lower than in the rooots.Translocation factor is too small, so spring barley is very interesting plant for phytoextraction. The results are shown in Table 4.

In acid soils, cadmium is more mobile and less able to return the adsorption to sediments and minerals, rocks and sand. Adsorption of cadmium dependents on its concentration, pH of the soil solution, soil type, duration of exposure and the concentration of complexing ligand. Cadmium is an element that is highly mobile in acidic soil. The mobility increases with decreasing pH, fertilizing by acid fertilizers and low content of organic matter in the soil **(Trebichalský et al., 2010)**.

Phytoextraction of heavy metals is a technology that has been studied for several years. It is more ecological and cheaper way how to clean our environment.

Several plant species are known becauce they hyperaccumulate a high contents of metals from the soil, they are able to store particularly high amounts of heavy metals in aboveground organs. The accumulators are mainly herbaceous species, crops and nowadays angiosperm trees with a high growth such as poplars or willows. Woody species now represent attractive models since they have a higher biomass and a more important root system to decontaminate soils deeper than herbaceous plants **(Saladin, 2015)**.

CONCLUSION

Periodical monitoring of plants should be encouraged especially crops from areas that are grown and harvested next to the mining or industrial areas and the geochemical anomalies. The heavy metals may enter the leaves via the stomata.

The effect of contamination by cadmium to germination, length of leaves and number of stomata on abaxial side of leaf was confirmed.The contaminated soil by cadmium do not pose a risk of heavy metal entry into the feed and food chain by spring barley (*Hordeum vulgare* L.).

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Contact address:

Miriama Kopernická, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: m.kopernicka@gmail.com.

Ján Tomáš, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jan.tomas@uniag.sk.

Tomáš Tóth, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: tomas.toth@uniag.sk.

Ľuboš Harangozo, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: harangozolubos@gmail.com.

Beáta Volnová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: bejatkavolnova@pobox.sk.

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MICROBIOLOGICAL QUALITY OF CHICKEN THIGHS MEAT AFTER APPLICATION OF ESSENTIAL OILS COMBINATION, EDTA AND VACCUM PACKING

Miroslava Kačániová, Margarita Terentjeva, Czeslaw Puchalski, Jana Petrová, Jana Hutková, Attila Kántor, Martin Mellen, Juraj Čuboň, Peter Haščík, Maciej Kluz, Rafal Kordiaka, Simona Kunová

ABSTRACT

The aim of the present work to monitoring chicken the microbiological quality of vaccum packaged thighs after treatment by ethylenediaminetetraacetate (EDTA), anise (*Pimpinella anisum*), spearmint (*Mentha spicata* var. *crispa*), thyme (*Thymus vulgaris* L.) oregano (*Origanum vulgare* L.) essential oils and stored in at 4 ±0.5 °C for a period of 16 days. The following treatments of chicken thighs were used: air-packaged control samples, control vacuum-packaged samples, vacuum-packaging with EDTA solution 1.5% w/w, control samples, vacuum-packaging after treatment with *Pimpinella anisum, Mentha spicata* var. *crispa* essential oil at concentrations 0.2% v/w, vacuum-packaging after treatment with *Thymus vulgaris* L., *Origanum vulgare* L. essential oil at concentration 0.2% v/w. The quality assessment of all samples was done microbiologically and following microbiological parameters were detected: the anaerobic plate count, *Enterobacteraceae* counts, lactic acid bacteria and *Pseudomonas* spp. counts. The number of anaerobic plate count ranged from 3.69 log CFU.g^{-1} in all tested group on 0 day to 5.68 log CFU.g^{-1} on 16 day in control group stored in air condition. The number of lactic acid bacteria ranged from 2.00 log CFU.g^{-1} in all tested group on 0 day to 4.82 log CFU.g^{-1} on 16 day in group with oregano, thyme essential oils combination. *Enterobacteriacea* counts in chicken thighs was 0.68 log CFU.g^{-1} on 0 day to 7.58 CFU.g^{-1} on 16 day in air-packed meat samples. The *Pseudomonas* spp. was not found in all tested samples. Among the antimicrobial combination treatments examined in this work, the as application of vacuum packaging, EDTA and essential oils treatment was the most effective against the growth of *Enterobactericeae*, inhibitory effect on anaerobic plate count also was observed. The results of this present study suggest the possibility of application the *Pimpinella anisum*, *Mentha spicata* var. *crispa*, *Thymus vulgaris* L., *Origanum vulgare* L. essential oil of as natural food preservatives and potential sources of antimicrobial ingredients for food industry for chicken thighs meat treatment.

Keywords: meat; microorganisms; essential oils; vaccum; EDTA

INTRODUCTION

Poultry meat is a very popular food commodity around the world due to its low cost of production, low fat content, high nutritional value, distinct flavor **(Barbut, 2001; Patsias et al., 2008)**. The diverse nutrient composition of meat makes it an ideal environment for the growth and prolifiration of meat spoilage micro-organisms, as well as food-borne pathogens **(Zhou et al., 2010)**. Therefore is essential to apply adequate preservation technologies to extend the shelf life of perishable meat products which is a major concern for the meat industries **(Wang et al., 2004)**.

Special attention in poultry meat production is paid to the fact that live animals are hosts to a large number of different microorganisms residing on their skin, feathers and in the alimentary tract. During the slaughter a majority of these microorganisms are eliminated, but subsequent contamination is possible at any stage of the production process including contamination from feather plucking and evisceration equipment, washing before storage, cooling or freezing. Microorganisms from the environment, equipment and operators' hands also can contribute to contamination of meat. During the processing the changes in the microflora of meat are reported from, in general, Gram-positive rods (micrococci) to Gram-negative bacteria including Enterobacteriaceae, *Pseudomonas* spp., which were isolated the most frequently. Industrial poultry slaughterhouses have a particular technological process, the individual stages of which are not in conformity with modern principles of hygienic meat production and processing. Factors, which alter the mcrobiological quality of poultry meat can occur during the all processing steps **(Kozačinski et al., 2006)**.

Naturally occurring antimicrobial compounds have good potential to be applied as food preservatives. Essential oils, other extracts from plants, herbs, spices, some of their constituents have shown antimicrobial activity against different food pathogens and spoilage microorganisms **(Bakkali et al., 2008; Burt, 2004; Holley and Patel, 2005)**. Plants, plants products have been claimed to have

health-promoting effects, which may be related to the antioxidant activity *in vivo* **(Ivanišová et al., 2013; Ivanišová et al., 2015a, b)**.

Anise (*Pimpinella anisum* L.), which belongs to the family *Apiaceae*, is an important spice, medicinal plant used for pharmaceutics, perfumery and food industry. The fruits as well as the essential oils are characterized by antispasmodic, antioxidant, antimicrobial, insecticidal and antifungal effects **(Gülcin et al., 2003; Özcan, Chalchat, 2006; Tepe et al., 2006; Tirapelli et al., 2007)**. Its fruits which are called aniseed contain around 1.5-5.0% of essential oil mainly composed of volatile phenylpropanoids like trans-anethole with around 90% **(Tabanca et al., 2005)**. In addition, the essential oil of the anise fruit also contains a small proportion of estragol, anisaldehyde, himachalene and cis-anethole **(Omidbaigi et al., 2003; Tabanca et al., 2006)**.

The genus *Mentha* of the family *Lamiaceae* comprises about 19 species, 13 natural hybrids, is widely distributed across the Europe, Africa, Asia, Australia and North America **(Kumar et al., 2011)**. *Mentha spicata* L., commonly known as spearmint, is a native of Africa, temperate Asia and Europe. It is an herbaceous, rhizomatous, perennial plant growing up to 40x130 cm in height. A literature review shows the antifungal effect of *M. spicata* EO (essential oil) against some food-poisoning fungi **(Sokovic et al., 2009)**, other storage insects **(Lee et al., 2002)**, but reports are lacking about this EO's ability to counter aflatoxin production.

Antimicrobial activity of thyme or oregano essential oil incorporated edible films have been evaluated by a number of researchers, however, limited data exist on the application of antimicrobial edible films incorporated with essential oils in real food systems **(Seydim and Sarikus, 2006; Chi et al., 2006; Oussalah et al., 2006; Du et al., 2008)**. Among *Lamiaceae* species, oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), wild thyme (*Thymus serpyllum* L.) have been studied widely for their antioxidant activity due to the high content of phenolic compounds **(Vichi et al., 2001; Zandi and Ahmadi, 2000)**.

The aim of this study was to investigate the effects of anise, spearmint, thyme, oregano essential oils and ethylenediaminetetraacetate in combination with vacuum packaging on the microbiological properties of chicken thighs.

MATERIAL, METHODOLOGY

Preparation of samples

To evaluate the antimicrobial activity of essential oils the chicken thigh with skin for each experimental group was taken. The chicken thigh fresh samples with were prepared as follow: for air-packaging (AC, control samples) chicken thigh fresh meat was packaged to polyethylene bags and stored aerobically at 4 ±0.5°C; for vacuum-packaged (VPC, control samples) chicken thigh fresh meat was packaged to polyethylene bags, stored anaerobically in vacuum at 4 ±0.5°C; for vacuum-packed samples with EDTA solution 1.5% w/w (VPEC, control samples) chicken thigh was treated with EDTA for 1 min, then packaged to polyethylene bags, stored anaerobically in vacuum at 4 ±0.5°C; for vacuum-packed samples treated with *Pimpinella anisum* + *Mentha spicata* var. *crispa* 0.20% v/w (VP+PAO+MSO) chicken thigh was treated with anise in combination with mint oil for 1 min, packaged to polyethylene bags, stored anaerobically in vacuum at 4 ±0.5°C; for vacuum-packed samples treated with *Thymus vulgaris* L. In combination with *Origanum vulgare* L. 0.20 % v/w (VP+TVO+OVO) chicken thigh was treated with essential oil for 1 min, packaged to polyethylene bags, stored anaerobically in vacuum at 4 ±0.5°C. For sample packaging, a vacuum packaging machine type VB-6 (RM Gastro, Czech Republic) was used. Each sample was packaged immediately after treatment. EDTA solution (pH 8.0, 99.5% purity, analytical grade, Invitrogen, USA) was prepared at final concentration of 50 mM and used in treatment of chicken thigs samples. Anise, spearmint, thyme and oregano essential oils (Hanus, Nitra, Slovakia) was added to coat the surface of chicken thigh on both sides of each sample using a micropipette. Final concentration of 0.2% v/w of EO was used for treatment.

Microbiological analysis

An amount of 10 g (10 cm^2) of the chicken thigh was sampled using sterile scalpels, forceps and immediately transferred into a sterile stomacher bag containing 90 mL of 0.1% peptone water (pH 7.0) and homogenized for 60 s in a Stomacher at room temperature. Sampling and microbiological testing was carried out after certain time intervals: 0, 4, 8, 12, 16 days of experiment. Chicken thighs were stored in vacuum packaging at 4 ±0.5 °C. Microbiological analyses were conducted with accordance to standard microbiological methods. Anaerobic plate count (APC) was determined on Plate Count Agar (PCA, Oxoid, UK) after incubation for 48 h at 35 °C in anaerobic conditions. For *Pseudomonas* spp., 0.1 mL from prepared chicken meat suspension was spread onto the Pseudomonas Isolation agar (PIA, Oxoid, UK). After inoculation PIA was incubated for 48 h at 25 °C. For lactic acid bacteria enumeration, a 1.0 mL of sample was inoculated onto Rogosa, Sharpe agar (MRS, Oxoid, UK), Inoculated agar was incubated for 48-78 h at 37 °C in an aerobic atmosphere supplemented with carbon dioxide (5% CO_2). For *Enterobacteriaceae* counts, a 1.0 mL of sample was transferred into 10 mL of molten (45 °C) Violet Red Bile Glucose agar (VRBL, Oxoid, UK). After setting, a 10 mL molten medium was added to cover the suspension. Inoculated VRBL agars were incubated at 37 °C for 24 h. All plates were examined for typical colony appearance and morphology characteristics associated with each medium applied for cultivation of microorganisms.

RESULTS, DISCUSSION

Essential oils have not only antibacterial properties, but their application in meat can affect some meat characteristics as well. Based on antibacterial properties of EOs, type of affected pathogen, some essential oils are better than others for application in meat industry. Concentration of essential oils, which should be added to meat in order to prevent the oxidation, proliferation of foodborne pathogens, or to extend shelf-life by inhibition of background microflora, is usually higher than one used

in *in vitro* conditions because of interaction with meat components **(Bošković et al., 2013)**.

Anaerobic plate count (AC) values for the tested groups of chicken thigh are showed in Figure 1. The initial anaerobic plate count value of chicken thigh was 3.69 log CFU.g^{-1} on 0 day and the number of microorganisms increases to 5.68 log CFU.g^{-1} on 16 day in control group stored in air condition. In control group stored in vacuum packaging the AC counts were from 3.69 log CFU.g^{-1} on 0 day to 5.12 log CFU.g^{-1} on 16 day of experiment. In control group stored in vacuum packaging and EDTA treated the AC ranged from 3.69 log CFU.g^{-1} on 0 day to 4.78 log CFU.g^{-1} on 16 day. In the group after treatment with anise and spearmint essential oils combination, AC ranged from 3.69 log CFU.g^{-1} on 0 day to 4.56 log CFU.g^{-1} on 16 day. In group after treatment with thyme and oregano essential oils combination, the AC ranged from 3.69 log CFU.g^{-1} on 0 day to 4.45 log CFU.g^{-1} on 16 day. The lowest number on APC on 16 days was found in the group treated with oregano and thyme essential oil combination (4.45 log CFU.g^{-1}).

In study of **Radha Krishnan et al., (2014)**, *Enterobacteriaceae*, a psychrotrophic facultative anaerobic bacterial group, formed a substantial part of the chicken meat microbial flora and reached the final counts of 4.68, 3.76 for samples from the initial count of 3.32 log10 CFU.g^{-1}. For other samples, final counts were obtained as 4.59, 4.41, 3.91, 4.26, 4.51, 4.01, 4.11, 3.84 log10 CFU.g^{-1} for, samples respectively. **Radha Krishnan et al., (2014)** confirmed that the bacterial counts obtained from spice treated samples were lower than those from the control samples. It is important to point out, that the samples treated with combination of different spice extracts showed lower counts in comparison with the samples treated with extracts of individual spices.

The results of **Kačániová et al., (2015)** study suggest the possibility of using the essential oil of *Pimpinella anisum* L. And *Mentha piperita* as natural food preservatives and potential source of antimicrobial ingredients for meat. Among the treatments of antimicrobial combination examined in this work, the application of vacuum packaging, EDTA and essential oils treatment were the most effective against the growth of lactic acid bacteria, *Enterobactericeae.* Inhbitory effect on total viable count also was observed. Based on microbiological analyses, treatments with *Pimpinella anisum* L. and *Mentha piperita* essential oils resulted in shelf-life extension in comparison with the control samples. The similar results were found in our study in group with combination of anise, spearmint essential oils were used.

The primary objective of chilling poultry is to reduce microbial growth to a level that will maximize both food safety and shelf life **(Popelka et al., 2014)**. Hovewer, psychrotrophic nature of lactic acid bacteria enhancing their survival and multiplying on meat and supporting the spoilage of products. Lactic acid bacteria (LAB) values for the tested groups of chicken thigh are showed in Figure 2. The initial TVC value of chicken thigh was 2.00 log CFU.g^{-1} on 0 day. The number of lactic acid bacteria ranged from 2.00 log CFU.g^{-1} in all tested group on 0 day to 4.82 log CFU.g^{-1} on 16 day in group treated with oregano and thyme essential oils combination.

In control group stored in air condition, the number of LAB ranged from 2.00 log CFU.g^{-1} on 0 day to 3.98 log CFU.g^{-1} on 16 day. In control group stored in vacuum packaging LAB counts ranged from 2.00 log CFU.g^{-1} on 0 day to 4.12 log CFU.g^{-1} on 16 day. In control group stored in vacuum packaging after EDTA treatment, LAB ranged from 2.00 log CFU.g^{-1} on 0 day to 4.23 log CFU.g^{-1} on 16 day.

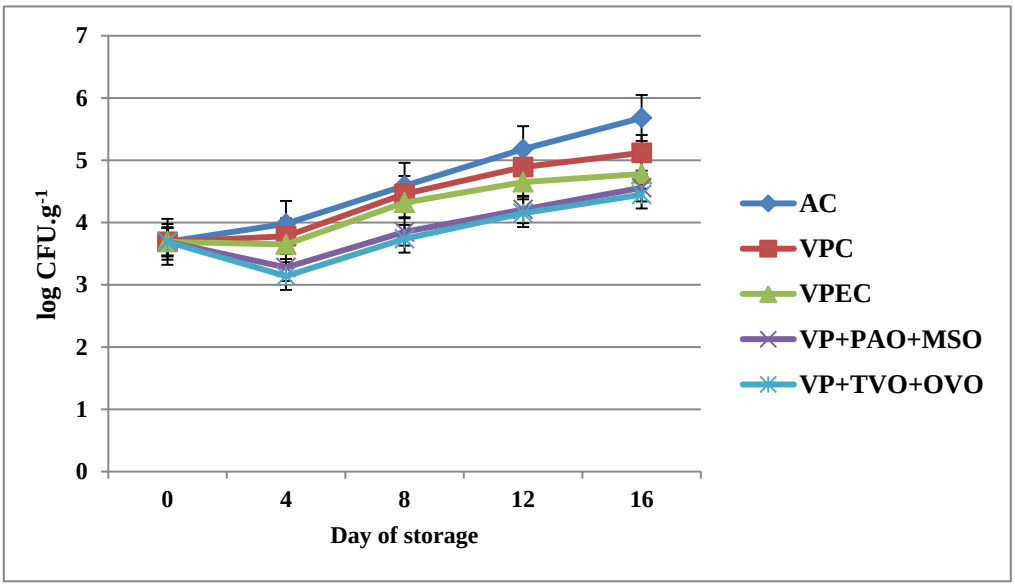

Figure 1 Changes (log CFU.g^{-1}) in population of anaerobic plate count in chicken thigh stored in air (AC); stored in vacuum (VPC); stored in vacuum packaging with EDTA (VPEC); stored in vacuum packaging after treatment with *Pimpinella anisum* + *Mentha spicata* var. *crispa* 0.20% v/w combination (VP+PAO+MSO); stored in vacuum packaging after treatment with *Thymus vulgaris* L. + *Origanum vulgare* L. 0.20 % v/w, combination (VP+TVO+OVO).

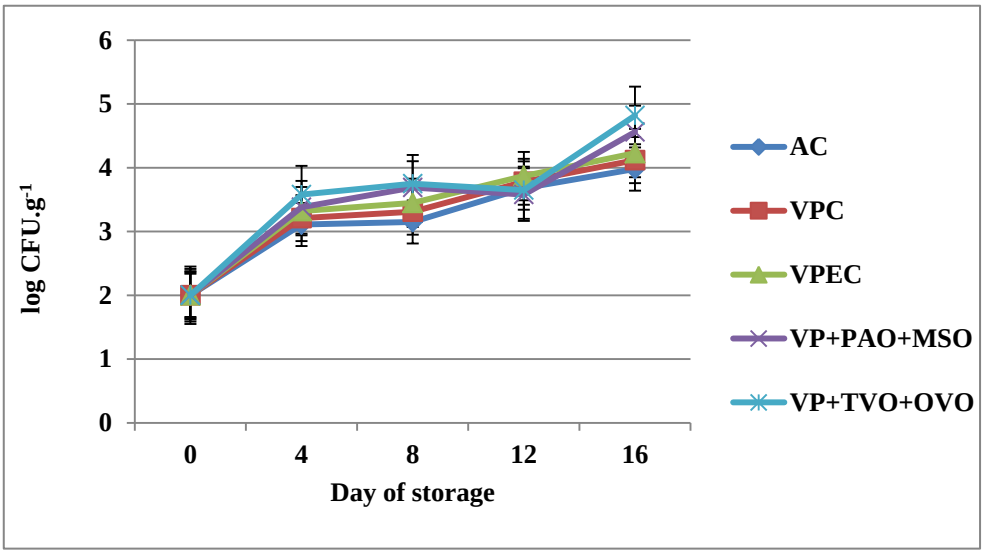

Figure 2 Changes (log CFU.g^{-1}) of lactic acid bacteria counts in chicken thigh stored in air (AC); stored in vacuum (VPC); stored in vacuum packaging with EDTA (VPEC); stored in vacuum packaging after treatment with *Pimpinella anisum* + *Mentha spicata* var. *crispa* 0.20% v/w combination (VP+PAO+MSO); stored in vacuum packaging after treatment with *Thymus vulgaris* L. + *Origanum vulgare* L. 0.20 % v/w, combination (VP+TVO+OVO).

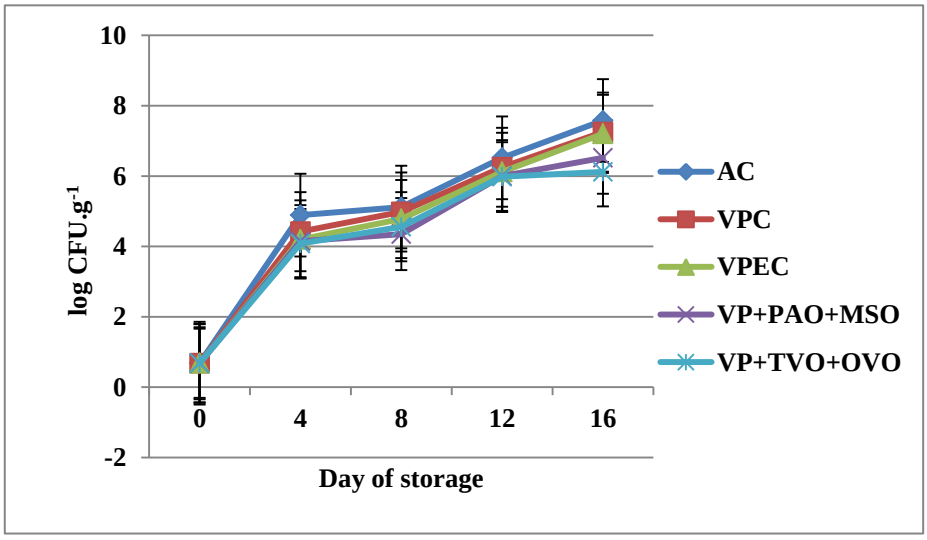

Figure 3 Changes (log CFU.g^{-1}) in population of *Enterobacteriaceae* in chicken thigh stored in air (AC); stored in vacuum (VPC); stored in vacuum packaging with EDTA (VPEC); stored in vacuum packaging after treatment with *Pimpinella anisum* + *Mentha spicata* var. *crispa* 0.20% v/w combination (VP+PAO+MSO); stored in vacuum packaging after treatment with *Thymus vulgaris* L. + *Origanum vulgare* L. 0.20 % v/w combination (VP+TVO+OVO).

In the group after treatment with anise and spearmint essential oils combination, number of LAB ranged from 2.00 log CFU.g^{-1} on 0 day to 4.56 log CFU.g^{-1} on 16 day. In the group after treatment with oregano and thyme essential oils combination ranged from 2.00 log CFU.g^{-1} on 0 day to 4.82 log CFU.g^{-1} on 16 day.

LAB behaves as facultative anaerobes and able to grow under high concentrations of CO_2. Thus they constitute a substantial part of the natural microflora of VP meats. LAB are recognized as the important competitors to other spoilage related microbial groups under VP/MAP conditions **(Castellano et al., 2004; Doulgeraki et al., 2011; Zhang et al., 2009)**. Particularly, *Lactobacillus* spp., *Carnobacterium* spp., *Leuconostoc* spp. are associated to the spoilage of refrigerated raw meat **(Nychas, Skandamis, 2005)**. More species of lactobacilli can be found during the storage under the vacuum at 4°C including *Lb. algidus* beyond *Lb. sakei*. The results of **Ntzimani et al. (2010)** indicate that LAB was an important part of the precooked chicken microflora, irrespective of the packaging conditions, the antimicrobial treatment combination. The latter observations could probably help to explain their rapid growth between days 0, 2 of storage. This is also in agreement with LAB growth in beef stored under MAP at 5°C **(Skandamis and Nychas, 2001)**.

Enterobacteriaceae counts of the tested groups of chicken thigh are showed in Figure 3. The initial *Enterobacteriacea* genera value of chicken thigh was 0.68 log CFU.g^{-1} on 0 day. Presences of these bacteria were found on all groups at 16 day. The number of *Enterobacteriaceae* genera ranged from 0.68 log CFU.g^{-1} in all tested groups of samples on 0 day to 7.58 log CFU.g^{-1} on 16 day in control group stored in air condition. In control group stored in air condition the number of *Enterobacteriaceae* genera ranged from 0.68 log CFU.g^{-1} on 0 day to 7.58 log CFU.g^{-1} on 16 day. In control group stored in vacuum packaging, *Enterobacteriaceae* counts ranged from 0.68 log CFU.g^{-1} on 0 day to 7.25 log CFU.g^{-1} on 16 day. In control group stored in vacuum packaging after EDTA treatment, *Enterobacteriaceae* counts ranged from 0.68 CFU.g^{-1} on 0 day to 7.20 log CFU.g^{-1} on 16 day. In the group of chicken thigh treated with anise and spearmint essential oils combination *Enterobacteriaceae* counts ranged from 0.68 log CFU.g^{-1} on 0 day to 6.52 log CFU.g^{-1} on 16 day. In the group of chicken thigh treated with oregano and thyme essential oils combination, *Enterobacteriaceae* counts ranged from 0.68 log CFU.g^{-1} on 0 day to 6.12 log CFU.g^{-1} on 16 day.

Enterobacteriaceae grew under vacuum packaging conditions at a slower rate than under aerobic packaging. This is in agreement with the results of **Chouliara et al., (2007)**, who reported that both MAP, oregano oil had a strong effect in the reduction of *Enterobacteriaceae* counts. On day 9 of storage, the use of oregano oil at its lower concentration (0.1%), had practically no effect on *Enterobacteriaceae* counts while the higher concentration (1%) gave a reduction of more than 6 log CFU.g^{-1}. On the same day, the *Enterobacteriaceae* counts were reduced by 1.5 log CFU.g^{-1} (MAP 1), 1.8 log CFU.g^{-1} (MAP 1, oregano oil 0.1%), more than 6 log CFU.g^{-1} (MAP 1, oregano oil 1%), 3.4 log CFU.g^{-1} (MAP2), 4.3 log CFU.g^{-1}g (MAP 2, oregano oil 0.1%), more than 6 log CFU.g^{-1} (MAP 2, oregano oil 1%).

Growth of the *Enterobacteriaceae* was completely inhibited after thyme essential oil treatment was applied and final counts (ca. 4.0 log CFU.g^{-1}) were reduced (ca. 3 log cycle) significantly (p <0.05) at the end of the storage period (day 12) in Giatrakou et al. (2010) study. The explanation of this was the antibacterial effects of the essential oils applied the study and this is in agreement with the results of the present study. Thymol essential oil treatment also produced the lower bacterial counts as compared to the control samples during the storage that is in agreement with our results.

Pseudomonas spp. were not isolated in the present study from all samples grou were tested. It is now well established that *Pseudomomas* spp. may form a significant part of the spoilage microflora of chicken meat stored under refrigeration **(Jay et al., 2005)**.

Among the treatments used for improving the shelf-life of products examined in the study of **Pavelkova et al., 2014**, the application of EDTA, oregano oil and thymus oil were the most effective against the growth of Gram-negative bacteria. Inhibitory effect on total viable count and LAB also was identified. Based on microbiological analyses, treatments with oregano and thymus oil combination produced a shelf-life extension of 8-9 days in comparison to the control samples. The ability of vacuum packaging to inhibit a growth of spoilage organisms is well documented, but many pathogenic organisms are less affected in this process. Therefore, the combined effect of essential oils as oregano and thymus including vacuum packaging on the safety of the meat could be investigated.

CONCLUSION

The results of the present study suggest the possibility of using the essential oil of anise, spearmint, thymol, oregano as natural food preservatives and potential source of antimicrobial ingredients for meat. Among the combinations of treatments, which may pose antimicrobial activity and examined in the present work, the use of modified storage condition as vacuum packaging, treatment with EDTA and essential oils were the most effective against the growth of lactic acid bacteria, *Enterobactericeae* family. Also the growth of anaerobic microorganisms were inhibited. Based on microbiological analyses, the treatment with anise, spearmint, thyme, oregano essential oils resulted in shelf-life extension as compared to the control samples. The combined effect of four essential oils, EDTA, vacuum packaging can significantly contribute the shelf-life and safety of the chicken thigh.

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Contact address:

Miroslava Kačániová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@uniag.sk.

Margarita Terentjeva, Latvia University of Agriculture, Faculty of Veterinary Medicine Institute of Food, Environmental Hygiene, K. Helmaņa iela 8, LV-3004, Jelgava, Latvia, E-mail: margarita.terentjeva@llu.lv.

Czeslaw Puchalski, University of Rzeszow, Faculty of Biology, Agriculture, Department of Bioenergy Technologies, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: cpuchal@univ.rzeszow.pl.

Jana Petrová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.petrova@uniag.sk.

Jana Hutková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.hutkova@uniag.sk.

Attila Kántor, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: attila.kantor@uniag.sk.

Martin Mellen, Hydina Slovakia, s. r. o., Nová Ľubovňa 505, 065 11 Nová Ľubovňa, Slovakia, E-mail: martin.mellen@gmail.com.

Juraj Čuboň, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: juraj.cubon@uniag.sk.

Peter Haščík, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.hascik@uniag.sk.

Maciej Kluz, University of Rzeszow, Faculty of Biology, Agriculture, Department of Biotechnology, Microbiology, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: kluczyk82@op.pl.

Rafal Kordiaka, University of Rzeszow, Faculty of Biology, Agriculture, Department of Biotechnology, Microbiology, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: rafal.kordiaka@wp.pl.

Simona Kunová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: simona.kunova@uniag.sk.

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HERBICIDAL EFFECT IN RELATION TO THE ACCUMULATION OF MACROELEMENTS AND ITS REGULATION BY REGULATORS OF POLYAMINE SYNTHESIS

Pavol Trebichalský, Tomáš Tóth, Daniel Bajčan, Alena Vollmannová, Petra Kavalcová

ABSTRACT

Stress effects of triazine herbicide on cumulating of important macroelements (phosphorus, potassium, calcium and magnesium) into the grain of barley variety Kompakt, as well as the elimination of its negative effect through the addition of regulators of polyamine synthesis (γ-aminobutyric acid and propylenediamine) were investigated in pot trial. These morphoregulators are degrading products of polyamines and hypothetically after foliar application they should support their biosynthesis which increased level act against stress in plants. Application of the herbicide alone in comparison to control variant reduced the contents of all mentioned macroelements in grain of barley and also in variants, where the mixtures of herbicide with regulators of polyamine biosynthesis were applied, also the values of contents of all macroelements (except of magnesium) in barley grain were reduced (in comparison to this variant). It could be summarized that the presence of regulators in mixtures with triazine herbicide in comparison to control variant had not positive effects on contents of these biogenic elements in grain. By the comparison of variant with the applied herbicide with variants, where also regulators of polyamine synthesis were applied, there was the most positive influence of these mixtures of morphoregulators on statistically non-significant accumulation of phosphorus into generative organs of spring barley and in the case of positive accumulation of magnesium into these plant tissues there was statistically significant relation only after application of mixtures of herbicide with propylenediamine. Positive influence on accumulation of calcium was evaluated only after using of mixtures of herbicide with propylenediamine (statistically significant relation was recorded at the dose 29.6 g.ha^{-1}).

Keywords: barley; polyamines; triazine herbicide

INTRODUCTION

Triazine herbicides are widely used against broad leaf weeds and crops and in tree seedling nurseries. Triazines are primarily soil applied herbicides. Further research showed that triazine herbicides when taken up by the root move rapidly to the top by apoplastic movement. It concentrates first in the internal veinal areas and finally in the margin of the leaf **(Parveen et al., 2002)**. They are photosynthetic inhibitors and cause chlorosis and desiccation of green tissues. However all these effects are observed in light and not in the dark. Atrazine is a chloroamino triazine herbicide. It is a selective, pre-emergence herbicide for control of many grasses and broad leaf weeds in maize, sorghum, sugar cane and many table crops and increases the yield of crop **(Shah et al., 2000)**.

The great majority of herbicides act by inhibiting a specific plant enzyme essential for metabolism, whereas the remainder, including auxinic herbicides, act as general inhibitors **(Powles and Yu, 2010; Cabrito et al., 2011)**.

Polyamines are small positively charged aliphatic molecules ubiquitous in almost all life forms. These compounds have been implicated in a wide range of life processes in plants including seed germination, growth, floral initiation, floral development, pathogen defenses, and environmental stress responses **(Martin-Tanguy and Aribaud, 1994; Walters, 2003; Palavan-Unsal, 1995)**. Despite extensive studies on polyamine metabolism, the exact role that these compounds play in plant physiology remains unclear **(Tiburcio et al., 1997)**. In plants, polyamines are involved in various physiological events such as development, senescence and stress responses. **(Gill and Tuteja, 2010; Ramakrishna and Ravishankar, 2011)**. Endogenous polyamines could contribute to plant stress tolerance as part of defense mechanisms or adaptation programs that help plant organism to cope with the negative stress consequences **(Todorova et al., 2015)**.

High cellular levels of polyamines correlate with plant tolerance to a wide array of environmental stresses. Moreover, as compared with susceptible plants, stress-tolerant ones generally have a large capacity to enhance polyamine biosynthesis in response to abiotic stress **(Gill and Tuteja, 2010)**.

Conversely, treatments with polyamine biosynthesis inhibitors reduce stress tolerance, but this effect is reversed by concomitant application of exogenous polyamines. The influence of polyamines on in vitro morphogenetic response and caffeine biosynthesis were reported in Coffea canephora. Apart from primary metabolic functions, external feeding of certain polyamines are known to act as elicitors **(Kumar et al., 2008)**.

In addition, uncommon polyamines, like homospermidine, 1,3-diaminopropane, cadaverine and canavalmine have been detected in a large number of biological systems, including plants, animals, algae and

bacteria. At the physiological pH, polyamines are found as cations. This polycationic nature of polyamines is one of their important properties effectuating their biological activities. Large body of evidence suggested that plant transformation with genes of polyamines biosynthetic enzymes or the exogenous application of polyamines such as putrescine, spermidine and spermine results in abiotic stress tolerance in various plants **(Valero et al., 2002)**.

Ali (2000) reported that exogenous application of putrescine reduced the net accumulation of Na^+ in different organs of *Atropa belladonna* subjected to salinity stress. Putrescine alleviated the adverse effect of NaCl during germination and early seedling growth and increased the alkaloids as well as endogenous putrescine of *A. belladonna*. **Lutts et al., (1996)** reported that putrescine increased the growth and the leaf tissue viability of salttreated plants in all cvs. of *Oryza sativa*. They suggested that this positive effect was associated with an increase in ethylene biosynthesis through an increase in ACC content and a suppression of NaCl-induced inhibition of ACC conversion to ethylene and suggested the involvement of putrescine in salinity tolerance in rice. **Ndayiragije and Lutts (2006)** studied the possible role of exogenous application of polyamines on *Oryza sativa* and noted that addition of polyamines in nutritive solution reduced plant growth in the absence of NaCl and did not afford protection in the presence of NaCl. Polyamine-treated plants exhibited a higher K^+/Na^+ ratio in the shoots, suggesting an improved discrimination among monovalent cations at the root level, especially at the sites of xylem loading. Putrescine induced a decrease in the shoot water content in the presence of NaCl, while spermidine and spermine had no effects on the plant water status. In contrast to spermidine, spermine was efficiently translocated to the shoots.

GABA is a non-protein amino acid with some functional properties for human health such as lowing blood pressure and regulating heart rate **(Mody et al., 1994)**. GABA is widely present in prokaryotic and eukaryotic organisms **(Yang et al, 2015)**. In recent years, GABA-enriched foods have become popular, such as GABA-tea **(Syu et al., 2008)**, GABA-brown rice **(Komatsuzaki et al., 2007)**, GABA-soy bean sprouts **(Guo et al., 2012)**. In plant cells, GABA is synthesized via the α-decarboxy1ation of glutamate (Glu) in an irreversible reaction which is cata1yzed by glutamate decarboxylase (GAD) **(Bown et al., 1997)**. This metabolic pathway is called GABA shunt. In addition, GABA can also be formed via γ-aminobutyraldehyde intermediate from polyamine degradation reaction where diamine oxidase (DAO) is the key enzyme **(Wakte et al., 2011)**. Researches on GABA accumulation in germinating seeds focus on GABA shunt **(Bai et al., 2009; Mae et al., 2012)**, but little information is available on polyamine degradation pathway **(Xing et al., 2007)**. In the majority of germinating seeds, stressful conditions such as hypoxia **(Guo et al., 2011)**, salt stress **(Widodo et al., 2009)** and drought **(Kramer et al., 2010)** can strongly increase GABA content. During fava bean germination under non-stress condition, GABA content increased slightly **(Yang et al., 2011)**, but it increased significantly when germinating under hypoxia stress **(Yang et al., 2013)**. Under these stressful conditions, the relationship between GABA shunt and polyamine degradation pathway is still not clear.

MATERIAL AND METHODS

In pot experiment 6 kg of substrate (soil:sand – 4:2) was weighed. Analyses done in soil used in experiment are shown in Table 1. It was sown 30 plants which were thinned into 20 pieces after post-emergence. At the phase of early tillering plants were foliar treated (after 25 days) in the control treatment with the water (Table 2), in other

Table 1 Agrochemical characteristics of soil (horizons 0 – 0.2m).

Soil reaction	Humus content	Content of nutrients			
		N_{an}	P	K	Mg
(pH/KCl)	(%)	(mg.kg^{-1})	(mg.kg^{-1})	(mg.kg^{-1})	(mg.kg^{-1})
7.03	2.34	8.7	54.3	178.35	407.8

Table 2 Variants of the pot experiment.

VARIANT NUMBER	FOLIAR TREATMENT
1	Control: 9.0 mL water
2	Triazine herbicide 0.5 l.ha^{-1}: 1.0 mL water solution of triazine herbicide +8.0 mL water
3	Triazine herbicide 0.5 l.ha^{-1} +GABA 500 g.ha^{-1}: 1.0 mL water solution of triazine herbicide +4.7 mL 20 mM solution GABA +3.3 mL water
4	Triazine herbicide 0.5 l.ha^{-1} +PDA 59.2 g.ha^{-1}: 1.0 mL water solution of triazine herbicide +3.8 mL 2 mM solution PDA +4.2 mL water
5	Triazine herbicide 0.5 l.ha^{-1} +PDA 29.6 g.ha^{-1}: 1.0 mL water solution of triazine herbicide +1.9 mL 2 mM solution PDA +6.1 mL water

NOTE: PDA – 1,3-propylenediamine, GABA – γ-aminobutyric acid.

variants with triazine herbicide alone (the active ingredient is cyanazine with chemical formula 2-(4-chloro-6-ethylamino-1,3,5-triazin-2-ylamino)-2-methylpropiono-nitrile), or its mixture with γ-aminobutyric acid (GABA) with dose 500 $g.ha^{-1}$, in another variant with 1,3-propylenediamine (PDA) with dose of 59.2 $g.ha^{-1}$, and in last variant with the PDA in the amount of 29,6 $g.ha^{-1}$. The plants were watered with constant volume in all pots.

Crops were harvested in full ripeness, 2 g of barley grain after homogenization were mineralized in 20 mL of nitric acid with 5 mL of perchloric acid and after filtration the filtrate was afterwards filled to volume 50 mL. Then the contents of potassium, calcium and magnesium were determined by method of flame AAS with VARIAN (AAS Varian AA Spectr DUO 240FS/240Z/UltrAA, manufacturer Varian Australia Pty Ltd, A.C.N. 004 559 540, Mulgrave, Australia). Phosphorus was determined by method of Gonzáles **(John, 1970)** – 0.5 mL of above mentioned solution in 50 mL volumetric flask was filled with water till mark, 1 mL of ascorbic acid was added and 4 mL of solution with extraction agent containing sulphuric acid, ammonium molybdate and potassium antimonyl tartrate hemihydrate. Solution was mixed and after two hours the absorbance at 670 nm on UVmini-1240, UV-VIS Spectrophotometer, SHIMADZU, Japan (UV-1800), was measured against distilled water. Final values of phosphorus content in barley grain were defined from calibration curve of standards absorbance.

Results were evaluated by statistical program Statgraphics 4.0 (Statpoint Technologies, Inc., Czech republic), the data were analyzed by means of one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Application of herbicide alone in comparison to control variant (Table 3) reduced the contents of all macroelements in barley grain (values of our tested macroelements in barley grain percentually declined in interval 13 – 29%) – statistically significant in the case of phosphorus and potassium and also in variants with applied mixtures of herbicide with regulators of polyamine biosyntheis; the values of all macroelements contents (except of magnesium) in grain of barley were also reduced (in comparison to first variant.

It could be summarized that the presence of regulators in mixtures with triazine herbicide in comparison to control variant had not positive effects on contents of these biogenic elements (P, K, Ca) in grain (Table 3). Evaluating of magnesium cumulating had following summarise: the most positive statistically significant influence was in variants with applied propylenediamine (PDA). By the comparison of variant with the applied herbicide with variants, where also regulators of polyamine synthesis were applied, there was the most positive influence of these mixtures of morphoregulators on statistically non-significant accumulation of phosphorus into generative organs of spring barley and in the case of positive accumulation of magnesium into these plant tissues there was statistically significant relation only after application of mixtures of herbicide with PDA. Positive influence on accumulation of calcium was evaluated only after using of mixtures of herbicide with PDA (statistically significant relation was recorded at the dose 29.6 $g.ha^{-1}$). Only the uptake of potassium into barley grain was not affected positively by regulators of polyamine synthesis when compared to variant, where triazine herbicide was used alone (in mentioned cases this influence was statistically non-significant).

Cereals in Slovak republic, as well as in European Union have important representation in structure of plant production **(Kračmár et al., 2014; Tomka et al., 2010)**. Cereal agricultural production is limited by a wide array of abiotic and biotic stress factors including weeds, drought, cold, heat, salinity, imbalances in mineral nutrition, viral, and others, often acting in combinations under field conditions.

Since pesticide stress has not yet been extensively examined at the grain macroelements level, no data on this topic are currently available in the literature. Therefore, this study provides the first data and a framework for further investigation. Several researches have suggested that crop selectivity to triazine herbicides or their residues might be improved by exploiting the natural variability that is clearly preset in plants, either by searching for varietal differences in triazine tolerance or by altering the genetic structure of the crop by repeated artificial selection for genuine resistance.

Treatment of spring barley variety Kompakt with triazine herbicide and its mixtures with regulators of polyamine biosynthesis affected the accumulation of macroelements. From the theoretical point of view it could be presumed that applied amounts of regulators of polyamine synthesis will not directly affect the content of nutrients in plant, because they do not contain mentioned inorganic macroelements (P, K, Ca, Mg), because they are organic compounds. Changes in accumulation of macroelements in spring barley grain are probably caused by influencing of translocation of nutrients in plant, or by influencing of

Table 3 Contens of macroelements in grain of barley.

Variant Number	Content of nutrients ($mg.kg^{-1}$)				Dry matter (%)
	P	K	Ca	Mg	
1	4672.9B	8627.7B	122.6A	1452.1A	94.10B
2	3652.6A	6316.9A	87.0A	1275.4A	94.00B
3	3926.5A	5990.5AB	80.2A	1406.7AB	93.85B
4	3674.7AB	5378.3AB	96.7AB	1481.1B	93.42A
5	3825.7B	4490.3A	106.1B	1622.3B	93.52B

NOTE: Letters in table stand for statistical significance in columns (p <0.01). Their conformity means that the values are statistically non-significant and different letters characterize statistically significance.

metabolism of compounds groups, or physiology of plant and subsequent change in ability of plant to uptake nutrients from soil solution.

Triazine herbicides affect biochemical processes in plant, also energetic processes in cells which with their presence in tissues are obviously inhibited. Macroelement phosphorus is the part of structures $H_2PO_4^-$ and HPO_4^{2-} that are the part of important compounds NADP and $NADPH^+$, as well as phosphate fragments are in macroergic bonds. Our experiment confirmed this fact and also there was minimal influence of PDA and GABA on reduction of stress induced by herbicide presence.

Function of potassium in metabolism of spring barley plants is versatile: affects managing with water and improves health state and grain quality. Triazine herbicide which acts in plants destructive has great impact also on its uptake into barley grain.

Similarly, Pakistani authors **(Perveen et al., 2002)** found out that the contents of potassium, phosphorus and sodium were in roots and in shoots of bean plants (*Vigna radiate* (L.)) decreased after the application of triazine herbicide. The authors have explained it by injuries of tissues in plants after application of these pesticides. Potassium is involved in the protein synthesis, cell membrane and ionic balance, opening of stomata and other plant movements **(Hale and Orcutt, 1987)**.

Not only macroelements contents have decreasing tendency after the application of triazine herbicide, but this decrease was evaluated also by other important organic compounds in plants. In experiment carried out by Indian scientists **(Khan et al, 2006)** it was obvious that the application of herbicide isoproturon significantly decreased the values of protein in grain of wheat *Triticum aestivum*. Also significant decline of chlorophyl content (with bound magnesium) was also recorded **(Yin et al, 2008**; **Nemat et al., 2008)**, even by low concentration of isoproturon in plants.

As well as in the case of phosphorus, also the cumulating of potassium into barley grain the morphoregulators have not reducing effect on stress induced by triazine herbicide presence.

The role of Ca^{2+} as one of the nutrients and as a key ion in maintaining the structural rigidity of the cell walls as well as in membrane structure and function has been known for a long time **(Reddy et al., 2011; Hepler, 2005)**. During the last three decades, numerous studies have shown that Ca^{2+} is an important messenger in eliciting responses to diverse signals, including many biotic and abiotic signals **(McAinsh and Pittman, 2009; DeFalco et al., 2010)**. It appears that plants use Ca^{2+} as a messenger more than any other known messengers in plants. This is evident from the fact that nearly all signals (developmental, hormonal, and stresses) cause changes in cellular Ca^{2+}, primarily in the cytosol and, in some cases, in the nucleus and other organelles.

These herbicides belong to groups of photosynthesis inhibitors – their effectiveness lies in inhibition of photosynthetic electron transfer by disabling of photochemic reaction II. level known as Hill reaction. The most probable place of chlorophyll inhibition by photosynthesis is 5-membered ring of chlorophyll. By the bond of herbicide on keto-, resp. enol- form of five 5-membered ring, the chlorophyll inhibits the transfer of electrons. Changes as consequence of destruction of photosynthetic apparatus (inhibition of photosynthetic electron process) in plant are induced. Main photoreceptors of green plants are chlorophylls a + b which contain magnesium complex of reduced porfirine. This fact could explain our decline in magnesium that is the part of these important organic structures. The experiment revealed more positive influence of regulators of polyamine biosynthesis on increase of magnesium content in generative organs of tested cereal, because they have more positive influence on protection of photosynthetic structure by inhibiting of chemical bonds of triazine herbicide formation with elements from chlorophyll.

Recently, GABA acts an important function in plant stress responses **(Saito et al., 2008)**. This compound inhibits not only the influence of herbicide on plants, but also reduces harm pathogens. In experiment of **Okada and Matsubara (2012)** where added GABA and arginine (0.1, 1% w/v) into the Fusarium root rot (Fusarium oxysporum f. sp. asparagi, MAFF305556, SUF1226) in vitro suppressed further rot extension. GABA was also patented in USA as important antistressor **(Plant Health Care Inc., 2009)**.

CONCLUSION

Negative influence of triazine herbicide on accumulation of tested macroelements (P, K, Ca, Mg) into barley grain variety Kompakt was recorded. The application of herbicide mixtures with regulators of polyamine biosynthesis in comparison to control variant did not improve this accumulation (except of macroelement magnesium content). In our experiment there was more positive influence of used PDA and GABA in combination with triazine herbicide only in comparison to variant, where the herbicide alone was applied and in the case of statistically non-significant accumulation of phosphorus, statistically significant relation of macroelements uptake into barley grain was evaluated, in the case of magnesium accumulation into barley grain with mixtures of herbicide with PDA and calcium only at the dose of this morphoregulator 29.6 $g.ha^{-1}$. The uptake of potassium into barley grain was not positively affected by regulators of polyamine synthesis in comparison to variant where triazine herbicide was used alone.

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Contact address:

Pavol Trebichalský, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: pavol.trebichalsky@uniag.sk.

Tomáš Tóth, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: tomas.toth@uniag.sk.

Daniel Bajčan, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: daniel.bajcan@uniag.sk.

Alena Vollmannová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: alena.vollmannova@uniag.sk.

Petra Kavalcová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: kavalcova.petra@gmail.com.

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YEAST DIVERSITY IN NEW, STILL FERMENTING WINE "FEDERWEISSER"

Attila Kántor, Jana Petrová, Jana Hutková, Miroslava Kačániová

ABSTRACT

The aim of this study was to isolate and identify yeasts in different new wine “federweisser” samples. We collected the samples at the end of the August 2015 and in the middle of the September 2015. Used 15 new wine samples in this study (5 white and 10 red) were from the local Slovak winemakers. Irsai Oliver (3), Moravian Muscat (2), Agria/Turan (1), Dornfelder (3), Blue Frankish (3), Pinot Noir (1) and Saint Laurent (2). Three cultivation media were used for detection of yeasts in “federweisser” samples. Malt extract agar base (MEA), Wort agar (WA) and Wild yeast medium (WYM) were used for the cultivation of yeasts. Cultivation was performed by spread plate method. Ethanol/formic acid extraction procedure was used for preparation of samples. MALDI-TOF Mass Spectrometer (Microflex LT/SH) (Bruker Daltonics, Germany) was used for the identification of yeasts. We identified seven different strains of *Saccharomyces cerevisiae* (23; 70%), two strains of *Kloeckera apiculata* [teleomorph *Hanseniaspora uvarum*] (7; 21%), and one strain of *Pichia kluyveri* (1; 3%), *Pichia occidentalis* [anamorph *Candida sorbosa*] (1; 3%) and *Metschnikowia pulcherrima* (1; 3%) in 15 new wine “federweisser” samples. *Saccharomyces cerevisiae* was dominant species in each new wine sample, and formed creamy convex colonies with circular edge. *Metschnikowia pulcherrima* formed convex to pulvinate, circular white-pink colored colonies, *Kloeckera apiculata* formed flat, circular smooth colonies with turquoise center with gray edge, *Pichia occidentalis* formed irregular pulvinate light-cream colored colonies, and *Pichia kluyveri* formed turquoise, convex, undulate and smooth colonies on Malt extract agar base with bromocresol green.

Keywords: new wine; yeasts; *Saccharomyces cerevisiae*; MALDI-TOF MS

INTRODUCTION

Federweisser wine is grape must which is just undergoing the process of fermentation. Grape must is the juice of the wine grapes which is gained after the pressing of grapes. After corresponding treatment and storing, the must would become wine after finishing the process of fermentation. Because of this, Federweisser is not specially produced as some kind of drink but as an early product of wine production. The fermentation causes the splitting of the fructose of the grapes in alcohol and carbon dioxide. Because of the yeasts and bacteria in the must, the fermentation goes on very quickly. That is why Federweisser is drinkable only a couple of days. But the cool storage can lengthen the process of fermentation. In the refrigerator, Federweisser can be kept about 10 days. The grape must is considered "Federweisser" wine as soon as the alcohol concentration is about 4 to 6%. At the beginning, it tastes quite sweet. During the process of fermentation, the sweetness subsides. Due to the concentration of carbon dioxide, Federweisser tastes very prickly and tangy. Because of the high carbon dioxide concentration, a corking or air tight closure of the Federweisser is not possible. Especially in the past, this caused a transportation problem. Federweisser could only be offered regionally and was limited. Grape must is inoculated with a pure culture of yeasts (*S. cerevisiae*), usually 10-20 g.100 L^{-1} of must. Federweisser is very good for cold or warm drinking **(Malík et al., 2012)**.

Yeasts are found throughout nature. However, they do not occur randomly, but are found in specific habitats where different species form communities **(Lachance and Starmer, 1998)**. Within the winemaking environment (habitat), the vineyard (grape surfaces) and cellar (equipment surfaces and must) can be considered specialized niches where the wine related yeasts can form communities **(Polsinelli et al., 1996)**. The yeast species found in different niches associated with grape growth (vineyards) and wine production (wineries, grape must, fermentation and wine) can be arbitrarily divided into two groups, i.e. the *Saccharomyces* group and the non-*Saccharomyces* group. The *Saccharomyces* group with its primary representative, *Saccharomyces cerevisiae*, is present on grape skins in low numbers **(Rankine, 1972; Török et al., 1996, König et al., 2009)**, and on winery equipment and in fermenting must in greater numbers **(Fugelsang et al., 2007)**. Non-*Saccharomyces* yeast are part of the natural microbiota present on grapes, and harvesting and winemaking equipment, and are present at least during the early stages of fermentation **(Fleet and Heard, 1993; Renouf et al., 2005, 2007)**. While generally incapable of completing alcoholic fermentation, their application in co-inoculation or sequential inoculation with *S. cerevisiae* is increasingly popular **(Ciani et al., 2006; Ciani and Maccarelli, 1998; Comitini et al., 2011; Jolly et al., 2006; Soden et al., 2000)**, particularly for their effects on wine composition, flavour and aroma **(Benito et al., 2011; Ciani et al., 2006; Comitini et al., 2011; Cordero Otero et al., 2003; Di Maio et al., 2012;**

Domizio et al., 2011; Garcia et al., 2002; Jolly et al., 2006, 2014; Magyar and Toth, 2011; Morata et al., 2012; Soden et al., 2000; Toro and Vazquez, 2002).

The fermentation of grape must is a complex microbiological process that involves interactions between yeasts, bacteria, and filamentous fungi **(Fleet, 2007; Fugelsang and Edwards, 2007)**. Yeasts, which play a central role in the winemaking process, are unicellular fungi that reproduce by budding **(Ribéreau-Gayon et al., 2006)**. More than 100 yeast species have been isolated from grapes, must and wine **(König et al., 2009)**. The predominant species on the grape is *Kloeckera apiculata*, which may represent more than 50% of the flora obtained from the fruit **(Fugelsang and Edwards, 2007)**. Other species of obligate aerobic or weakly fermentative yeasts with very limited alcohol tolerance may also be found in lesser proportions. These belong to the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Rhodotorula* **(Fleet and Heard, 1993; Ribéreau-Gayon et al., 2006)**. The growth of these species, known collectively as non-*Saccharomyces* yeasts (or wild yeasts), is limited to the first 2 or 3 days of fermentation, after which they die as a result of ethanol toxicity. As these yeasts disappear, highly fermentative strains of the species *Saccharomyces cerevisiae* and *Saccharomyces bayanus* begin to multiply until they become solely responsible for alcoholic fermentation. The yeasts present in the must during the first few hours after filling the tanks belong to the same genera as those found on the grapes, predominantly *Kloeckera* (*Hanseniaspora*). In these spontaneous vinification conditions, *Saccharomyces* yeasts begin to develop after around 20h and are present alongside the grape-derived yeast flora. After 3rd or 4th day of fermentation, *Saccharomyces* yeasts predominate and are ultimately responsible for alcoholic fermentation **(Ribéreau-Gayon et al., 2006)**. This change in the yeast population is linked to the increasing presence of ethanol, the anaerobic conditions, and the use of sulfites during harvesting and in the must, the concentration of sugar, and the greater tolerance of high temperatures shown by *S. cerevisiae* compared with other yeasts **(Fleet and Heard, 1993; Fleet, 2007)**. *S. cerevisiae* comprises numerous strains with varying biotechnological properties **(Ribéreau-Gayon et al., 2006)**.

The aim of this study was to isolate and identify yeasts in different Slovak new wine "federweisser" samples.

MATERIAL AND METHODOLOGY

Federweisser samples, Spread plate method and Cultivation media

Samples of new wine "federweisser" were collected at the end of the August 2015 and in the middle of the September 2015 from local Slovak winemakers. Samples (apx.100 mL) were collected into 200 mL sterile plastic bottles with screw caps, and immediately stored at 8 ±1 °C in refrigerator. Bottle caps have been released, because the carbon dioxide (CO_2) was still produced by yeasts. Collected and stored samples (No. 15) were diluted with sterile physiological saline (0.85%), and dilution 10^{-4} and 10^{-5} were used for next analysis. 100 µL each dilution (10^{-4}, 10^{-5}) was placed on the surface of solidified agar media. The spread plate method was used for isolation of yeasts in federweisser samples. Samples were obtained from white (5) and red new wines (10). Irsai Oliver (3), Moravian Muscat (2), Agria/Turan (1), Dornfelder (3), Blue Frankish (3), Pinot Noir (1) and Saint Laurent (2). Three cultivation media were used for detection of yeasts in federweisser samples. Malt extract agar base (MEA) (BioMark™, India); Wort agar (HiMedia®, India) and Wild Yeast medium (HiMedia®, India). MEA has been enriched with glucose (CentralChem®, Slovakia) (50 g.L^{-1}), yeast extract (Conda, Spain) (3 g.L^{-1}) and acid base indicator bromocresol green (Sigma-Aldrich®, USA) (0.020 g.L^{-1}) (pH 3.8-5.4 yellow to blue). Yeasts were cultivated on Petri dishes at 25 °C for 5 days in aerobic conditions.

Identification of yeasts

We used MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany) for identification of yeasts isolated from federweisser samples. After incubation of yeasts at 25 °C for 5 days, isolated colonies were picked and suspended in 300 µL of sterile distilled water and mixed thoroughly. 900 µL of absolute ethanol was added. The mixture was centrifuged at 13 000 × g for 2 min. After the supernatant was discarded, the pellet was centrifuged again. Residual ethanol was completely removed by pipetting and the pellet was allowed to dry at room temperature. Subsequently 10 µL of formic acid (70%) was added and mixed with the pellet with a sterile toothpick. Next, 10 µL of acetonitrile (100%) was added and mixed thoroughly. The solution was centrifuged at maximum speed for 2 minutes again, and 1 µL of the supernatant was spotted on a polished MALDI target plate (Bruker Daltonics, Germany). Immediately after drying 1 µL of the matrix solution was added to each spot and allowed to air dry. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, Germany) dissolved in 50% acetonitrile with 0.025% trifluoroacetic acid (TFA). The matrix solution preparation (2.5 mg of HCCA) contains 500 µL of acetonitrile, 475 µL of ultra-pure water and 25 µL of trifluoroacetic acid. Next added 250 µL of this solution to the 2.5 mg of HCCA. Samples were then processed in the MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics, Germany) with flex Control software and results obtained with Real-time Classification software (RTC) by used database "Taxonomy" (Bruker Daltonics, Germany).

RESULTS AND DISCUSSION

After cultivation time, we obtained results from number of CFU (colony forming unit) in 100 µL of new wine sample of each used decimal dilutions. For better interpretation of results logarithmic conversion was applied on numerical results. Natural logarithm (Log_e) was used in Microsoft® Office Excel program by function LN. The highest number of yeasts cultivated on malt extract agar (MEA) was found in sample number thirteen Pinot Noir 6.43 log CFU.100 µL^{-1} and the lowest number of yeasts cultivated on MEA was present in the third sample Moravian Muscat 4.62 log CFU.100 µL^{-1}. The highest number of yeasts cultivated on Wort agar (WA) was found also in sample number 13 Pinot Noir 6.39 log CFU.100 µL^{-1}, but the lowest number of yeasts

Table 1 Number of yeasts in federweisser samples in log CFU.100 μL^{-1}.

	Cultivation media	MEA		WA		WYM	
No.	**Variety**	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}
1.	*Agria/Turan*	6.12	5.29	6.34	5.74	6.04	5.39
2.	*Irsai Oliver*	6.07	5.35	6.27	5.69	6.00	4.75
3.	*Moravian Muscat*	5.91	4.62	6.07	5.57	6.03	5.08
4.	*Irsai Oliver*	6.32	5.89	6.18	5.38	5.74	5.51
5.	*Blue Frankish*	6.30	ND	6.20	ND	5.63	ND
6.	*Irsai Oliver*	6.27	5.81	6.28	5.54	6.29	6.05
7.	*Blue Frankish*	6.31	ND	6.25	ND	6.33	ND
8.	*Moravian Muscat*	6.38	5.98	6.30	5.78	5.70	5.24
9.	*Blue Frankish*	6.37	6.06	6.03	5.75	5.82	5.61
10.	*Dornfelder*	6.39	6.09	6.28	5.76	5.66	5.12
11.	*Saint Laurent*	5.98	5.07	6.18	5.77	4.25	2.89
12.	*Dornfelder*	6.23	5.86	6.24	5.75	5.56	4.57
13.	*Pinot Noir*	6.43	6.14	6.39	6.18	ND	ND
14.	*Saint Laurent*	6.51	6.19	6.29	5.77	5.11	4.72
15.	*Dornfelder*	6.01	5.84	5.92	5.65	5.25	4.20

NOTE: **MEA**: Malt extract agar, **WA**: Wort agar, **WYM**: Wild yeast medium, **ND**: not detected.

cultivated on WA was present in the fourth sample Irsai Oliver 5.38 log CFU.100 μL^{-1}. The highest number of yeasts cultivated on Wild yeast medium (WYM) was found in sample number seven Blue Frankish 6.33 log CFU.100 μL^{-1} and the lowest number of yeasts cultivated on WYM was present in the fifteenth sample Dornfelder 4.20 log CFU.100 μL^{-1}. Table 1 contains results from microbiology of new wines obtained by spread plate method with used specific decimal dilutions 10^{-4} and 10^{-5}. Yeasts were countable at these two used dilutions. In this study we identified seven different strains of *Saccharomyces cerevisiae* (23), two strains of

Table 2 Yeast species in new wine "federweisser" samples.

No.	Variety	Species identified by MALDI-TOF MS
1.	**Turan/Agria**	*Saccharomyces cerevisiae* WS LLH
		Saccharomyces cerevisiae 991400574
		Kloeckera apiculata DSM 70788
2.	**Irsai Oliver**	*Saccharomyces cerevisiae* WS LLH
		Saccharomyces cerevisiae DSM 1334
3.	**Moravian Muscat**	*Saccharomyces cerevisiae* DSM 3798
		Saccharomyces cerevisiae WS LLH
		Kloeckera apiculata DSM 2768
4.	**Irsai Oliver**	*Saccharomyces cerevisiae* DSM 70868
5.	**Blue Frankish**	*Saccharomyces cerevisiae* DSM 1334
		Metschnikowia pulcherrima CBS 610NT
		Pichia kluyveri MY890_09
		Kloeckera apiculata DSM 2768
		Saccharomyces cerevisiae WS LLH
6.	**Irsai Oliver**	*Kloeckera apiculata* DSM 2768
		Pichia occidentalis CBS 1910
		Saccharomyces cerevisiae WS LLH
		Saccharomyces cerevisiae DSM 1334
7.	**Blue Frankish**	*Kloeckera apiculata* DSM 70788
		Saccharomyces cerevisiae DSM 1334
		Saccharomyces cerevisiae WS LLH
8.	**Moravian Muscat**	*Saccharomyces cerevisiae* WS LLH
		Kloeckera apiculata DSM 2768
		Saccharomyces cerevisiae CBS 1171
9.	**Blue Frankish**	*Kloeckera apiculata* DSM 2768
		Saccharomyces cerevisiae CBS 1171
10.	**Dornfelder**	*Saccharomyces cerevisiae* DSM 1334
11.	**Saint Laurent**	*Saccharomyces cerevisiae* DSM 1334
12.	**Dornfelder**	*Saccharomyces cerevisiae* DTY3
		Saccharomyces cerevisiae DSM 1334
13.	**Pinot Noir**	*Saccharomyces cerevisiae* DSM 70868
14.	**Saint Laurent**	*Saccharomyces cerevisiae* CBS 1171
15.	**Dornfelder**	*Saccharomyces cerevisiae* DSM 70868

Kloeckera apiculata (7), and one strain of *Pichia kluyveri* (1), *Pichia occidentalis* (1) and *Metschnikowia pulcherrima* (1) in fifteen federweisser samples. *Pichia kluyveri* was identified in Blue Frankish sample number five and *Pichia occidentalis* (anamorph *Candida sorbosa*) in sample number six (Irsai Oliver). We also identified one strain of *Metschnikowia pulcherrima* in sample number five (Blue Frankish).

The most common species in new wine samples was *Saccharomyces cerevisiae* and we identified seven different strains namely: DSM 1334, DSM 3798, DSM 70868, DTY3, CBS 1171, WS LLH and strain 991400574. Second most common species in new wine samples was *Kloeckera apiculata* (*Hanseniaspora uvarum*). *K. apiculata* was found in 7 new wine samples, two different strains (DSM 2768 and DSM 70788). Seven different strains of *Saccharomyces cerevisiae* was found in 15 new wine samples, what can be seen in Table 2.

S. cerevisiae is the most important yeast for wine production and is responsible for the metabolism of grape sugar to alcohol and CO_2. For these reasons *S. cerevisiae* is often simply referred to as "the wine yeast" **(Fleet, 1993; Pretorius et al., 1999; Swiegers and Pretorius, 2005)**. From all of identified yeasts, *Saccharomyces cerevisiae* was the dominant species, and we identified this species in all 15 new wine samples (70%). Grapes contain different species of yeast belongs to non-*Saccharomyces* yeasts such as *Kloeckera* (dominant genera), *Metschnikowia, Candida, Pichia, Rhodorotula, Aureobasidium* etc. *Saccharomyces* yeasts are not present in grape surface, or present in very low levels (less than 50 $CFU.mL^{-1}$) **(Prakitchaiwattana et al., 2004; Combina et al., 2005; Raspor et al., 2006; König et al., 2009)**.

When alcoholic fermentation starts non-*Saccharomyces* yeast population decrease. After the start of alcoholic fermentation when the ethanol concentration reaches 5 to 6% these yeast will be die **(Fugelsang et al., 2007)**. As fermentation progresses, the levels of these yeasts decrease, while that of *Saccharomyces* increases **(Fleet and Heard 1993)**. By the end of fermentation, *Saccharomyces* is the majority of the yeasts found, and often the only yeast isolated. New, still fermenting wine contains 4 to 6% ethanol and mostly contains only *Saccharomyces cerevisiae*, which is always predominant in new wines. But yeasts as *Kloeckera, Metschnikowia, Candida, Pichia* etc. can be identified in new wine samples in low populations. Some winemakers use commercial pure cultures and the others prefer to encourage the growth of some non-*Saccharomyces* yeasts early in alcoholic fermentation but eventually inoculate with *Saccharomyces* **(Fugelsang et al., 2007)**.

We identified except *S. cerevisiae* also *Kloeckera apiculata* in 7 new wine samples (21%) in lower population. Very interesting was that we isolated and identified only 3 another species of yeasts: *Metschnikowia pulcherrima* (3%), *Pichia kluyveri* (3%) and *Pichia occidentalis* (3%). In study **Kántor et al., (2015)** bromocresol green was used as a supplement also in Malt extract agar base (MEA) from BioMark™ (India). But in that study, cultivation media was not supplemented with yeast extract and glucose, only bromocresol green was added. After sterilization by autoclaving, that medium had a dark blue color. However in this study we supplemented Malt extract agar base (BioMark™, India) with yeast extract and glucose, and after sterilization by autoclaving had medium olive-green color. Malt extract agar base (BioMark™, India) contains only malt extract (30 $g.L^{-1}$), mycological peptone (5 $g.L^{-1}$) and agar (15 $g.L^{-1}$). Supplementation was desired in this case with glucose, yeast extract and bromocresol green. Yeasts grow very well in this modified medium, and bromocresol green is very helpful in differentiating of yeasts. Figure 1 shows the different colony morphology of 4 yeast species grown on MEA in sample number 5 (Blue Frankish). As you can see, the number of *Saccharomyces cerevisiae* was the highest, then *Kloeckera apiculata* and after that *Pichia kluyveri* and

Figure 1 Yeast species isolated from new wine "Federweisser" (Sample no. 5, dilution 10^{-4}).

only one colony on this petri dish belonged to *Metschnikowia pulcherrima*. *M. pulcherrima* produced maroon colored pigment called pulcherrimin, which was visible from the bottom of the petri dish. *Metschnikowia pulcherrima* formed convex to pulvinate, circular white-pink colonies. *Kloeckera apiculata* formed flat, circular smooth colonies with turquoise center with gray edge. *Pichia kluyveri* formed turquoise, convex, undulate and smooth colonies and *Pichia occidentalis* formed irregular pulvinate light-cream colonies.

CONCLUSION

In this study we isolated and identified yeast species in 15 Slovak new wine "Federweisser" samples. We identified the yeast isolates by MALDI-TOF mass spectrometry biotyper (Bruker Daltonics, Germany). The most dominant species was *Saccharomyces cerevisiae* which was isolated from all 15 new wine samples, which was a very good result. By mass spectrometry we identified 7 different strains of *S. cerevisiae*. The second most common species was *Kloeckera apiculata* (*Hanseniaspora uvarum*) found in 7 new wine samples (2 strains). We also identified other non-*Saccharomyces* yeasts such as *Metschnikowia pulcherrima* (1 strain), *Pichia occidentalis* (1 strain) and *Pichia kluyveri* (1 strain).

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Contact address:

Attila Kántor, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Trieda A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: kantor.spu@gmail.com.

Jana Petrová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Trieda A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.petrova@uniag.sk.

Jana Hutková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Trieda A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: rapcanova.pk@gmail.com.

Miroslava Kačániová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Trieda A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@gmail.com.

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RISK OF CONTAMINATION OF WILD BERRIES FROM UPPER ORAVA REGION BY CADMIUM

Stanislav Zupka, Alena Vollmannová, Ľuboš Harangozo, Marek Slávik, Michal Medvecký

ABSTRACT

The upper Orava region is located at the North Slovakia, near of potential sources of environmental contamination due by mining of coal, zinc and lead ores. The aim of the study was to evaluate the risk of consumption of wild forest fruit from Upper Orava region from the aspect of cadmium content. Ten sampling points were found by random search. From these points samples of soil, leaves and fruits of wild berries (9 samples of blueberries *Vaccinium Myrtillus* and 1 sample of strawberries *Fragaria Vesca*) were collected. In soil samples the active soil reaction (pH/H_2O) ranged from 3.53 (strong acidity) to 4.56 (extremly strong acidity), and the determined percentage of humus ranged from 1.66 (low humic soil) to 4.90 (high humic soil). In two soil samples the total content of cadmium determinated in soil extracts by *aqua regia* exceeded limit 0.70 mg.kg^{-1} given by the legislation in tne Slovak Republic. In three soil samples the determined content of cadmium mobile forms determined in soil extracts by NH_4NO_3 exceeded the limit 0.10 mg.kg^{-1}. The content of Cd determined in leaves as well as in fruits was evaluated according to Food Codex of the Slovak Republic. Only in one sample of leaf samples the limit 1.00 mg.kg^{-1} was exceeded. The other leaf samples are safely when used as an ingredient in tea mixtures. On the other hand even in 7 fruit samples the limit 0.05 mg.kg^{-1} was exceeded. This fruit can pose a risk for the human organism when is directly consumed as well as may negatively affect the human health when is used as raw materials in the food industry.

Keywords: Upper Orava; heavy metals; cadmium; soil; wild berries; leaves

INTRODUCTION

The upper Orava region is located on the North Slovakia, surrounded by Orava reservoir, near South Poland border (under 20 km).

In South Poland, close to the Silesia-Cracow region, seven of twenty-seven ecological hazardous areas of Poland are located. Mining of coal, zinc and lead ores cause a great additional threat to the natural environment and the human population. A considerable set of data is available on the air pollution in the city of Cracow and the whole area of Katowice voivodship **(Godzik, 1993)**.

These two towns are from the investigated area 120 km respectively 140 km far. So, the air pollution caused by long-distance transfer from these industrial centres can be a potencial source of contamination by heavy metals in the environment of the Upper Orava region.

Heavy metal pollution is released into the environment by various anthropogenic activities, such as industrial manufacturing processes, domestic refuse and waste materials **(Guala et al., 2010)**. Soils contaminated with heavy metals cause many environmental and human health problems calling for an effective technological solution. Many sites around the world remain contaminated because it is expensive to clean them up by available technologies.

Anthropogenic pollution caused by heavy metals entering into the plant is subsequently passed into the food chain with the consequence in hazards to human health **(Krížová, 2009)**.

Cadmium, a by-product of zinc production, is one of the most toxic elements to which man can be exposed at work or in the environment. Once absorbed, Cd is efficiently retained in the human body, in which it accumulates throughout life. Cd is primarily toxic to the kidney, but it can also cause prostate and renal cancer as well as the bone demineralization **(Bernard, 2008)**.

In Slovakia there are many areas with natural resources of some forest fruit, such as raspberries, blueberries, blackberries, lingonberries, etc. These fruits contain vitamins, minerals and polyphenolics compounds which are resistant against unsuitable climatic conditions and they can adapt to more severe soil-climatic conditions. Upper Orava region belongs to the Slovakian areas with an occurrence of wild forest berries. Forest fruit such as blueberries, forest strawberries, raspberries, cranberries etc. are often collected by people for their flavor, color and bioactive components which have a positive effect to the human health **(Nile and Park 2014)**. According to **Häkkinen et al., (1999**) small forest fruits, both wild or bred, are traditional part of Finnish consumers, with significant content of biological active non-nutrients, but also of essential nutritive components. The essential elements (K, Ca, P, Mg, Al, B, Cu, Fe, Na, Mn and Zn) are important components of highbush blueberries, while suitable fact for human organism is low content of Na **(Bushway et al., 2006)**. **Prior et al., (1998)** consider blueberries as one of the richest sources of antioxidant

phytonutrients, while composition and content of phenolic compounds in blueberries have changed in relation to variety, period, as well as to locality of growing **(Giovanelli and Burati, 2009)**.

The aim of the study was to evaluate the risk of consumption of wild forest fruit from Upper Orava region from the aspect of Cd content.

MATERIAL AND METHODOLOGY

The experiment was realized in region Upper Orava, in cadasters of villages: Malé Borovce, Habovka, Zábiedovo, Brezová, Vitanová and area near Orava reservoir (Figure 1). Samples were collected in June 2014. The exact coordinates of sampling sites are presented in Table 1. The average annual temperature is 6 °C (12.5 °C during vegetation) and the average annual rainfall is 800 – 900 mm (550 mm during vegetation).

Samples of soil, fruits (9 samples of blueberries (*Vaccinium Myrtillus*) and 1 sample of wild strawberries (*Fragaria vesca*)) and leaves (can be used as a tea mixture) were taken from individual sampling points. The soil samples were taken from the upper horizon.

The active soil reaction pH/H_2O was determined electrometrically (691 pH Meter, Metrohm, Swiss), and content of oxidizable carbon (C_{OX}, %) was determined using volumetric method according to Tjurin (H_2SO_4: Merck, Germany, $K_2Cr_2O_7$: Merck, Germany; $(NH_4)_2Fe(SO_4)_2$•$6H_2O$: Merck, Germany) while a content of humus (Hum., %) was calculated from C_{OX} content.

Pseudototal content of cadmium including all the form besides residual metal fraction was assessed in soil extract by *aqua regia* (HCl: CentralChem, Slovakia, HNO_3: Merck, Germany) and content of mobile forms in soil extract by NH_4NO_3 (c = 1 mol.dm^{-3}, Merck, Germany)). Used analytical method was flame AAS (AAS Varian AA Spectr DUO 240 FS/240Z/UltrAA, Varian, Australia).

The determined values were compared with limits given by **European Commission Regulation no. 1881/2006** as well as **Slovak decree no. 220/2004 of coll.**

Homogenized berry samples (4 g) were mineralized in a closed system of microwave digestion using Mars X-Press 5 (CEM Corp., USA) in a mixture of 5 mL HNO_3 (Suprapur, Merc, Germany) and 5 mL deionized water (0.054 μS.cm^{-1}) from Simplicity 185 (Millipore, UK). Metal determinations were performed in a Varian AA240Z (Varian, Australia) atomic absorption spectrometer with Zeeman background correction. The graphite furnace technique was used for the Cd determination. The obtained results were expressed as mg.kg^{-1} FM. Gained results were evaluated according to hygienic limit for Cd content in fruit given by the **Food Codex of the Slovak Republic**. Each analysis was done in 4 repetitions.

Statistical processing of the results was carried out using software Statgraphics Centurion XVI.I. One-way analysis of variance (α = 0.05) was used. Mean comparisons between investigated parameters were done by the LSD test.

RESULTS AND DISCUSSION

Soil samples:

In soil samples the active soil reaction (pH/H_2O) ranged from 3.53 (strong acidity) to 4.56 (extremly strong acidity), data are available in Table 1. **Römkens et al. (1998) and Barančíková (1998)** reported that Cd and Zn solution concentrations were higher in forest soils and were strongly increased below pH 5.5 even despite the low total metal content. **Kawabata et al., (2011)** presented similar pH values to our results in soil used for blueberry production. On the other hand, **Maliníková et al., (2013)** presented higher pH values of forest soil (5.28-7.67). The

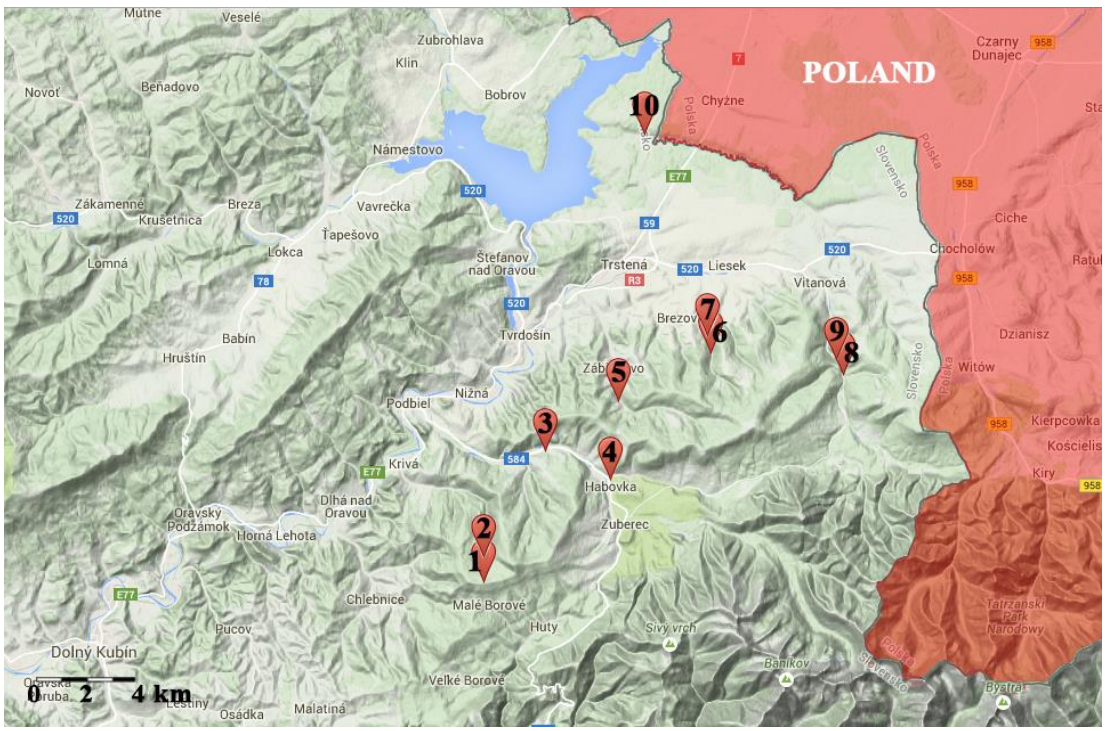

Figure 1 Investigated area and sampling points.

% of humus in soil samples was in range 1.66 (low humic soil) to 4.90 (high humic soil), data are available in Table 1. **Fanrong (2011)** reported a positive correlation between organic mater content and bioavailability of heavy metals in soil. The total cadmium content in soil samples (determinated in soil extracts by *aqua regia*) was in the range 0.12 – 1.45 mg.kg^{-1} (Table 1). In two soil samples (Samples no. 1 and 3) the total content of cadmium exceeded the limit value 0.70 mg.kg^{-1} given by **European Commission Regulation no. 1881/2006** as well as **Slovak decree no. 220/2004 of coll.** (1.45 and 0.73 mg.kg^{-1} respectively). Several studies focused on the monitoring of the environmental contamination of the soils in Slovakia were published. **Musilová et al., (2015)**, **Vilček et al., (2012), Tomáš et al., (2009)** determined the total Cd content in soil samples in intervals 0.65 – 6.73 mg.kg^{-1}, 0.36 – 12.26 mg.kg^{-1}, 0.34 – 0.40 mg.kg^{-1} respectively. **Taraškevičius et al., (2013)** presented values of the Cd contents in soil extracts of *aqua regia* in some European soils in range 0.07 – 12.8 mg.kg^{-1}. In Table 1 also values of content of mobile cadmium forms determined in soil extracts by NH_4NO_3 are presented. The values were compared to the limit value 0.10 mg.kg^{-1} given by **European Commission Regulation no. 1881/2006** as well as **Slovak decree no. 220/2004 of coll**. In Sample no. 1 (0.15 mg.kg^{-1}), Sample no. 2 (0.10 mg.kg^{-1}) and Sample no. 8 (0.13 mg.kg^{-1}) the determined content of cadmium mobile forms exceeded the limit. **Musilová et al. (2015), Vilček et al. (2012), Tomáš et al. (2009)** also determined contents of cadmium mobile forms in soils of Slovakia. They determined values in range 0.029 – 0.236 mg.kg^{-1}, 0.02 – 0.78 mg.kg^{-1}, 0.5 – 0.7 mg.kg^{-1} respectively, similar to our results.

Leaf samples:

The determined Cd content in leaves of investigated forest fruit (Table 2) was compared to hygienic limit 1.00 mg.kg^{-1} for Cd content in tea mixtures given by the **Food Codex of the Slovak Republic**. Only in Sample no. 6 (2.02 mg.kg^{-1} DM) the limit was exceeded, all other leaf samples have the determined Cd content under the hygienic limit. Despite that finding our results indicate a significantly higher degree of Cd accumulation in leaves than in fruits.

Fruit samples:

On the other hand even in 8 fruit samples the limit 0.05 mg.kg^{-1} given for small berries by the **Food Codex of the Slovak Republic** was exceeded (Table 2), whereas in 1 Sample (no. 2) the Cd content was lower than the detection limit. **Von Hoffen et al., (2014)** determined Cd content of blackberries in range 0.004 – 0.18 mg.kg^{-1} FM. On the other hand, **Reimann et al., (2001)** presented significantly lower values (0.009 mg.kg^{-1}) of Cd content in blueberries compared to our results. According to **Wieczorek et al., (2010)** the concentration of Cd in wild berries, ranged from 6 to 49 μg.kg^{-1} fresh weight.

Cadmium (Cd) is a toxic heavy metal that can accumulate in the human body and the environment for

Table 1. Analysis of soil samples from Upper Orava region, July 2014.

No.	Sample	GPS coordinates	Active soil reaction [pH H2O]	Cox [%]	% of humus in soil samples [%]	Total Cd content in soil samples [mg.kg^{-1} DM]		Content of mobile forms of Cd in soil samples [mg.kg^{-1} DM]	
						Average	SD	Average	SD
1	Blueberries	N 49° 14.186´´ E 19° 31.819´´	3.53	2.84	4.90	1.45	±0.01	0.15	±0.02
2	Blueberries	N 49° 14.791´´ E 19° 31.826´´	4.11	1.79	3.09	0.37	±0.01	0.10	±0.01
3	Blueberries	N 49° 17.265´´ E 19° 33.997´´	4.48	1.54	2.66	0.73	±0.01	0.08	±0.00
4	Blueberries	N 49° 16.613´´ E 19° 36.374´´	4.56	2.53	4.36	0.34	±0.01	0.03	±0.01
5	Blueberries	N 49°18.442´´ E 19° 36.637´´	4.23	1.56	2.69	0.27	±0.02	0.05	±0.01
6	Blueberries	N 49° 19.607´´ E 19° 40.058´´	4.31	1.39	2.39	0.25	±0.01	0.05	±0.01
7	Blueberries	N 49° 19.957´´ E 19° 39.881´´	4.35	1.47	2.54	0.14	±0.01	0.06	±0.02
8	Blueberries	N 49° 19.053´´ E 19° 44.552´´	3.89	2.26	3.90	0.57	±0.02	0.13	±0.02
9	Blueberries	N 49° 19.412´´ E 19° 44.520´´	4.44	0.97	1.66	0.12	±0.01	0.07	±0.01
10	Strawberries	N 49° 24.775´´ E 19° 37.613´´	4.00	1.61	2.78	0.17	±0.01	0.09	±0.01
	Limit value *					**0.70**		**0.10**	

NOTE: * limit given by European Commission Regulation no. 1881/2006 as well as Slovak decree no. 220/2004 of coll.

Table 2. Cd contents in samples collected in Upper Orava region (fruits and leaves) and transfer factors (soil-fruit and soil-leaves), July 2014.

No.	Sample	Cd content in fruit samples [mg.kg^{-1} FM]		Cd content in leaves samples [mg.kg^{-1} DM]		Transfer factors soil - fruit	Transfer factors soil - leaves
		Average	SD	Average	SD		
1	Blueberries	0.09 e	±0.01	0.60 cd	±0.01	1.70	0.25
2	Blueberries	UDL**	-	0.89e	±0.02	-	0.11
3	Blueberries	0.05 c	±0.01	0.57 bc	±0.01	1.73	0.14
4	Blueberries	0.08 e	±0.00	0.92 e	±0.01	0.30	0.03
5	Blueberries	0.05 c	±0.01	0.52 a	±0.01	0.92	0.09
6	Blueberries	0.07 d	±0.01	2.02 f	±0.06	0.78	0.03
7	Blueberries	0.04 b	±0.01	0.61 d	±0.03	1.54	0.09
8	Blueberries	0.05 bc	±0.01	0.56 b	±0.01	2.82	0.23
9	Blueberries	0.05 c	±0.01	0.60 d	±0.02	1.53	0.12
10	Strawberries	0.07	±0.00	0.97	±0.01	1.35	0.09
	P-value	0.0000		0.0000			
	F-ratio	42.19		1413.93			
Limit value *		**0.05**		**1.00**			

NOTE: Average values marked with the same letter are not significantly different ($p < 0.05$)
* limit value given by Food Codex of Slovakia.
** Cd content under detection limit.

lengthy periods **(Zhang et al., 2014)** and due its exposure the toxic effects in a variety of structures such as kidneys, liver and central nervous system including proteinuria, glucosuria, and aminoaciduria with final renal dysfunction are confirmed **(Xu et al., 2013)**.

The high concentration of heavy metals in soils is usually reflected by higher concentrations of metals in plants, and consequently in animal and human bodies **(Buszewski et al., 2000)**.

The transfer of soil pollutants into the plants causes many physiological disorders. The degree of heavy metal mobility, activity and bioavailability and consequently plant uptake is influenced by many factors such soil reaction, temperature, redox potential, cation exchange capacity of solid phase, competition with other metal ions, ligation by anions, composition and quantity of the soil solution **(Wopereis et al., 1988)**.

To characterize quantitatively the transfer of an element from soil to plant, the soil–plant Partition Coefficient or Transfer Factor (TF) or Concentration Ratio or Biological Accumulation Coefficient (BAC) that expresses the ratio of contaminant concentration in plant parts to concentration in dry soil is used **(Chojnacka et al., 2005)**. In Table 2 the calculated values of TF are presented. Generally, the transfer factors calculated for soil-leaves transfer were low. This may be because only the accumulation of metals in the leaves were studied more metals could have accumulated in the root **(Olayinka et al. 2011)**. The transfer factors calculated for soil-fruit transfer were higher. The higher the value of the TF, the more mobile/available the metal is **(Olayinka et al., 2011)**.

CONCLUSION

In two soil samples from the Upper Orava region the total content of cadmium exceeded limit 0.70 mg.kg^{-1} and in three soil samples the determined content of cadmium mobile forms exceeded the limit 0.10 mg.kg^{-1}, which can be put into context with extremely strong soil reaction. This factor increases the release of mobile forms in the soil environment. Wild berries such as blueberries (*Vaccinium Myrtillus*) and wild strawberries (*Fragaria Vesca*) have a positive effect to the human health because of their content of bioactive and chemoprotective components as well as an antioxidant activity. On the other hand it is necessary to monitor content of cadmium or other heavy metals in this fruit which is affected by soil-ecological conditions. Heavy metals become toxic for the human organism, when they entering into the food chain. Eating wild berries from the region of Upper Orava may present a potential risk for the human health. Our results indicate a significantly higher degree of Cd accumulation in leaves than in fruits, even though the limit for tea mixture was exceeded only in one sample.

It is necessary to monitor the soil content of hazardous elements in territory of Upper Orava as well as their transfer into plants and the food chain because of food safety.

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Contact address:

Stanislav Zupka, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zupkastanislav@yahoo.com.

Alena Vollmannová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: alena.vollmannova@uniag.sk.

Ľuboš Harangozo, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: lubos.harangozo@uniag.sk.

Marek Slávik, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marek.slavik@uniag.sk.

Michal Medvecký, Krivá 62, 02755 Krivá, Slovakia, E-mail: michal.medvecky@uniag.sk.

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THE EXTENSION OF SHELF-LIFE OF CHICKEN MEAT AFTER APPLICATION OF CARAWAY AND ANISE ESSENTIAL OILS AND VACUUM PACKAGING

Maciej Kluz, Margarita Terentjeva, Czeslaw Puchalski, Jana Hutková, Attila Kántor, Jana Petrová, Martin Mellen, Juraj Čuboň, Peter Haščík, Rafal Kordiaka, Simona Kunová, Miroslava Kačániová

ABSTRACT

The effect of caraway (CEO) and anise (AEO) essential oils as well as vacuum packaging (VP) in extending of the shelf life of fresh chicken breast meat stored at 4 °C was investigated. CEO and AEO were used at concentrations 0.2% v/w with and without VP. Microbiological properties of chicken breast meat were monitored over a 16 day period. The microbiological parameters as the anaerobic plate count (AC), *Enterobacteraceae*, lactic acid bacteria and *Pseudomonas* spp. counts were detected. The anaerobic plate counts ranged from 2.77 log CFU.g^{-1} in all tested group on 0 day to 5.45 log CFU.g^{-1} on 16 day in control group stored in air condition. The number of lactic acid bacteria ranged from 3.20 log CFU.g^{-1} in all tested group on 0 day to 4.75 log CFU.g^{-1} on 16 day in control group stored in air condition. *Enterobacteriaceae* counts ranged from 0.00 to 4.25 log CFU.g^{-1} on 16 day in control group stored in air condition. The number of *Pseudomonas* spp. ranged from 0.00 log CFU.g^{-1} in all tested group on 0 day to 2.65 log CFU.g^{-1} on 16 day in control group stored in air condition. Statistically significant differences ($p \leq 0.001$) were found among tested group in all tested microorganisms. Among the antimicrobial combination treatments were examined in the study, the as application of vacuum packaging, EDTA, and essential oils were the most effective against the growth of lactic acid bacteria and *Enterobactericeae* and to a less extent on anaerobic plate count. The results of this present study suggest the possibility of using the essential oil of caraway and anise as natural food preservatives and potential source of antimicrobial ingredients for chicken breast meat.

Keywords: bacteria; caraway and anise essential oils; vacuum; EDTA; chicken breast

INTRODUCTION

Special attention in poultry meat production is paid to the fact that live animals are hosts of a large number of different microorganisms residing on their skin, feathers or in the alimentary tract. Majority of these microorganisms are eliminated during the slaughter, but subsequent contamination is possible at any stage of the production process. Contamination may occur from feather plucking and evisceration equipment, washing prior to storage as cooling, or during the freezing. Microorganisms from the environment, equipment and operators' hands can contaminate meat. During the slaughter, the changes in composition of microflora occur from, in general, Gram-positive rods and micrococci to, most frequently, Gram-negative bacteria, including *Enterobacteriaceae*, *Pseudomonas* spp. Industrial poultry slaughterhouses have a particular technological process, the individual stages of which are not in conformity with modern principles of hygienic meat production and processing so there are various possibilities for contamination of chicken meat **(Kozačinski et al., 2006)**. Poultry meat is a highly perishable food commodity providing an almost perfect medium for microbial growth including both spoilage and pathogenic microorganisms **(Jay et al., 2005**) therefore the microbial contamination during the poultry meat processing is very crucial.

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. Moreover, meat and poultry products have frequently been found to be contaminated with pathogens **(Mor-Mur and Yuste, 2010)**. The pathogens ability to grow at refrigerator temperatures helps the organism to evolve from a low initial to an infective dose level during the storage of refrigerated foods, including those originally harbouring the pathogen and those, post-heat treatment, contaminated **(Ray, 2001)**.

It is well known that packaging makes food more convenient and gives the food greater safety assurance from microorganisms, biological and chemical changes so that the packaged foods may have a longer shelf life. As a result, packaging has become an indispensable element in the food manufacturing process. In order to meet the huge demand of the food industry, there has been a remarkable growth in the development of food packaging in the past decades **(Tsigarida and Nychas, 2001)**.

Aromatic plants and herbal products have been used worldwide as natural additives for medicinal purposes because they have been accepted by consumers. Various biological activitive compounds sharing antioxidative, anticoccidial, immunostimulating or antimicrobial properties have been identified in these plants **(Ivanišová et al., 2013; Ivanišová et al., 2015 a,b)**.

Carum carvi, which is also known as caraway, is one of the oldest spices cultivated in Europe. Nowadays, it is cultivated from northern temperate to tropical climates, including countries such as Jamaica, India, Canada, the United States of America and Australia. In India, this spice is known as *Kashmiri jeera*. The dried ripe fruits (schizocarp) of *C. carvi* L. family *Apiaceae* (*Umbelliferae*) are extensively being used in folk medicine as a carminative, found to be effective against spasmodic gastrointestinal complaints, irritable stomach, indigestion, lack of appetite and dyspepsia in adults, and in relieving flatulent colic of infants. The volatile oils from *C. carvi* have also been used as an anti ulcerogenic, antitumor, antiproliferative and antihyperglycemic agent. The seeds of *C. carvi* have been used in alternative medicine as a laxative, in colic treatment, and as a mouth freshener **(Thippeswamy et al., 2013)**.

Anise (*Pimpinella anisum* L.) a member of the *Apiaceae* family, is an annual aromatic plant, native to Iran, India, Turkey and many other warm region in the world. Anise seed possesses eugenol trans-anethole, methylchavicol, anisaldehyde, estragole, coumarins, scopoletin, umbelliferone, estrols, terpene hydrocarbons, polyenes, and polyacetylenes. Most of the plant parts such as fruits, seeds, and essential oil contain compounds with proven antiparasitic and digestion stimulating, antifungal and antipyretic, antioxidant, antimicrobial, anthelmintic and hypocholesterolemic properties **(Yazdi et al., 2014)**.

The present study was undertaken to determine the effect of vacuum packaging combined with caraway or anise essential oil tratment on microbiological properties of chicken breast meat stored at 4 °C.

MATERIAL AND METHODOLOGY

Preparation of samples

Chicken breast samples (totally 30) for microbiological analysis were used in this study.

To evaluate the antimicrobial activity of essential oils the chicken breast with skin of each experimental group was taken. The chicken breast fresh samples were prepared as follow: for air-packaging (AC, control samples) chicken breast fresh meat was packaged to polyethylene bags and stored aerobically at 4 °C; for vacuum-packaged (VC, control samples) chicken breast fresh meat was packaged to polyethylene bags and stored anaerobically in vacuum at 4 °C; for vacuum-packed samples with EDTA solution 1.5% w/w (VPEC, control samples) chicken breast fresh meat was treated with EDTA for 1 min and packaged to polyethylene bags and stored anaerobically in vacuum at 4 °C; for vacuum-packed samples with *Carvum carvi* 0.20% v/w (VP+CEO) chicken breast fresh meat was treated with caraway oil for 1 min and packaged to polyethylene bags and stored anaerobically in vacuum at 4 °C; for vacuum-packed samples with *Pimpinella anisum* L. 0.20% v/w, (VP+AEO) chicken breast fresh meat was treated with anise oil for 1 min and packaged to polyethylene bags and stored anaerobically in vacuum at 4 °C. For sample packaging a vacuum packaging machine type VB-6 (RM Gastro, Czech Republic) was used and each sample were packed immediately after treatment.
EDTA solution (pH 8.0, 99.5% purity, analytical grade, Invitrogen, USA) was prepared at final concentration of 50 mM Caraway and anise essential oils (Calendula, Nová Ľubovňa, Slovakia) was added to coat chicken breast surface (both sides) of each sample using a micropipette.

Microbiological analysis

An amount of 10 g (10 cm^2) of the chicken breast was sampled using sterile scalpels and forceps, immediately transferred into a sterile stomacher bag, containing 90 mL of 0.1% peptone water (pH 7.0), and homogenized for 60 s in a stomacher at room temperature. Sampling was carried out on 0, 4, 8, 12 and 16 days of experiment. Microbiological analyses were conducted by using standard microbiological methods. Anaerobic plate count (AC) was determined using Plate Count Agar (PCA, Oxoid, UK) after incubation for 48 h at 35 °C under anaerobically condition. For *Pseudomonas* spp., 0.1 mL from serial dilutions of chicken homogenates was spread onto the surface of Pseudomonas Isolation agar (PIA, Oxoid, UK). *Pseudomonas* spp. enumerated after incubation for 48 h at 25 °C. For lactic acid bacteria, Rogosa and Sharpe agar (MRS, Oxoid, UK) was inoculated with a 1.0 mL of sample suspension. Inoculated plates were incubated for 48-78 h at 37 °C in an aerobic atmosphere supplemented with carbon dioxide (5% CO_2). For *Enterobacteriaceae*, a 1.0 mL of sample was transferred into 10 mL of molten (45 °C) Violet Red Bile Glucose agar (VRBL, Oxoid, UK). Inoculated plates were incubated at 37 °C for 24 h. All plates were examined for typical colony types and morphology characteristics associated with each medium applied for incubation. Enumeration of all tested groups of bacteria was performed in triplicate.

Figures were created Microsoft® EXCEL 2013. Data for the mean from each replication was calculated and all data were log transformed. Statistical analysis were done with STATGRAPHICS 5 software (UMEX GmbH Dresden, Germany). Confectionary Student's Tukey HSD test was calculated for differences in numbers of bacteria and samples were accepted as significantly different at $p \leq 0.001$.

RESULTS AND DISCUSSION

Food contamination by microorganisms and their development and, hence, the food decontamination possibilities represent a serious problem. Chemical agents to prevent microbial growth and various additives that are used in food industries are considered to be potentially harmful to human health. In seeking of possible alternatives, the antimicrobial compounds of natural origin sharing antibacterial activities originated from plants currently are studied intensively worldwide.

Spices are aromatic plants that are widely used in the food industry and culinary food preparation for flavouring. However, their essential oils and extracts can contribute to control of the growth of harmful microorganisms. It is

necessary the spice to be effective enough to ensure that the product is safe and also have acceptable sensory characteristics **(Dimič et al., 2012)**. The primary objective of chilling poultry safety enssurance is to reduce microbial growth to a level that will improve both food safety and shelf life **(Popelka et al., 2014)**. The anaerobic plate count ranged from 2.77 log CFU.g^{-1} in all tested group on 0 day to 5.45 log CFU.g^{-1} on 16 day in control group stored in air condition. In control group stored vacuum packaged AC ranged from 2.77 log CFU.g^{-1} on 0 day to 5.25 log CFU.g^{-1} on 16 day. In control group stored vacuum packaged after EDTA treatment, AC ranged from 2.77 log CFU.g^{-1} on 0 day to 5.21 log CFU.g^{-1} on 16 day. After treatment with caraway essential oil, AC ranged from 2.77 log CFU.g^{-1} on 0 day to 4.20 log CFU.g^{-1} on 16 day and after treatment with anise essential oil ranged from 2.77 log CFU.g^{-1} on 0 day to 4.15 log CFU.g^{-1} on 16 day. Statistically significant differences ($p \leq 0.001$) of anaerobic plate count were found among all tested group at all tested days except AC and VP+CEO, VC and VP+CEO, VC and VP+AEO, VP+CEO and VP+AEO on 4th day; VC and VPEC on 8th and 16th day; VP+CEO and VP+AEO on 16th day. Anaerobic plate count (AC) values for the tested groups of chicken breast are showed in Figure 1.

Many herbs and spices have been recognized for their preservative or medicinal properties for millennia. Essential oils present in plant matter have been attributed as principal sources of compounds exhibiting antimicrobial activity, which has been illustrated against bacteria and fungi. The understanding of antimicrobial mechanisms of action of EO has led to increased interest to the specific compounds responsible for this activity, specifically those phenolic in nature **(Davidson et al., 2013)**.

The initial LAB value of chicken breast was 3.20 log CFU.g^{-1} on 0 day. In AC samples the LAB counts ranged from 3.20 log CFU.g^{-1} to 4.75 log CFU.g^{-1} on 16 day. In control group VC samples LAB count ranged from 3.20 log CFU.g^{-1} on 0 day to 4.52 log CFU.g^{-1} on 16 day. In VPEC samples LAB ranged from 3.20 log CFU.g^{-1} on 0 day to 4.38 log CFU.g^{-1} on 16 day. In VP+CEO samples the counts of LAC ranged from 3.20 log CFU.g^{-1} on 0 day to 3.05 log CFU.g^{-1} on 16 day and in VP+AEO ranged from 3.20 log CFU.g^{-1} on 0 day to 3.00 log CFU.g^{-1} on 16 day. Lactic acid bacteria (LAB) values for the tested groups of chicken breast are showed in Figure 2. Statistically significant differences ($p \leq 0.001$) of lactic acid bacteria numbers were found among all tested group at all tested days except VP+CEO and VP+AEO on 16th day.

Lactic acid bacteria are found to be more resistant to the cytotoxic effects of essential oils. **Rodríguez et al., (2009)** suggest that a fact that LAB are present and grow on phenol containing plants, and therefore have adapted in order to successfully colonize such antagonistic substrates like one of the reason of LAB resistance to phenolics is because. Degradation capabilities of phenolic compounds by LAB have also been described, although the number of studies is still limited.

The use of spices and spice blends in many food products that already contain high levels of similar seasonings has also been examined. Ideally, reengineering of food formulations that contain high levels of spices such as oregano or thyme seasonings could take advantage of the already present sources of essential oils. Unfortunately, some work has shown that spices stimulate the growth and acid production of LAB **(Shelef, 1983)**.

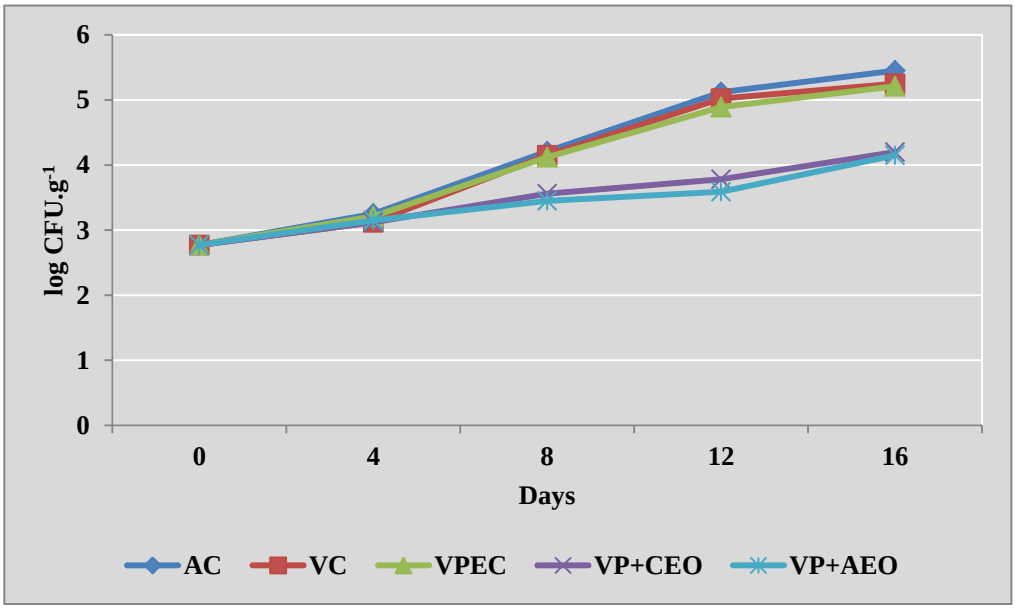

Figure 1 Changes (log CFU.g^{-1}) in population of anaerobic plate count in chicken breast stored in air (AC); stored in vacuum (VC); stored vacuum packaged with EDTA (VPEC); stored under vacuum packaged with *Carvum carvi* 0.20% v/w (VP+CEO); stored vacuum packaged with *Pimpinella anisum* L. 0.20% v/w (VP+AEO).

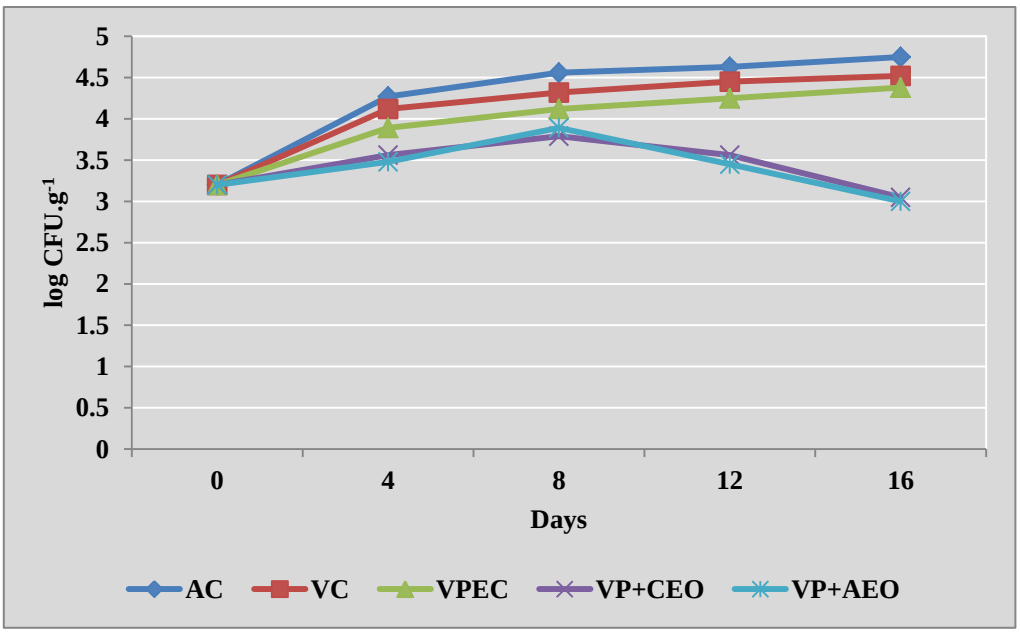

Figure 2 Changes (log CFU.g^{-1}) of lactic acid bacteria in chicken breast stored in air (AC); stored in vacuum (VC); stored vacuum packaged with EDTA (VPEC); stored vacuum packaged with *Carvum carvi* 0.20% v/w (VP+CEO); stored vacuum packaged with *Pimpinella anisum* L. 0.20% v/w (VP+AEO).

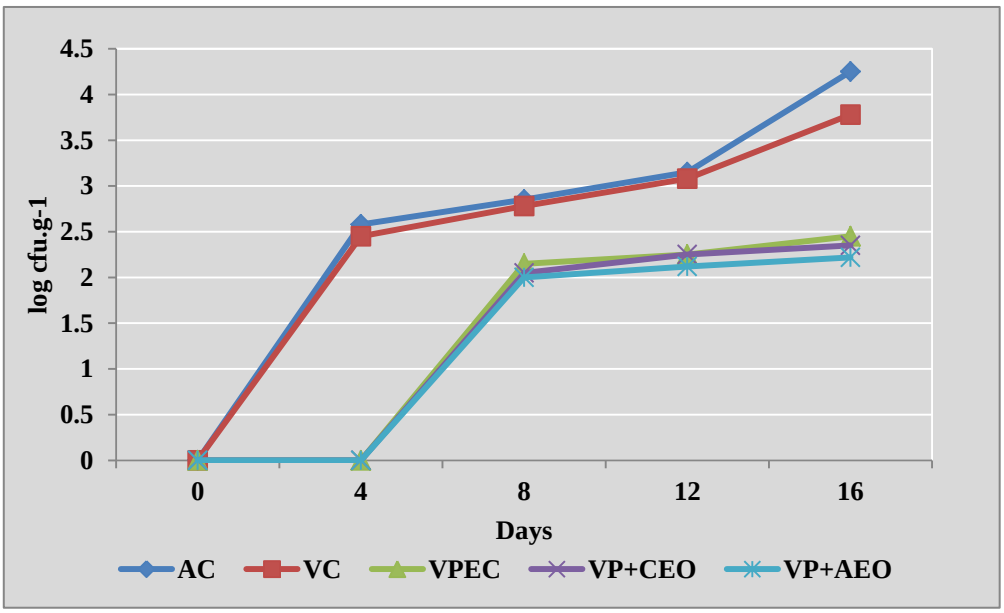

Figure 3 Changes (log CFU.g^{-1}) in *Enterobacteriaceae* counts in chicken breast stored in air (AC); stored in vacuum (VC); stored vacuum packaged with EDTA (VPEC); stored vacuum packaged with *Carvum carvi* 0.20% v/w (VP+CEO); stored vacuum packaged with *Pimpinella anisum* L. 0.20% v/w (VP+AEO).

The chemical composition and physical characteristics of meat makes it a suitable environment for bacterial growth, which includes bacteria such as LAB, *Pseudomonas*, and foodborne pathogens. LAB spoilage in meats is a relevant problem as they are facultative anaerobes that can grow and continue to spoil foods under chilled conditions **(Fratianni et al., 2010; Pyrgotou et al., 2010)**.

Fratianni et al. (2010) treated fresh strips of chicken breast meat with an agar slurry solution containing 0.5% thyme and balm essential oils for 15 min. Samples were stored for 21 days at 4 °C. Thyme was incredibly effective to control the LAB growth for the period of 16 days; 21-day counts were only 0.8×10^3 CFU.mL^{-1}, which was consistent throughout the entire 3 weeks of experiment. The antibacterial effect of balm oil was much less evident until the day 21, with balm oil closely matching the untreated control up until that point. *Salmonella* on the treated chicken was very sensitive to balm oil, while thyme oil very effectively reduced the growth of *E. coli*.

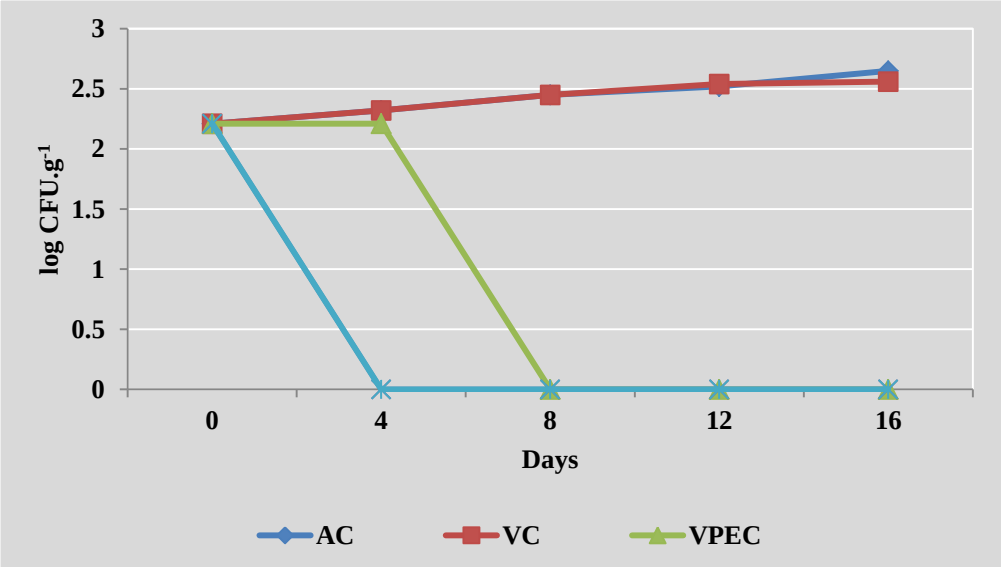

Figure 4 *Pseudomonas* count (log CFU.g^{-1}) in chicken breast stored in air (AC); stored in vacuum (VC); stored in vacuum packaging with EDTA (VPEC); stored vacuum packaged with *Carvum carvi* 0.20% v/w (VP+CEO); stored vacuum packaged with *Pimpinella anisum* L. 0.20% v/w (VP+AEO).

Enterobacteriaceae counts ranged from 0.0 log CFU.g^{-1} in all tested group on 0 day to 4.25 log CFU.g^{-1} on 16 day in AC group. In VP group *Enterobacteriaceae* counts ranged from 0.00 log CFU.g^{-1} on 0 day to 3.78 log CFU.g^{-1} on 16 day. In VPEC group *Enterobacteriaceae* counts ranged from 0.00 log CFU.g^{-1} on 0 day to 2.45 log CFU.g^{-1} on 16 day. In the group with caraway essential oil treatment *Enterobacteriaceae* counts ranged from 0.00 log CFU.g^{-1} on 0 day to 2.35 log CFU.g^{-1} on 16 day and in group treated with anise essential oil from 0.00 log CFU.g^{-1} on 0 day to 2.22 log CFU.g^{-1} on 16 day. Statistically significant differences ($p \leq 0.001$) of *Enterobacteriaceae* genera number were found among all tested group at all tested days except VPEC and VP+CEO, VPEC and VP+CEO, VP+CEO and VP+AEO on 4th day; AC and VC, VPEC and VP+CEO, VPEC and VP+CEO, VP+CEO and VP+AEO on 8th; VPEC and VP+CEO on 12th day. *Enterobacteriaceae* genera values for the tested groups of chicken breast are showed in Figure 3.

Generally, the Gram-positive bacteria were more sensitive to essential oils or antibacterial compounds than Gram-negative bacteria, which is in agreement with previous reports **(Dorman and Deans, 2000; Burt, 2004; Shan et al., 2007)**. This resistance could be ascribed to the structure of the cellular walls of Gram-negative bacteria, mainly with regard to the presence of lipoproteins and lipopolysaccharides that form a barrier to restrict entry of hydrophobic compounds **(Cox and Markham, 2007)**.

Pseudomonas spp. counts ranged from 0.00 log CFU.g^{-1} in all tested group on 0 day to 2.65 log CFU.g^{-1} on 16 day in AC group. In VC group *Pseudomonas* spp. ranged from 0.00 log CFU.g^{-1} on 0 day to 2.56 log CFU.g^{-1} on 16 day. In another tested groups on 16 day *Pseudomonas* spp. were not found. Statistically significant differences ($p \leq 0.001$) of anaerobic plate count were found among all tested group at all tested days except AC and VC on 4th, 8th 12th day; VPEC and VP+CEO, VPEC and VP+CEO on 8th, 12th, 16th day; VP+CEO and VP+AEO on 4th, 8th 12th, 16th day. *Pseudomonas* spp. values for the tested groups of chicken breast are showed in Figure 4.

Numerous studies documented the inhibitory effects of some essential oils and extracts of spices, plants, or their major active constituents on the bacteria - *Escherichia coli*, *Aeromonas* spp., *Enterococcus faecalis*, *Salmonella enterica Typhimurium*, *Staphylococcus aureus*, *Shigella* spp., *Bacillus* spp., *Listeria monocytogenes*, *Micrococcus spp.*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Streptococcus* spp., *Lactobacillus* spp., *Enterobacter* spp. with Gram-positive bacteria as generally more sensitive than Gram-negative bacteria **(Amensour et al., 2010; Bagamboula et al., 2003; Baidar et al., 2004; Celiktas et al., 2007,Faleiro et al., 2003; Moreira et al., 2005; Skočibušić et al., 2006; Sokmen et al., 2004; Veldhuizen et al., 2007; Viuda-Martos et al., 2008)**.

CONCLUSION

Caraway and anise essential oils exhibited good antimicrobial properties against anaerobic bacteria, lactic acid bacteria and *Enterobacteriacea* at 0.2% concentration. Essential oils and their components may provide a solution for the growing demand of natural preservation methods that require minimal processing of meat. Even more exciting is the fact that these essential oils are already approved for use in foods, meaning that once the issues of application and concentration are resolved and food producers can almost immediately begin using essential oils in their food formulations. Future work must comprise studies that determine which essential oils are most appropriate for preservation, what concentrations and delivery methods are most appropriate and effective, and what foods or packaging methods are most ideal for reformulation or reengineering to take advantage of the antimicrobial activity of essential oils.

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Contact address:

Maciej Kluz, Faculty of Biology and Agriculture, University of Rzeszow, Department of Biotechnology and Microbiology, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: kluczyk82@op.pl.

Margarita Terentjeva, Latvia University of Agriculture, Faculty of Veterinary Medicine Institute of Food and Environmental Hygiene, K. Helmaņa iela 8, LV-3004, Jelgava, Latvia, E-mail: margarita.terentjeva@llu.lv.

Czeslaw Puchalski, Faculty of Biology and Agriculture, University of Rzeszow, Department of Bioenergy Technologies, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: cpuchal@univ.rzeszow.pl.

Jana Hutková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.hutkova@uniag.sk.

Attila Kántor, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: attila.kantor@uniag.sk.

Jana Petrová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.petrova@uniag.sk.

Martin Mellen, Hydina Slovakia, s.r.o., Nová Ľubovňa 505, 065 11 Nová Ľubovňa, Slovakia, E-mail: martin.mellen@gmail.com.

Juraj Čuboň, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: juraj.cubon@uniag.sk.

Peter Haščík, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.hascik@uniag.sk.

Rafal Kordiaka, Faculty of Biology and Agriculture, University of Rzeszow, Department of Biotechnology and Microbiology, Zelwerowicza St. 4, 35-601 Rzeszow, Poland, E-mail: rafal.kordiaka@wp.pl.

Simona Kunová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: simona.kunova@uniag.sk.

Miroslava Kačániová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@uniag.sk.

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COMPARABLE EFFICIENCY OF DIFFERENT EXTRACTION PROTOCOLS FOR WHEAT AND RYE PROLAMINS

Peter Socha, Marián Tomka, Kvetoslava Kačmárová, Blažena Lavová, Eva Ivanišová, Barbara Mickowska, Dana Urminská

ABSTRACT

The identification and quantification of cereal storage proteins is of interest of many researchers. Their structural or functional properties are usually affected by the way how they are extracted. The efficiency of extraction process depends on the cereal source and working conditions. Here, we described various commonly used extraction protocols differing in the extraction conditions (pre-extraction of albumins/globulins, sequential extraction of individual protein fractions or co-extraction of gluten proteins, heating or non-heating, reducing or non-reducing conditions). The total protein content of all fractions extracted from commercially available wheat and rye flours was measured by the Bradford method. Tris-Tricine SDS-PAGE was used to determine the molecular weights of wheat gliadins, rye secalins and high-molecular weight glutelins which are the main triggering factors causing celiac disease. Moreover, we were able to distinguish individual subunits (α/β-, γ-, ω-gliadins and 40k-γ-, 75k-γ-, ω-secalins) of wheat/rye prolamins. Generally, modified extraction protocols against classical Osborne procedure were more effective and yields higher protein content in all protein fractions. Bradford measurement led into underestimation of results in three extraction procedures, while all protein fractions were clearly identified on SDS-PAGE gels. Co-extraction of gluten proteins resulted in appearance of both, low-molecular weight fractions (wheat gliadins and rye secalins) as well as high-molecular weight glutelins which means that is not necessary to extract gluten proteins separately. The two of three extraction protocols showed high technical reproducibility with coefficient of variation less than 20%. Carefully optimized extraction protocol can be advantageous for further analyses of cereal prolamins.

Keywords: extraction; prolamins; wheat; rye

INTRODUCTION

Cereal baked products are predominantly manufactured from wheat or rye flours. Storage non-enzymatically active proteins (prolamins), namely gliadins in wheat and secalins in rye, together with glutelin polymers represent the main triggering factor of celiac disease **(van den Broeck et al., 2011)**. Celiac disease is an inflammatory disorder that mainly affects the small intestine with typical gastrointestinal or extraintestinal symptoms **(Kaukinen et al., 2014)**. So far, the only therapy for celiac disease is lifelong gluten-free diet avoiding any products from wheat, rye, barley, their crossbred varieties and possibly oats **(Zingone et al., 2010)**. Majority of patients following strict gluten-free diet continue to suffer from symptoms, therefore to avoid contamination of gluten-free products by gluten and tighten labeling of such products is a priority.

According to the mobility in polyacrylamide gels, wheat gliadins are subdivided into α/β-, γ- and ω-subunits **(Wieser, 2007)** while rye secalins comprise from γ- and ω-subunits **(Shewry, 2004)**. Wheat α/β- and γ-gliadins as well as rye 40k-γ-secalins belong to the group of monomeric polypeptides with low molecular weight of approx. 28-45 kDa. Wheat ω-gliadins, rye 75k-γ-secalins and rye ω-secalins have molecular weight of approx. 50-80 kDa **(van Eckert et al., 2010)**. Glutelin polymers of wheat are generally subdivided into low-molecular weight glutenin subunits (LMW-GS) and high-molecular weight glutenin subunits (HMW-GS) **(van den Broeck et al., 2009)**, and glutelins of rye are represented by the HMW secalins **(Wieser, Koehler, 2008)**. These proteins have elevated content of two amino acids, glutamine (35% in wheat) and proline (15% in wheat), which makes them highly resistant to degradation by gastrointestinal proteolytic enzymes **(Gregorini et al., 2009)**. Therefore, analysis of structural or functional properties of wheat/rye prolamins requires an appropriately optimized extraction protocol.

Generally, based on different solubility, cereal proteins can be classified into water/salt-soluble albumins and globulins, alcohol-soluble prolamins, and high-molecular weight glutelins soluble in diluted acid/base solutions **(Osborne, 1924; Mamone et al., 2011)**. Various extraction protocols usually consisted initially of removing albumins and globulins **(Kruger et al., 1988)** or exploited co-extraction of gluten proteins (wheat gliadins and glutenins) without pre-extraction of salt soluble proteins **(van den**

Broeck et al., 2009). The differences among prolamin extraction methods involved various temperature conditions as well. Extractability of prolamins is almost unaffected by heating up to 75-80 °C **(Wieser, 1998)** which is the major problem of heat-processed foods. One way how to increase the extractability of prolamins is using a reducing agent to the extractant. However, reducing conditions are more suitable for low-molecular weight α/β- and γ-gliadins in wheat bearing 3-4 intramolecular disulphide bonds comparing to cysteine-free ω-gliadins **(Wieser, 1998)**.

Here, we investigated various extraction protocols of wheat/rye flour proteins involving different sequential extraction steps (pre-extraction of albumins and globulins, co-extraction of gluten proteins, sequential extraction of gliadins/secalins and glutelins) as well as different conditions (heating and non-heating, reducing and non-reducing).

MATERIAL AND METHODOLOGY

Biological Material

Commercially available wheat and rye flours were obtained from mill house Vitaflora (Kolarovo, Slovakia). Gliadin standard was purchased from Sigma-Aldrich (St. Louis, USA).

All extraction protocols were optimized for milligram quantities in Eppendorf tubes and extractions were performed in technical duplicates.

Extraction of cereal proteins according to Osborne (1924)

Cereal proteins were extracted using 1.5 ml of solvent per 50 mg flour by continuous mixing (Roller Mixer SRT9D, Stuart, Staffordshire, UK) at 60 rpm for 1 hour at room temperature. Albumin and globulin fractions were extracted with 0.5 M NaCl, the salt was then removed by distilled water, and finally, prolamins were extracted with 70% (v/v) aqueous ethanol. After each step, supernatant was centrifuged at 9000 x *g* for 15 min at room temperature.

Extraction of cereal proteins according to Osborne (1924) and further modified by Weiss et al. (1993)

To obtain salt soluble protein extract, flours (375 mg) were firstly extracted with 1.5 ml 50 mM Tris-HCl (pH 8.8) containing 1.5% (w/v) polyvinylpolypyrrolidone for 1 hour at 4 °C with vortexing at 15-min intervals. Centrifugation was carried out at 20,000 x *g* for 20 min at 4 °C. The extraction step for salt soluble proteins was repeated at the same conditions. Supernatants were pooled and referred to as "albumin/globulin" fraction. To remove buffers, pellet was re-suspended and washed in distilled water. Alcohol soluble proteins were extracted twice with 1.5 mL of 75% (v/v) aqueous ethanol by continuous mixing (Roller Mixer SRT9D, Stuart, Staffordshire, UK) at 60 rpm for 2 hours at room temperature. After centrifugation at 20,000 x *g* for 20 min at 4 °C, both supernatants referred to as "prolamin" fraction were pooled. The rest of ethanol was removed by re-suspending the pellet in distilled water. Finally, the "glutelin" extract was obtained by addition of 1.5 mL of SDS-DTT buffer (50 mM Tris-HCl, pH 8.8, 1% SDS 0.5% DTT) and extracted for 1 hour at room temperature with vortexing at 15-min intervals, followed by centrifugation at 20000 x *g* for 20 min at 4 °C.

Extraction of cereal proteins according to van den Broeck et al. (2009)

The two-step gluten extraction procedure was carried out at protein sample/extraction buffer ratio 1:10 (w/v). Pre-extraction of wheat gliadins and rye secalins was performed with 50% aqueous iso-propanol (v/v) by continuous mixing (Roller Mixer SRT9D, Stuart, Staffordshire, UK) at 60 rpm for 30 min at room temperature, followed by centrifugation at 10,000 x *g* for 10 min at room temperature. The residual pellet was extracted twice with 50% aqueous iso-propanol, 50 mM Tris-HCl, pH 7.5 containing 1% (w/v) DTT (ratio 1:10) for 30 min at 60 °C with vortexing every 5-10 min, followed by centrifugation at 10,000 x *g* for 10 min at room temperature. After each step, samples were properly re-suspended by mixing and sonicated for 10 min in an ultrasonic bath (Sonorex Digitec, Bandelin, Berlin, DE). Supernatants were pooled and referred to as "two-step gluten extract".

Measurement of total protein content

All protein fractions from each extraction protocol were divided into few aliquots (300 µL) and precipitated with 5 volumes of ice-cold 1 M ammonium acetate in methanol incubated at -30 °C overnight. The next day precipitate was centrifuged at 5,000 x *g* for 10 min at 4 °C, washed 2-times with ice-cold 1 M ammonium acetate in methanol, and pellet was dried using vacuum concentrator (Concentrator Plus, Eppendorf, Hamburg, DE). One aliquot was reconstituted in 100 µL of solubilisation buffer (8 M Urea, 50 mM DTT) and used to determine the protein concentration using Bradford Solution for Protein Determination (Applichem, Darmstadt, DE) according to manufacturer´s instructions with BSA as a standard. Protein quantification was performed in technical duplicate (n = 2) using BioDrop DUO spectrophotometer (Biochrom Ltd, Cambridge, UK).

SDS-PAGE analysis

The second aliquot after ammonium acetate precipitation was reconstituted in 100 µL of buffer for electrophoresis (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) and analyzed by Tris-Tricine SDS-PAGE under reducing conditions according to the Schägger-von Jagow method **(Schägger and von Jagow, 1987)**. Proteins (10 µg/lane) were separated using BioRad MiniProtean Tetra Cell system (Bio-Rad Laboratories, Hercules, USA), followed by silver staining **(Blum et al., 1987)**. Gels were scanned using a Bio-Rad GS-800 Densitometer (Bio-Rad Laboratories, Hercules, USA) and saved as TIFF format.

RESULTS AND DISCUSSION

The aim of our work was to assess the efficiency of various extraction protocols focusing on wheat and rye flour prolamins. Both flours are routinely used in Slovak bakery industry. Many extraction protocols have recently been developed **(Singh et al., 1991; Weiss et al., 1993; DuPont et al., 2005; van den Broeck et al., 2009)** for cereal proteins, mainly wheat gliadins.

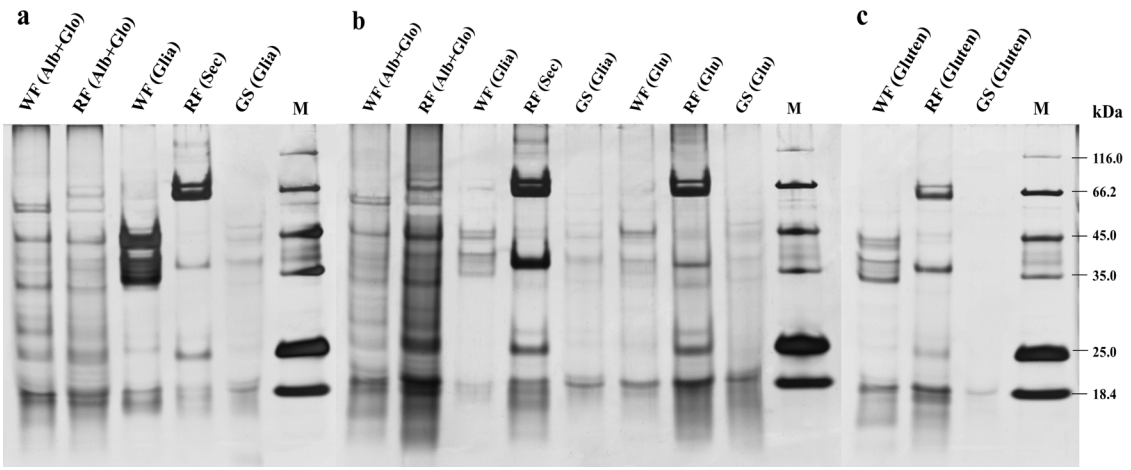

Figure 1 SDS-PAGE analysis of wheat (WF) and rye (RF) flour protein fractions followed by silver staining using different extraction protocols (a) Osborne, 1924; (b) Osborne, 1924 further modified by Weiss et al., 1993 and (c) van den Broeck et al., 2009. Gliadin standard (GS) was used as a control. Abbreviations in parenthesis refer to the protein fractions of wheat and rye flour as well as gliadin standard after each step of extraction: Alb+Glo – albumins and globulins; Glia – gliadins; Sec – secalins; Glu – glutelins; Gluten – gluten extract; M – marker.

However, pilot study of **Osborne (1924)** based on different solubility of proteins is most widely used extraction protocol. Generally, albumin and globulin fractions are soluble in water/salt solutions, prolamins are soluble in alcohols and high-molecular weight glutelins are soluble in diluted acid/base solutions. According to **Osborne (1924)** procedure the average protein content (n = 2) of wheat gliadins was only 0.03 mg.mL^{-1} (±0.01) and rye secalins 0.03 mg.mL^{-1} (±0.00) after removal of salt soluble albumins/globulins (Figure 2). To evaluate the efficiency of gliadin/secalin extraction we assigned gliadin standard as a control sample for measurement. Ethanol extraction of gliadin standard resulted in average protein content (n = 2) of 2.89 mg.mL^{-1} (±0.01) suggesting incomplete extraction of wheat/rye prolamins from flours.

SDS-PAGE analysis revealed that Osborne procedure was successful in extraction of prolamins (Figure 1a) indicating few strong bands of approx. 30-45 kDa (α/β- and γ-gliadins) and 66.2 kDa (75k-γ-secalins) in wheat and rye flours, respectively. Electrophoretic profile of gliadin standard is poorly visible (Figure 1a), most likely due to the incomplete solubility in SDS-PAGE buffer. The average protein content (n=2) of albumins/globulins was 0.06 mg.mL^{-1} (±0.02) and 0.18 mg.mL^{-1} (±0.00) in wheat and rye flours, respectively. The obtained results suggested that Bradford determination underestimates real amount of protein content comparing to SDS-PAGE analysis. Contrasted differences between Bradford measurement and electrophoretic profiling could also be assigned to very low technical reproducibility (e.g. 23% error between wheat gliadins duplicates in Bradford measurement).

The Osborne procedure was further modified **(Weiss et al., 1993)** by separated extraction of low-molecular weight subunits (gliadins in wheat, secalins in rye) and high-molecular weight glutelins, as well as by the addition of reducing agent at non-heated conditions. The average protein content (n = 2) after two sequential extraction steps was 1.76 mg.mL^{-1} (±0.01) and 0.13 mg.mL^{-1} (±0.01) of wheat gliadins and rye secalins, respectively. Albumins/globulins were also sequentially extracted; after pooling the average protein content (n = 2) was 1.08 mg.mL^{-1} (±0.06) in wheat and 0.76 mg.mL^{-1} (±0.02) in rye flours. These results indicated that salt soluble albumins/globulins in rye flour are more abundant comparing to secalins (Figure 2). Glutelins were most represented in both, wheat and rye flours (Figure 2) with average protein content (n=2) of 2.32 mg.mL^{-1} (±0.07) and 0.87 mg.mL^{-1} (±0.04), respectively.

However, SDS-PAGE analysis revealed that glutelins extracted at reducing conditions have similar molecular weights (Figure 1b) but with less intensity of α/β- and γ-gliadins (35-40 kDa) as well as 40k-γ-secalins (one band of approx. 37 kDa). These results are in agreement with general statement that glutelins are high-molecular weight subunits **(van den Broeck et al., 2009)** and are co-extracted together with monomeric gliadins and secalins. After glutelin extraction using reducing agent, rye 75k-γ-secalins (two bands of approx. 66.2 kDa) represented dominant fraction (Figure 1b) with highest protein content, 0.87 mg.mL^{-1} (±0.04). The same conclusions were also achieved in a study of **Gellrich et al., (2003)**. While the average protein content (n = 2) of gliadin standard was very high, 8.03 mg.mL^{-1} (±0.37) in gliadin extract and 2.44 mg.mL^{-1} (±0.01) in glutelin extract, electrophoretic profile indicated its impaired solubility in SDS-PAGE buffer (Figure 1b).

The last protocol used in our study **(van den Broeck et al., 2009)** differs from previous in simultaneous two-step co-extraction of gluten proteins (gliadins/secalins and glutelins) under reducing conditions at higher temperature (60 °C) and without removal of albumins/globulins. The average protein content (n = 2) in wheat gluten extract was markedly lower, 0.77 mg.mL^{-1} (±0.01), comparing to **Weiss et al., (1993)** modification protocol (Figure 2), probably due to the underestimation of results using Bradford measurement. In case of rye gluten extract, the average protein content (n = 2) was 0.50 mg.mL^{-1} (±0.08).

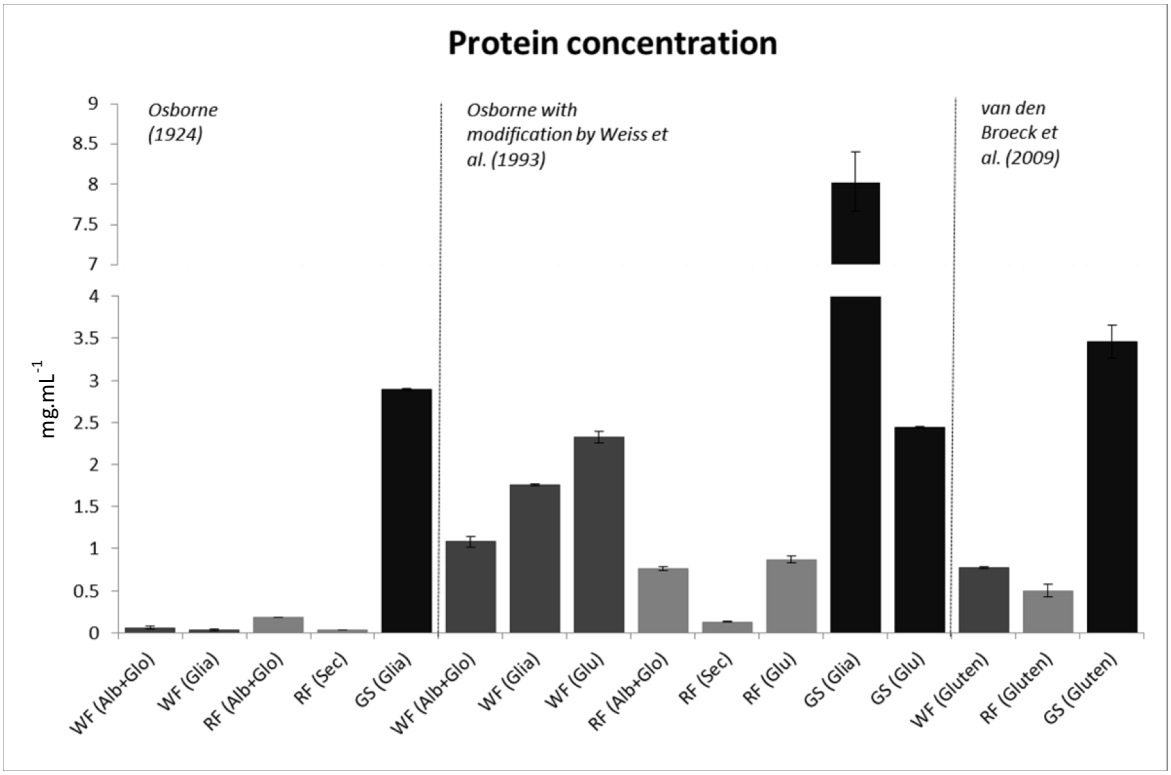

Figure 2 Protein concentration (mg.mL^{-1}) of wheat (WF) and rye (RF) flour protein fractions using Bradford solution for protein determination in three different extraction protocols. Gliadin standard (GS) was used as a control. Abbreviations in parenthesis refer to the protein fractions of wheat and rye flour as well as gliadin standard after each step of extraction: Alb+Glo – albumins and globulins; Glia – gliadins; Sec – secalins; Glu – glutelins; Gluten – gluten extract. Data presented is averages ± standard deviation (n = 2). All error bars are included.

SDS-PAGE analysis revealed typical bands of approx. 30-45 kDa (α/β- and γ-gliadins) and 37 kDa (40k-γ-secalins) in wheat and rye flours, respectively (Figure 1c). Moreover, 75k-γ-secalins (two bands of approx. 66.2 kDa) were also detected (Figure 1c) in rye gluten extract. The average protein content (n = 2) of gliadin standard in gluten extract was 3.46 mg.mL^{-1} (±0.20). Similarly, to previous protocols, separation of gliadin standard on polyacrylamide gel was insufficient due to the incomplete solubility in SDS-PAGE buffer (Figure 1c).

Precisely optimized extraction protocol is a critical step to analyze cereal proteins many of which causing allergies or food intolerances. In our study we aimed to compare different extraction protocols for wheat/rye prolamins as a main triggering factor in celiac disease. Generally, wheat contain higher amount of prolamins than rye which was proved by e.g. fractionation of protein complex **(Mickowska et al., 2012)**. Both, wheat and rye prolamins has highest content of two amino acids, glutamine and proline **(Mickowska et al., 2012)** suggesting their poor digestibility by gastric enzymes. As a result, Glu- and Pro-rich peptides containing T-cell stimulating epitopes are occurred that can cause celiac disease. Ancient varieties of wheat and rye could also be harmful **(Ciclitira et al., 2005; Hybenova et al., 2013)** as they are genetically similar with amino acid composition comparable to modern varieties. However recent studies **(van den Broeck et al., 2010)** revealed that e.g. presence of the Glia-α9 epitope was lower in the wheat landraces.

In our study we aimed to analyze prolamin extract from commercially available wheat and rye flours. According to Bradford method, the total protein content was slightly lower comparing to other studies **(van den Broeck et al., 2009)**. The differences could be attributed by using the different wheat varieties. Moreover, wheat/rye flours used here were milled during different conditions (procedure not described) which probably resulted in a loss of proteins, for instance, wheat ω-gliadin fractions with molecular weight of 50-80 kDa were almost unable to detect in SDS-PAGE gels. Contrary, **van den Broeck et al., (2009)** described that ω-gliadins/D-type LMW-GS fractions were abundantly presented in all wheat varieties. In summary, the efficiency of extraction protocol depends not only on the cereal protein source, but also on working conditions and analytical method of their identification/quantification.

CONCLUSION

Various extraction protocols with different working conditions examined here were generally efficient in extraction of wheat/rye flour prolamins. Although, the pilot Osborne procedure yields in lower protein content using Bradford measurement comparing to SDS-PAGE analysis, it is still considered as an effective method due to its rapid and simple nature. Up to date, several modifications of extraction conditions are under investigation using multiple extraction steps or reducing agents. These protocols are usually time consuming, however, carefully optimized conditions can reduce not only time but also can increase the protein content in extracts. In some cases, the huge amount of starting material is required for analysis. Therefore, in our study we optimized all extraction

protocols for milligram quantities using Eppendorf tubes. Except the Osborne procedure, the two protocols used here showed high technical reproducibility according to Bradford with coefficient of variation less than 20%. Assuming above mention facts, van den Broeck protocol is a good choice for simultaneous co-extraction of gluten proteins from wheat/rye flours.

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Contact address:

Peter Socha, Slovak University of Agriculture in Nitra, Research Centre AgroBioTech, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.socha@uniag.sk.

Marián Tomka, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marian.tomka@uniag.sk.

Kvetoslava Kačmárová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A.

Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xkacmarova@is.uniag.sk.

Blažena Lavová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: blazena.lavova@uniag.sk.

Eva Ivanišová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Plant Products Storage and Processing, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: eva.ivanisova@uniag.sk.

Barbara Mickowska, University of Agriculture, Faculty of Food Technology, Malopolska Centre of Food Monitoring, Balicka 122, 30-149 Krakow, Poland, E-mail: bmickowska@ar.krakow.pl.

Dana Urminská, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Institute of Chemistry, Slovak Academy of Sciences, Center for White-Green Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: dana.urminska@uniag.sk.

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EVALUATION OF PRIMARY AND SECONDARY METABOLITES IN SELECTED VARIETIES OF POTATOES

Ján Mareček, Eva Ivanišová, Helena Frančáková, Janette Musilová, Tomáš Krajčovič, Andrea Mendelová

ABSTRACT

The aim of study was to determine primary and secondary metabolites in selected varieties of potatoes. Potatoes (*Solanum tuberosum* L.) are good source of bioactive compounds, mainly phenols as one of the most important components. The chemical composition with reducing sugar, starch, ascorbic acid, total polyphenol and flavonoid content were analysed in five potato varieties (Agria, Marabel, Red Anna, Picasso, Princess). Values of dry matter content ranged from 20.34 to 23.64%. In terms of tubers storage, its content above 20% is required. The highest level of starch was detected in variety Princess (16.82%). The lowest reducing sugar content was recorded by variety Marabel (0.08%). Similarly, low values reached varieties Princess (0.12%), Agria (0.14) and Red Anna (0.16%). These would be appropriate to use for food processing and for production of fried potato chips or fries. Variety Red Anna reached the highest amount of vitamin C (73.72 mg.kg^{-1}). The lower levels of this vitamin showed tubers of varieties Picasso (35.02 mg.kg^{-1}) and Princess (36.89 mg.kg^{-1}). The antioxidant activity was measured with radical scavenging assays using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as well as phosphomolybdenic assay. Potato varieties contained high levels of total polyphenols (0.474 – 1.550 mg GAE per dry weight) and flavonoids (1.407 – 15.933 µg QE per dry weight). The consumption of potatoes can provide nutritional value along with antioxidant potential that can be helpful for proper functioning of the body physiological systems. Statistical evaluation by the single factor analysis of variance detected high significant impact of variety on the content of all the analytical parameters in evaluated varieties of potato tubers.

Keywords: starch; reducing sugars; antioxidant activity; polyphenols; flavonoids

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fifth most important crop worldwide after sugar cane, maize, wheat and rice with production of >364 million tons in 2012 **(FAO, 2014)**. Potato spread to Europe from the America in the late 1500s **(Camire et al., 2009)** and immediately became very important for human nutrition in the "Old Word" as well. Nowadays, potatoes are cultivated in more than 160 countries with more than 4000 cultivars **(Hils and Pieterse, 2007)**. Potatoes are rich in carbohydrate and provide significant quantities of proteins, minerals (iron) and vitamins (B complex and vitamin C), dietary fiber, and antioxidants which vary with variety, storage conditions, growing season, soil type, and pre-harvest nutrition **(Singh and Kaur, 2009)**. Nowadays, potatoes have received substantial interest as a valuable source of antioxidants because they contain a variety of secondary metabolites including phenols and are consumed in relatively high amounts **(Wegener and Jansen, 2013)**. Phenols have been associated with certain health benefits such as inhibition of cholesterol accumulation in blood, reduction of the risk of coronary heart disease, prevention of some types of cancer, and retardation of macular degeneration among others **(Kita et al., 2013)**. In potatoes, most of the phenols are present between their cortex and peel, while their content reduces towards the center of the tuber **(Friedman, 1997)**. Chlorogenic acid and caffeic acid are two of the most prominent phenolic acids reported in potato followed by protocatechuic acid, *t*-cinnamic acid, *p*-coumaric acid, ferulic acid, vanillic acid, gallic acid, syringic acid, and salicylic acid **(Reddivari et al., 2007)**.

Antioxidant activity and total phenolics are different between potato cultivars. Bioactive composition of potato compared to other vegetables is low but since potato form a substantial part of our daily diet, it is therefore important to screen and identify those genotypes which are high in antioxidants **(Kaur and Aggarwal, 2014)**.

The aim of this study was to evaluate primary (reducing sugar content, starch content), secondary metabolites (polyphenols, flavonoids, ascorbic acid content) and antioxidant activity in selected varieties of potatoes.

MATERIAL AND METHODOLOGY

Plant material

Potatoes were grown on a field nursery at the Department of Environmental Protection and Organic Farming (DEPOF) in Spišská Belá (Slovakia). The used genotypes of potatoes were: Agria, Marabel, Red Anna, Picasso and Princess. This list includes one of the most cultivated varieties in Slovak Republic. Samples with peel were before the measurement crushed to mash, lyophilized

(IISHIN Freeze Dryer, IISHIN lab. Co. Ltd.) and then stored at 4°C in refrigerator. These varieties from the crop year of 2014 were assessed 4 weeks after the harvest. Storage of tubers was carried out in cooling box at 6 °C and under relative humidity 85%. Material samples were weighed on Mettler Toledo Analytical Balances.

Chemicals

All chemicals used were of analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

Sample preparation

0.5 g of milling fractions was extracted with 20 mL of 80% ethanol for 20 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 20 min, the supernatant was used for measurement (antioxidant activity, polyphenols, flavonoids).

Dry matter content

Dry matter content of potato varieties was measured in samples of around 10 g by pre-drying at 65 °C for 3 hours and by drying at 105 °C for 3 hours to the constant weight (WTB Binder drying oven). Weight of the dried sample was converted to the initial fresh mass.

Starch content

10 g of sample was hydrolysed in a boiling water bath at 100 °C using 100 mL of 1.422% hydrochloric acid. Each solution was then treated and cleaned with 2 mL of 15% potassium ferrocyanide and 2 mL of 30% zinc sulphate solution. Optical activity of filtrate sample was measured on P3001RS Automatic Digital Polarimeter (°Sx1.775), (A. KRÜSS Optronic GmbH, Germany).

Reducing sugar content

Determination of reducing sugars was performed by Schoorl method using Fehling´s solutions I and II. There was used around 5 g of weighed sample. Titration of sample was done with sodium thiosulfate solution. Reducing sugar content was determined by the consumption difference between the blank titer and average sample titer (Schoorl table).

Ascorbic acid content

Vitamin C was extracted from homogenized samples by the metaphosphoric acid solution. Dehydro-L-ascorbic acid was reduced to L-ascorbic acid. The total vitamin C content was determined by HPLC method with UV detection at 265 nm (Agilent 1220, Agilent Technologies, USA). Dosing of samples was realized on Agilent Autosampler (Agilent Technologies, USA) by injection.

Radical scavenging activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(Yen and Chen, 1995)**. The extracts (0.5 mL) were mixed with 2 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). Absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, Jenway, Staffordshire, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10-50 $mg.L^{-1}$; R^2 = 0.983) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Reducing power

Reducing power of samples was determined by the phosphomolybdenum method of **Prieto et al., (1999)** with slight modifications. The mixture of sample extract (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90 °C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, Jenway, Staffordshire, England). Trolox (10-1000 $mg.L^{-1}$; R^2=0.998) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Total polyphenol content

Total polyphenol content of potato extracts was measured by the method of **Singleton and Rossi, (1965)** using Folin-Ciocalteu reagent. 0.2 mL of each sample extract was mixed with 0.2 mL of the Folin-Ciocalteu reagent, 2 mL of 20% (w/v) sodium carbonate and centrifugated at 10000 g (Neofuge VS – 100 BN, China) for 10 min. After 30 min. in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, Jenway, Staffordshire, England). Gallic acid (5-250 $mg.L^{-1}$; R^2 = 0.999) was used as the standard and the results were expressed in $mg.g^{-1}$ gallic acid equivalents.

Total flavonoid content

Total flavonoids were determined using the modified method of **(Quettier – Deleu et al., 2000)**. 2 mL of sample extract was mixed with 0.4 mL of 5% (w/v) ethanolic solution of aluminium chloride. After 30 min. in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, Jenway, Staffordshire, England). Quercetin (0.5-20 $mg.L^{-1}$; R^2 = 0.999) was used as the standard and the results were expressed in $\mu g.g^{-1}$ quercetin equivalents.

Statistical analysis

Statistical software SAS 9.2 and Enterprise Guide 3.0 was used for the statistical evaluation. On the other hand, single factor analysis of variance was used to assess the impact of variety on dry matter, starch, reducing sugar, vitamin C, polyphenol and flavonoid content. Statistical significance was measured with Tukey´s test (p <0.5).

RESULTS AND DISCUSSION

Dry matter content

Dry matter content of potato tubers ranges in Slovak climate conditions from 20 to 25%. Its higher content is in terms of storage a stabilizing factor. However, it may have negative effect on potato taste properties. It usually also correlates with the starch content. Our evaluated cultivars reached values of dry matter content from 20.34% to 23.64 (Table 2) while the highest amount showed variety Princess that is therefore suitable for long-term storage. Dry matter content is affected by the climate conditions, fertilization and variety **(Poberezny and Wszelaczynska, 2011)**.

Starch content

Starch content is highest in tubers after harvest. During the storage, its content gradually decreases because of degradation to simple sugars and it is also consumed

during breathing of tubers. Its values in assessed cultivars were in the range from 14.13 to 16.82% (Table 2). These values can be rated in growing conditions of Central European region as appropriate or greater. Higher starch content is suitable for the production of potato chips. During the heat treatment, starch starts to gelatinize and tubers may rupture **(Šimková et al., 2013)**.

Reducing sugar content

Reducing sugars are an important factor in food processing of potato tubers for fried chips. To help prevent the Maillard reaction accompanied by undesirable sensoric symptoms, low reducing sugar content is appropriate. Ideal are values up to 0.20%. This requirement was, except for Picasso (0.25%), fulfilled by all of the varieties (Table 2). These can be used for the production of potato chips. Lower storage temperatures support an increase in the content of reducing sugars **(de Quadros et al., 2010)** which must be therefore monitored during the storage.

Ascorbic acid content

Vitamin C is nutritional ingredient that characterizes potatoes as an important crop with antioxidant activity **(Külen et al., 2013)**. The highest content is in the fresh tubers, whilst heat treatment reduces its amount. Among the assessed cultivars the highest content showed tubers of variety Red Anna (Table 2) with purple skin (73.72 $mg.kg^{-1}$). Conversely, variety Picasso reached its lowest value (35.02 $mg.kg^{-1}$). The amount of vitamin C decreases due to storage conditions.

Antioxidant activity

The antioxidant potential of potato cultivars was determined on the basis of scavenging activity of the stable free radicals DPPH and reducing ability by phosphomolybdenum assay (Figure 1 and Figure 2). Agria, Red Anna and Princess cultivars contained highest antioxidant activity – 1.556; 1.316 and 1.028 mg TEAC per 100 g^{-1} dry matter for DPPH and phospomolybdenum method (19.071; 23.450 and 23.428 mg TEAC per 100 g^{-1} dry matter) respectively. Among the tested potatoes Marabel and Picasso cultivars showed lower antioxidant potential. Red Anna belongs to the cultivars with red peel; Agria, Marabel, Picasso and Princess are potatoes with brown peel. Extract prepared from red peel potatoes have stronger antioxidant activity than brown peel, probably due to the strong effect of anthocyanins. Previously, it was expounded that the antioxidant activity of ethanolic and aqueous potato extract has activity 62.3% and 62.5%, respectively **(Kaur and Kapoor, 2002)**. **Karadeniz et al., (2005)** reported similar activity (70%) of the potato extracts 70% for same sample weight. **Ezekiel et al., (2013)** reported that potatoes showed 94% scavenging activity towards hydroxyl radicals, and almost complete inhibition of superoxide radicals. Antioxidant activity of potatoes is mainly caused by their chlorogenic, protocatechuic and caffeic acid content. Chlorogenic acid from potatoes has been found to be an effective inhibitor of lipid oxidation **(Al-Shaikan et al., 1995)**. Numerous investigations reported that potato has applicable amount of antioxidant that possess significant inhibition ability **(Karadeniz et al., 2005)**. Genotype and growth conditions, such as water availability, light quality and temperature, affect the synthesis and accumulation of antioxidants in potatoes. Peeling the potato considerably reduced its antioxidant activity. According to **Ezekiel et al., (2013**) potatoes contain relatively low amount of total phenolic acids, but they have high antioxidant activity compared to other fruits and vegetables.

Total polyphenol content

The results of the Folin–Ciocalteu assay are shown in Table 1. Among the evaluated cultivars Agria and Red Anna had the highest gallic acid equivalent (1.550 $mg.g^{-1}$; 0.977 $mg.g^{-1}$), followed by Princess and Marabel cultivars (0.675 $mg.g^{-1}$ and 0.524 $mg.g^{-1}$). In Picasso variety was observed the lowest value of total polyphenol content – 0.474 $mg.g^{-1}$. The variations of polyphenol content between varieties may result from genotypes and harvest locations that influence the accumulation of phenolic compounds by synthesizing different quantities and/or types of phenolics **(Lachman et al., 2009)**. Earlier, **Karadeniz et al., (2005)** reported that the polyphenol content of potato is 32.44 ±6.07 mg GAE.100 g^{-1}. Previously, **Al-Saikhan et al. (1995)** also published that potato contains 11.41 –27.47 mg GAE.100 g^{-1} total polyphenol content. Generally, it is considered that edible part of potato accounts 40% of the total polyphenol content **(Chu et al., 2002)**, while amount of conjugated polyphenols in potato is 57.9±13.4% **(Vinson et al., 1998)**.

Polyphenols are distributed mostly between the cortex and peel tissues of the potato. Potato peel contains about ten times as much polyphenols as potato flesh. About 50% of the phenolic compounds may be found in peel and adjoining tissues while the rest decreases in concentration from the outside towards the centre of the potato tuber. Chlorogenic acid constitutes up to 90% of the total polyphenolic content of potatoes **(Friedman 1997)**. Other phenolic acids include protocatechuic, sinapic, coumaric and vanillic acids. Other polyphenolic compounds present in potatoes include anthocyanins, flavanones (naringenin and eriodictyol), flavan-3-ols (catechin and epicatechin) and flavonols (kaempferol and sometimes quercetin glycosides) **(Lewis, 1999)**. Many of these compounds are present in fairly low concentrations. The phenols can be recovered from the skin portion, which is discarded as waste during potato processing and can be used for value addition in different food products **(Navarre et al., 2009; Bončíková et al., 2012; Musilová et al., 2015)**.

Total flavonoid content

Flavonoid content of evaluated potatoes (Table 1) ranged from 1.417 to 15.933 µg/g QE. Red Anna variety contains the highest flavonoid content, due to the presence of anthocyanins in the peel. Anthocyanins are a sub-group within the flavonoids and present in substantial amounts in pigmented flesh potatoes. Anthocyanin levels between 5.5 and 35 mg/100 g fresh weight in potatoes have been reported **(Brown, 2008)**. Red peel and purple or red-fleshed cultivars has twice of the flavonoid concentration of white-fleshed cultivars and their concentrations are considerably higher in skin. In potatoes was reported presence of these flavonoids: catechin, epicatechin, erodictyol, kaempeferol, naringenin and rutin. The potato flavonols content is not significantly high, but these can be considered as a valuable source of these compounds because of their high consumption **(Tudela et al., 2002)**.

Table 1 Total polyphenol and flavonoid content in selected varieties of potatoes.

Sample	Total polyphenols content (mg GAE.g^{-1})	Total flavonoids content (µg QE.g^{-1})
Agria	1.550 ±0.08	14.709 ±1.91
Marabel	0.524 ±0.05	6.301 ±0.71
Red Anna	0.977 ±0.01	15.933 ±1.47
Picasso	0.474 ±0.04	1.417 ±0.27
Princess	0.675 ±0.03	3.547 ±0.95

NOTE: GAE (gallic acid equivalent); QE (quercetin equivalent); ± (standard deviation of the mean).

Table 2 Components contained in potato tubers.

Sample	Dry matter content (%)	Starch content (%)	Reducing sugar content (%)	Vitamin C (mg.kg^{-1})
Agria	22.49 ±0.20	16.12 ±0.14	0.14 ±0.02	56.32 ±1.15
Marabel	21.92 ±0.11	15.72 ±0.25	0.08 ±0.01	49.60 ±0.84
Red Anna	20.34 ±0.06	14.31 ±0.03	0.16 ±0.01	73.72 ±2.59
Picasso	20.98 ±0.26	14.79 ±0.28	0.25 ±0.01	35.02 ±1.33
Princess	23.64 ±0.19	16.82 ±0.16	0.12 ±0.01	36.89 ±0.89

NOTE: ± (standard deviation of the mean).

Table 3 Single factor analysis of variance for selected parameters of potato tubers (Tukey test), Major effect: variety.

Sample	Degrees of Freedom	Sum of Squares	Mean Squares	F-test (F-ratio)	Significance (p-value)
Dry matter	4	19.96473333	4.99118333	52.95	0.0001^{+++}
Starch	4	13.29029333	3.07257333	27.79	0.0001^{+++}
Reducing sugar	4	0.04710667	0.01177667	28.49	0.0001^{+++}
Vitamin C	4	3995.890067	748.972517	110.87	0.0001^{+++}
Total polyphenols	4	1469.900440	367.475110	279.13	0.0001^{+++}
Total flavonoids	4	0.32515160	0.08128790	89.15	0.0001^{+++}

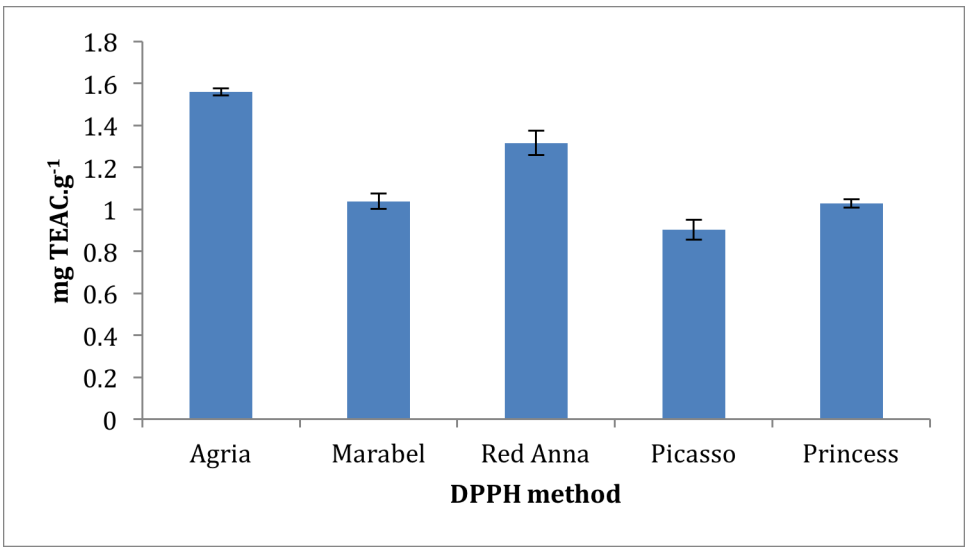

Figure 1 Antioxidant activity of potatoes determined by DPPH method (TEAC – Trolox equivalent antioxidant capacity).

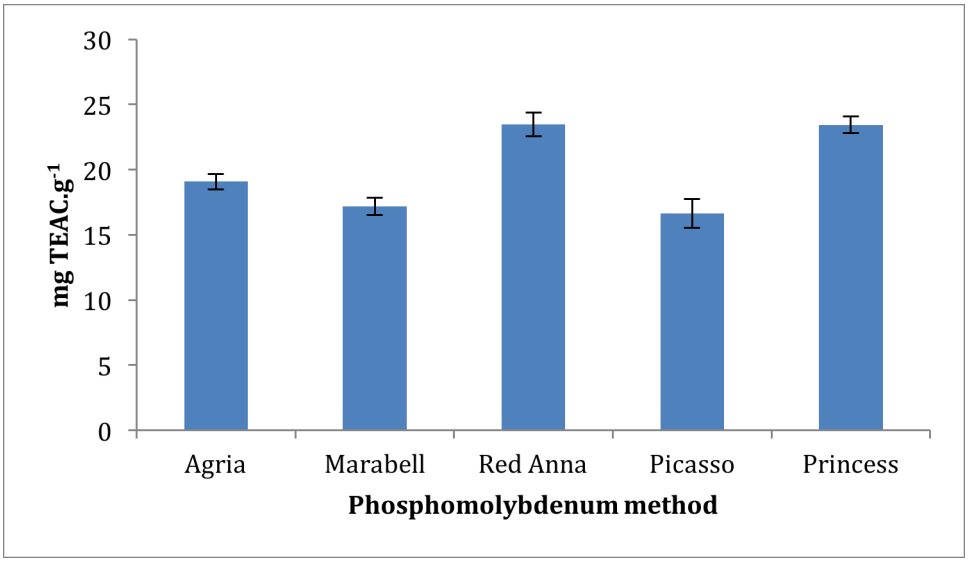

Figure 2 Antioxidant activity of potatoes determined phosphomolybdenum method (TEAC – Trolox equivalent antioxidant capacity).

It can be stated, that the amounts of flavonoids are not proportional to total polyphenol content in evaluated samples. Each potato cultivar contained different levels of these bioactive compounds, which is in general agreement with results of other authors, because the content of phenolic compounds depends on potato cultivar, weather, soil and agrotechnical conditions **(Hamouz et al., 1999; Gumul et al., 2011)**. The results of statistical evaluation are listed in Table 3. Statistical evaluation using the single factor analysis (ANOVA, SAS 9.2) confirmed highly statistically significant influence on the content of all the analyzed compounds.

CONCLUSION

From the data in this study, it can be concluded that potatoes are rich sources of primary and secondary metabolites. The variety Red Anna and Agria showed higher biological activity (antioxidant activity, polyphenols and flavonoids) in comparison with other varieties. The content of dry matter and starch measured in evaluated tubers predetermines these cultivars for long-term storage. Low values of reducing sugar content in varieties Agria, Marabel, Princess and Red Anna is desired parameter for the production of potato chips. Potato tubers are in addition a valuable source of vitamin C (variety Red Anna). Phytochemicals in potatoes can be used for development of functional foods or nutraceuticals. Considering the large quantities in which potatoes are consumed throughout the world, they could be a very good vehicle for addressing some health related problems.

There was statistically high significant impact of variety on dry matter, starch, reducing sugar, vitamin C, polyphenol and flavonoid content. The results confirmed a significant influence of varietal characteristics on examined components of potato tuber cultivars. Above results are an original compact research focused on technological parameters, biologically active compounds and antioxidant activity of selected potato varieties grown in Slovakia. The results provide innovative findings in the area of potato quality research.

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Contact address:

Ján Mareček, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storing and Processing of Plant Products, Tr. A. Hlinku 2, 94976 Nitra, Slovakia, E-mail: Jan.Marecek@uniag.sk.

Eva Ivanišová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storing and Processing of Plant Products, Tr. A. Hlinku 2, 94976 Nitra, Slovakia, E-mail: eva.ivanisova@uniag.sk.

Helena Frančáková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storing and Processing of Plant Products,

Tr. A. Hlinku 2, 94976 Nitra, Slovakia, E-mail: Helena.Francakova@uniag.sk.

Janette Musilová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 94976 Nitra, Slovakia, E-mail: Janette.Musilova@uniag.sk.

Tomáš Krajčovič, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storing and Processing of Plant Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: krajcovic.thomas@googlemail.com.

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LUNASIN DETECTION IN COLOURED WHEAT GENOTYPE

Milan Chňapek, Dušan Siman, Zdenka Gálová

ABSTRACT

Lunasin is a biologically active protein, composed of 43 amino acid residues. There has been proven many health-promoting effects of lunasin peptide. The most important health benefits include: anti-hypertension, antioxidant activity, cancer prevention or therapy. It was also demonstrated anti-inflammation, hypocholesterolemic activity, anti-obesity and immunomodulation. The focus of our research is to summarize the discovery, characterization and biological activities of lunasin, which will provide a reference for the future development and utilization of lunasin, and a basis for exploring the underlying mechanisms of these health-beneficial functions. Lunasin was first isolated in 1987 at Niigata University School of Medicine in Japan, during the screening of protease inhibitors from soybean seeds. It was subsequently found in other beans, grains and herbal plants, including wheat, barley, rye, triticale. Concentration of lunasin is ranging from 0.013 to 70.5 mg protein lunasin/g of protein. Big step forward in the understanding of the lunasine operating mechanism in the fight against cancer has arisen after study on cloning of the soybean lunasin gene and subsequent transfection into mammalian cells which led to the discovery that the lunasin gene can disrupt mitosis and induce chromosome breakage, ultimately leading to cell apoptosis. The main goal of our work was to evaluate collection of wheat with unusual grain colour for presence of lunasin gene. DNA was extracted by commercial kit and lunasin gene was detected by PCR reaction. Our results showed presence of lunasin gene detected by 3 combinations of 2 sets of primer pair and indicated lunasin peptide presence in cereal grains. These findings are necessary to confirmed by proteome analysis.

Keywords: cancer; coloured wheat; gene detection; lunasin; PCR

INTRODUCTION

Civilization diseases are one of the most worldwide problems of mankind. Cancer is the largest and the most widespread illness. Surgical treatment was the most effective, in past, but there are a lot of less invasive methods of cancer healing, in presence. New substances originated from plants or animals, which show chemopreventive effects, are shown by ongoing studies **(Hernández-Ledesma et al., 2009)**.

Carcinogenesis is a process which consists of combination of multiple heritable and environmental factors. Epidemiological studies shown, that cancer appearance and mortality significantly varied across the world. Cancer remains the main cause of mortality in western world. These parts of world where is diet centered on plant foods tending to have a lower rate of cancer, but prevalence of cancer is rising rapidly in one generation after their emigration to the western countries. This indicates that genetic factors are not the primary factors that cause cancer and modification of nutritional habits and lifestyle, as well as, consumption of foods containing bioactive components can offer a significant protection against carcinogenesis **(Hernándes-Ledesma et al. 2009)**.

Lunasin is one of these substances, which produce not only a lot of positive effects on human organism, but also anticancer activity. Lunasin is biological active peptid, which consist of 43 amino acids. There has been confirmed, that lunasin protected cells against chemical transformation induced by chemical carcinogens and virus and ras oncogenes. Mechanism of lunasin action is based on balance influence between acetylation and deacetylation of histones. This mechanism may cause cell death, because in this case lunasin acts as a tumour suppressor which is tightly bounded on deacetylated histones in cell nucleus and have ability to influence cancer cells apoptotically and cytotoxic **(Chang et al., 2014)**.

In vitro studies, animal treatment and epidemiologic researches showed that soy consumption is in connection with decreasing of some cancer types **(Hernández-Ledesma et al., 2009)**.

Hsieh et al., (2010) reported, that the first animal model confirmed preventive properties against chemical carcinogen-induced skin cancer in mice. Lunasin also play role as an active cancer preventive agent in treatment of human breast cancer. Lunasin in combination with aspirin arrest the cell cycle in the S- and G-phases, respectively, acting synergistically to induce apoptosis which was achieved by modulating the expression of genes encoding G1 and S-phase regulatory proteins.

Lunasin is a soybean derived peptide with a MW of 5.5 kDa and contains 9 aspartic acid residues on C domains, cell adhesion motifs consit of 3 amino acids residues (arginin – glycine – aspartic acid) and predicted helix with structural homology to a conserved region of chromatin binding

proteins. Lunasin is not fully digested in gastrointestinal system, but is absorbed intact, reaching target tissues. The biological activity of lunasin depends on cultivar, environmental factors and processing conditions, which in turn affected its concentration **(Wang et al., 2008)**.

Lunasin has been discovered in most of soybean varieties and its concentration ranged from 4.4 to 70.5 mg lunasin in one gram of protein **(Hernándes-Ledesma et al., 2009)**.

Jeong et al., (2007) detected lunasin in wheat using mass spectrometry. They determined 14 amino acids fragment (KQLQGVNLTPCEKH) with m/z 656, 8640 Da. This fragment corresponded to 12-25 amino acids fragment of soy albumin subunits, which was identified as a lunasin peptide.

The main goal of our research was to detected lunasin gene in collection of coloured wheat grain.

MATERIAL AND METHODOLOGY

There was analysed collection of 8 genotypes of wheat grain (Table 1) with unusual grain colour.

DNA isolation was performed from wheat grain by commercial kit GeneJET TM (Fermentas). Isolation protocol was modified for isolation DNA not only from fresh tissue, but also from grain. Modification contains supplementation of Lysis buffer A with 2% (w/v) polyvinylpyrrolidone. Wheat grains of each cultivar (up to 100 mg) were grinded in liquid nitrogen using a mortar and pestle. Then were grounded plant tissue powder transferred into the tubes with the prealiquoted Lysis buffer A (with PVP). The rest of extraction steps were held according standard procedure with usage of Lysis buffer B, RNase A. Purificatrion of extracted DNA were realized in spin column tubes with utilization of Plant g-DNA binding solution which anchored extracted DNA on spin column membrane. Wash buffer I and Wash buffer II purified anchored DNA. Elution of DNA from membrane to solution was realized by Elution buffer. The quantity and quality of puried DNA were measured by nanophotometer and visualisated by 1.5% horizontal agarose electorphoresis.

GoTaq® Green Master Mix from Promega Company was used for Polymerase chain reaction (PCR) DNA amplification. GoTaq® Green Master Mix is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. PCR amplification protocol of DNA fragments were realized using primer pairs (Table 2) and their combination (Table 3) according to **Dinelli et al., (2014)**. 25 µL of reaction mix was prepared on ice and contained 12.5 µL of GoTaq® Green Master Mix, 2.5 µL of 10 µM upstream primer, 2.5 µL of 10 µM downstream primer, 2.5 µL of nuclease-free water and 5 µL of 0.025 $ng.\mu L^{-1}$ DNA template. Standard PCR procedure consists of 2 min initial denaturation step at 95 °C for activation of reaction mix. Product amplification contained 3 subsequent steps. 20 s denaturation of DNA at 95 °C, 30 s anelation of primers at 54 °C and 30 s polymerization of DNA fragmets at 72 °C. These 3 steps repeat 50 times. The last step of PCR procedure is 5 min final extension at 72 °C. Quality of amplyfied product were confirmed by 1.5% horizontal agarose electrophoresis.

DNA fragments were separated in 8% vertical polyacrylamide gel electrophoresis for 360 min at 500V in Hoeffer SE 660 electrophoretic system and visualised by silver staining.

Table 1 List of analysed genotypes.

	Name	Species	Originated	Colour
1	Barevná 25 – modrá	*Triticum aestivum* L.	CZ	**Blue**
2	Trojzrnka	*Triticum aestivum* L.	CZ	**Red**
3	Mnohokvietková	*Triticum aestivum* L.	CHINA	**Red**
4	Tr.Etiopicum Jakubz	*Triticum aestivum* L.	Ethiopia	**Purple**
5	Tr. Etiopicum araratica-červená	*Triticum aestivum* L.	Ethiopia	**Purple**
6	UC 66094	*Triticum aestivum* L.	USA	**Blue**
7	Koniny-červená	*Triticum aestivum* L.	CZ	**Red**
8	Modré zrno	*Triticum aestivum* L.	CZ	Blue

Table 2 Nukleotid sequences of used primers.

Name	Primer type	Sequence
Lun1	forward	**AAATGGCANCACCAGNA**
	revers	**CGTCATCATCATNATCGTNA**
Lun2	forward	**GATANCTGCCNCAAGCA**
	revers	**TCTTNTCCATNATGTGCTTCTC**

Table 3 Primer pairs combinations.

Number	Primer pair combination
1	Lun1 F x Lun1 R
2	Lun1 F x Lun2 R
3	Lun2 F x Lun1 R
4	Lun2 F x Lun2 R

Electrophoretic separation and visualisation of DNA fragments were performed according **Bassam et al., (1991)**.

Visualised DNA fragments were captured by UVP digital imagine system and detected by Doc-IT LS software from UVP Jena, Germany.

RESULTS AND DISCUSSION

Lunasin is a novel, cancer-preventive peptide whose efficacy against chemical carcinogens and oncogenes has been demonstrated in mammalian cells and in a skin cancer mouse model. Isolated and characterized in soy, lunsin peptide is also documented in barley, wheat, tritikale, rye and oat **(Lumen et al., 2005)**.

The characterisation of cDNAs encoding lunasin shows that it corresponds to the small subunit of the soybean 2S albumin. The biological activity of lunasin has led to searches for related peptides in other plant species, including reported isolation from *Solanum*, amaranthus seeds, Brazil nut, sunflower and cereal seeds.

The identity of the peptides in wheat was confirmed by partial sequences which match exactly to the soybean sequence over stretches of 14 amino acids **(Mitchell et al., 2013)**.

We therefore decided to search for lunasin gene sequence aroud colour wheat genotype by utilization of 2 sets of primers developed by **Dinelli et al., (2014)**.

Our results show that utilization of primer pair Lun1 forward and Lun1 revers showed presence of 121 bp length DNA fragment. This primers pair combination seems to be suitable for lunasin gene detection, because we were able to detect lunasin gene in all of genotypes (Figure 1 and Figure 2).

Primer pair combination Lun1 forward and Lun2 revers not provide any fragment with desirable length 85 bp Using of this primer pair combination is contradictory and has to be tested in future.

Primer pair combination Lun2 forward and Lun1 revers was used for detection of DNA fragment with length 103 bp. There were obtained presence of desired DNA fragment in each genotype of evaluated wheat collection (Figure 3 and 4).

Application of primer pair combination Lun2 forward and

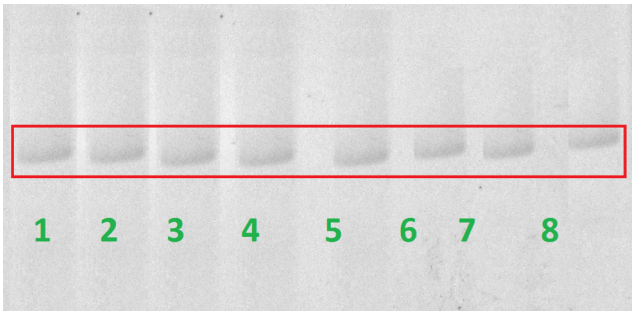

Figure 3 Lunasin gene detection with primer pair combination F2R1 – 103 bp.

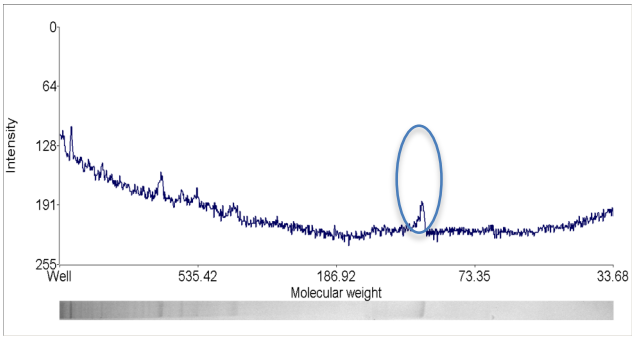

Figure 4 Barevná 25 – F2R1.

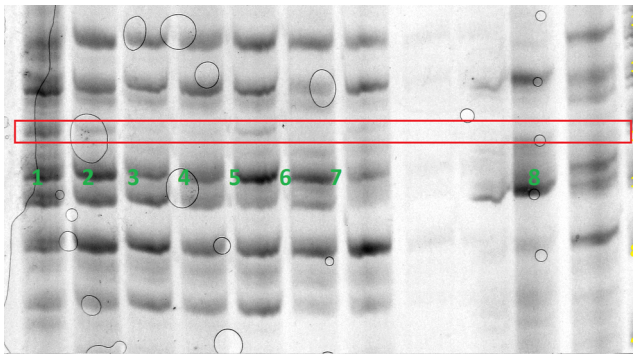

Figure 1 Lunasin gene detection with primer pair combination F1R1 – 121 bp.

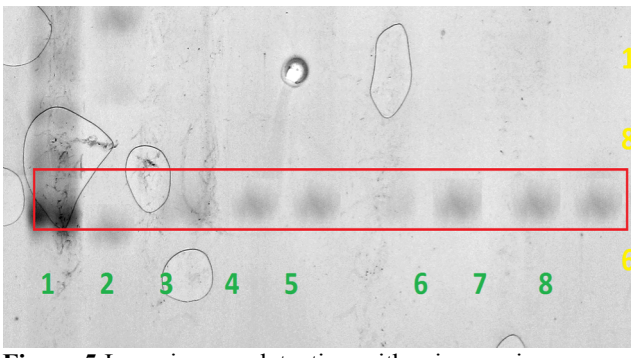

Figure 5 Lunasin gene detection with primer pair combination F2R2 – 67 bp.

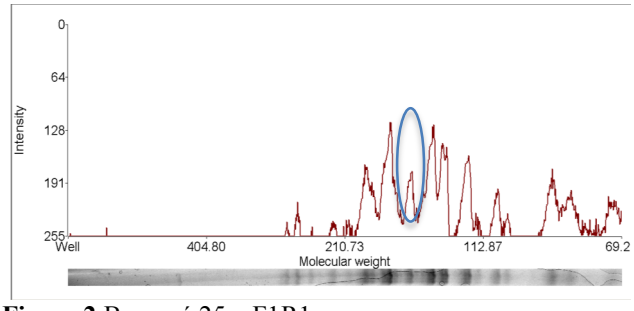

Figure 2 Barevná 25 – F1R1.

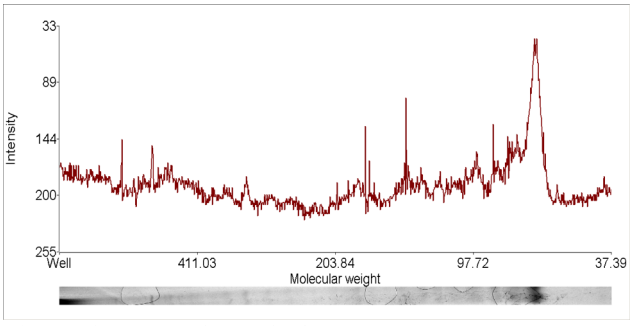

Figure 6 Barevná 25 – F2R2.

Lun2 revers provide detection of lunasin gene fragment with length 67 bp. This primer pair combination was suitable for detection of lunasin gene fragment in all genotype (Figure 5 and Figure 6).

We focused on confirmation of **Dinelli et al., (2014)** results in our research. **Dinelli et al., (2014)** monitored, that although there was positive presence of lunasin peptide in wheat proteome analysis, no gene coding this peptide was detected. However, **Jeong et al., (2009)** and **Maldonado-Cervantes et al., (2010)** observed lunasin peptide in proteome analysis and postulated also presence of gene coding this peptide.

Our results showed presence of gene coding lunasin peptide, but are in controversy with **Dinelli et al., (2014)** results. These lunasin gene detection findings require analysis of wheat proteome for detection of lunasin peptide to confirm expression of monitored DNA fragment.

Nakurte et al., (2012) and **Mitchell et al., (2013)** observed presence of lunasin peptide in cereals and their results indicated importance of mass spectrometry in cereal proteome analysis to confirm lunasin gene detection.

Presence of lunasin in triticale (X *Triticosecale* Wittmack) confirmed by **Nakurte et al., (2012)** indicated, that triticale is the most lunasin-rich cereal. The greatest lunasin content was 6.46 $mg.g^{-1}$ in the grain of triticale genotype 0002-26. In comparison, the highest lunasin content in rye variety Dankovske Diament was 1.5 $mg.g^{-1}$ of grain and the highest lunasin content in the winter wheat variety Fredis was 0.23 $mg.g^{-1}$ of grain. They conclude that triticale can play significant role as functional food, with great potential for the use of triticale products in human and animal diets.

Results of **Nakurte et al., (2012)**, which detected lunasin peptide in wheat and triticale corresponds to our observation about presence of lunasin gene in colour wheat genotype.

Jeong et al., (2009) focused their research on identification of lunasin peptide in rye (*Secale cereale* L.) cultivars. Lunasin was present in 15 out of 21 cultivars of analyzed rye cultivars. Lunasin present in rye crude protein preparation was stable to pepsin and pancreatin in *in vitro* digestion. They concluded that lunasin in rye is bioavailable and that consumption of rye may play an important role of cancer prevention in rye consuming population. Wheat is close relative to rye and therefore is possibility of wheat utilization in cancer prevention. Our results indicate presence of lunasin gene in wheat. Although lunasin peptide presence in wheat is contradictory, observation obtained by **Nakurte et al., (2012)** and **Jeong et al., (2007)** are in agreement with our observations.

Lunasin peptide detection in oat genotypes (*Avena sativa* L.) was performed by **Nakurte et al., (2013)**. Lunasin was detected using LC-MS/MS analysis. They observed genotype-related fluctuations in the lunasin content. The highest lunasin level was 0.197 $mg.g^{-1}$ of grain. There was also no correlation between lunasin and protein content, but genotype-dependent variations of the lunasin content was demonstrated during different years. Therefore, is very important to study influence of farming system, crop management and climate conditions on lunasin content in cereals as well as clarifying if consumption of lunasin-containing foods plays as important role in cancer and cardiovascular disease prevention.

Jeong et al., (2010) also elucidated role of cereals in cancer prevention. They reported the prevalence; bioavailability and bioactivity of lunasin from barley.

The liver and kidney of rats were fed with lunasin-enriched barley and inhibits the activities of histone acetyl transferases.

These findins and our results indicated that lunasin is prevalent in cereals and is bioavailable and bioactive. Consuption of cereals could play an important role of cancer prevention in cereal-consuming populations.

Recombinant production of the therapeutic peptide lunasin was widely studied by **Kyle at al., (2012)**. They used a pET28 vector to express cellulose binding domain (CBD)-lunasin fusion with a hexahistidine tag and Tobacco Etch Virus protease site, to allow protease-mediated release of native lunasin. The use of CBD as a fusion partner gave high protein yields by autoinduction, with lunasin release by TEV protease cleavage. This approach could provide a potentially valuable route for production of this therapeutic peptide.

CONCLUSION

Utilization of 2 sets of primer pair in 4 combinations showed suitability of F1R1, F2R1 and F2R2 primer pair combination for detection of lunasin gene. Identification of lunasin gene may be used for chromosome site identification and genetic manipulation with promotor to enhance gene activity and production of desirable level of peptide.

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Contact address:

Milan Chňapek, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: Milan.Chnapek@uniag.sk.

Dušan Siman, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xsiman@uniag.sk.

Zdenka Gálová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: Zdenka.Galova@uniag.sk.

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EFFECT OF THE ADDITION OF HYDROCOLLOIDS ON THE RHEOLOGICAL AND BAKING PROPERTIES OF THE PRODUCTS WITH ADDED SPELT FLOUR (*TRITICUM SPELTA* L.)

Tatiana Bojňanská, Jana Šmitalová, Alena Vollmannová

ABSTRACT

The paper presents the results of the evaluation of the effect of additives on the rheological properties of composite flour made of wheat flour in the amount of 70% and spelt flour at 30%. As additives guar gum (0.5% by weight of flour) and xanthan gum (0.16% by weight of flour) were used. Properties of produced control dough and doughs with hydrocolloids were evaluated by means of rheological appliances by Farinograph, Extenzograph, Amylograph and Rheofermentometer. Based on the observed results it can be concluded that the addition of xanthan gum has a positive effect on increasing of farinographic water absorption capacity, extension of dough development time and dough stability and generally positively affected farinographic properties. The addition of guar gum has improved especially extensographic properties as extensographic energy and extensographic resistance. Based on amylographic evaluation of control doughs and doughs with additives it can be stated that in the dough with guar gum the amylographic maximum has slightly increased. Hydrocolloid guar gum contributed to an increased retention capacity of dough observed. Based on our measurements we can indicate that addition of guar and xanthan gum contributed to an increased rheological quality of doughs prepared with addition of flour from spelt wheat. With reference to the baking experiment it was found that the use of hydrocolloids has a positive effect on the improvement of the baking properties, in particular larger volume, specific volume, and the volume yield of the dough with the addition of guar and xanthan gum compared to the control. Our results showed that aditives significantly influenced rheological qualities of dough and a baking quality of products. These findings thus allow optimizing the recipe in order to increase the technological quality of leavened bakery products.

Keywords: hydrocolloids; guar gum; xanthan gum; rheological properties; frozen dough

INTRODUCTION

Spelt wheat (*Triticum spelta* L.), family (Poaceae) is like common wheat (*Triticum aestivum* L.) classified as hexaploid wheat with 42 chromosomes and a six-rowed ear. Contrary to the common wheat spelt wheat has the husk that protects the grain against pests, insects and microbial contamination **(Krkošková et al., 2011; Filipčev et al., 2014)**. Spelt wheat can be described as old European cultural wheat that is currently grown in Western Europe, in Austria, Germany, Belgium, Switzerland and northern Spain. Its popularity is increasing due to the lower cultivation demands, and it is also suitable to be grown in foothill areas with poor soil and excess rainfall. It is used in various forms - such as grains, also for production of pasta, crackers, bread and beer **(Bojňanská et al., 2002)**.

The dry matter of common wheat grain is composed of saccharides in an amount of about 70%, with the most important part of the saccharides being starch at 60% – 65%, which consists of amylose (26% – 28%) and amylopectin (72% – 74%). The proportion of oligosaccharides of the total saccharides content is about 2% to 3%. The amount of total dietary fibre in grains varies from 9% to 12%, of which about 2% are made up of soluble fibre and the rest being insoluble fibre **(Feillet, 2000; Pruska-Kedzior et al., 2008; Escarnost et al., 2012)**.

The seed of spelt wheat contains about 15% of protein and the albumin portion is 13% of the total protein content, 3% is of globulins, 40% of prolamins, and 45% glutelins **(Krkošková et al., 2005; Pruska-Kedzior et al., 2008)**. It was found that the digestibility of spelt proteins is better in comparison to wheat protein, but the difference is minimal **(Ranhotra et al., 1995)**. The amount of essential amino acids of the total amino acids in common wheat and spelt wheat differs only marginally, and this difference is not statistically significant suggesting a very similar protein quality **(Grela, 1996; Abdel-Aal et Hucl, 2002). Bojňanská et al. (2002)** indicated that the amount of gluten varies in the range from 30% to 52%.

The grain of spelt wheat contains approximately 3% of fat, predominantly located in the embryo, aleurone, and at the lesser degree in endosperm (about 1.5% of the total lipids) **(Delcour et Hoseney, 2010)**. Fatty acids are represented mainly by linoleic acid (60%) and palmitic acid (17%), and these are lower amounts compared to their content in common wheat. Another major fatty acid is

oleic acid (18% in spelt wheat which is 6% more than in common wheat) **(Escarnot et al., 2012)**.

Spelt wheat is similarly to the common wheat a source of B group vitamins with approximately 1.5% concentrated mainly in germ. **Ranhort et al., (1995)** found out comparable amounts of thiamine and riboflavin in the grains of spelt wheat and common wheat. According to findings by **Bojňanská et al., (2002)** the amount of minerals varies between 1.8% and 2.3% depending on the variety and the year. The similar results reported also **Ranhort et al., (1995)** and **Ruibal-Mendiet et al., (2005)**.

Based on these findings it can be concluded that spelt wheat is therefore technologically and nutritionally suitable raw material for the use in bakery products affecting also their sensory characteristics.

Hydrocolloids are high molecular and hydrophilic biopolymers performing several functions in the food industry. The most significant features are their ability to control the rheological properties and texture of food. In the baking industry they are added in particular to stabilize emulsions, suspensions and foams and to improve the processing properties. Among other properties they have the ability to inhibit starch retrogradation, retain moisture, improve the overall structure, and slow down aging of products. They can be used as a substitute for fat and eggs **(Arozarena et al., 2001; Collar et al., 1999; García - Ochoa et al., 2000; Příhoda et al., 2003; Kohajdová et al., 2008 a, b; Magala et al., 2011; Rodge et al., 2012; Šedivý et al., 2013; Eduardo et al. 2014; Qiu et al., 2015)**. An important positive feature is that the use of hydrocolloids even in small quantities (less than 1% by weight of flour), has a significant impact on enhanced ability of dough to bind water, increase the volume of products, slow retrogradation of starch and thus extend the shelf life of bakery products **(Collar et al., 1999; Khan et al., 2007; Škara et al., 2013)**.

Guar gum is defined as the ground endosperm of the seeds of natural strains of the guar plant, *Cyamopsis tetragonolobus* (L.) Taub. (family *Leguminosae*) and it consists of hydrocolloidal polysaccharides of high molecular weight composed of galactopyranose and mannopyranose combined through glycosidic linkages, which may be described chemically as galactomannan **(FC SR, Decree 1)**. The plant grows mostly in India and Pakistan, but since 1950 it has also been grown in Texas and Arkansas as a commercial commodity **(Achayuthakan et Suphantharika, 2008). Kohajdová et al., (2008a)** reported that bread with addition of guar gum had after baking better baking properties, such as a higher volume and improved sensory quality, particularly more attractive appearance and aroma, softer crumb and firmer crust.

Xanthan gum is an exocellular polysaccharide of microbial origin that is produced by aerobic fermentation of sugar by the bacterium *Xanthomonas campestris*. China is its largest producer in the world **(Hojerová et al., 2005; Achayuthakan et Suphantharika, 2008; FC SR Decree 2; Tao et al., 2012)**. The main chain of xanthan gum is formed by β-D- (1,4) glucose units and the side chains are composed by residues of D-glucuronic acid and two mannose moieties D **(Velíšek, 2002)**. Xanthan gum has an impact on strengthening links between flour proteins and thereby firming the dough structure. Baked products with a xanthan gum have larger volume, and optimum shape in comparison to products without this additive. During dough preparation xanthan gum binds to the starch and by that slows down its retrogradation, which is to be said by experts one of the main reasons of products staling **(Collar et al., 1999; Arozarena et al., 2001; Rosell et al., 2001; Gimeno et al., 2004; Ashwini et al., 2009)**.

MATERIAL AND METHODOLOGY

In the study, the effect of the addition of hydrocolloids of xanthan and guar gum to composite flour from spelt wheat on the rheological properties of dough was addressed. The results were compared with objective baking properties of baked products.

To prepare control loaves wheat flour T 650 was used. The second group of loaves was made from composite flour, based on wheat flour T 650 in an amount of 70% (Mlyn Pohronský Ruskov a.s., Hlavná 76, 935 62 Pohronský Ruskov, Slovakia) with an addition of spelt wholemeal flour at 30% (company J. Vince s.r.o., 925 91 Kráľová nad Váhom 320, Slovak Republic). According to recipe fresh compressed yeast was used (Trenčianske droždie, Old Herold Hefe, s.r.o., Bratislavská 36, 911 05 Trenčín, SR). In wheat and composite flour, the moisture was determined (%) **(ICC Standards No. 110/1 (1976))**, as well as content of crude protein (%) **(ICC Standard No. 105/2, (1994))** and content of ash (%) **(ICC Standard No. 104/1 (1990))**. Rheological measurements of prepared composite flour and wheat flour were made by means of *Farinograph-E*, Brabender OhG, Duisburg, Germany **(ICC Standrd 115/1 (1992), AACC Method 54-21 (1995))**. Based on these measurements following characteristics were determined: farinographic flour water absorption capacity (%), dough development time (min), dough stability (min), farinographic quality number. By means of *Extensograph-E*, Brabender OhG, Duisburg, Germany **(ICC - Standard 114/1 (1992), AACC Method 54-10 (1995))** extensographic energy (cm^2), extensographic tensibility (mm) and extensographic maximum (EU) were determined. By means of *Amylographe-E,* Brabender OhG, Duisburg, Germany **(ICC-Standard 126/1, AACC Method 22-10 (1995))** the initial gelatinization temperature (°C), the maximum gelatinization temperature (°C) and the amylographic maximum (AU) were determined.

The recipe of dough, which was tested in rheofermentometer consisted of 250 g of composite flour, fresh yeast in an amount of 2.8%, which was dispersed in water and added to flour during dough preparation in farinograph. After the first minute salt was added in an amount of 2%, and mixing continued for six minutes. The volume of added water depended on flour water absorption capacity to produce dough of optimal consistency. 315 g of dough was then inserted into Rheofermentometer Rheo F4 (Tripette & Renaud Chopine, Villeneuve-la-Garenne, France) **(AACC Method 89-01.01)** in order to determine during a three-hour test *total volume* (cm^3), *volume of* CO_2 *lost* (cm^3), *retention volume* (cm^3), *retention coefficient R* (%) (ratio of the volume that was detained in the dough to the total volume of produced CO_2).

Experimental loaves were prepared from a mixture of flour (350 g of wheat and 150 g of soy flour), sucrose (5 g), salt (9 g), yeast (20 g) and water addition based on

farinographic water absorption capacity. Bread experiment was carried out without the use of enzyme-active substances and other improvement agents. The development of dough took place in a laboratory mixer Diosna SP 12. After that the dough was elaborated and formed into loaves that stayed yeasted in a yeasting room for 20 minutes at temperature of 30 °C and were baked in an oven Miwe Condo at 240 °C with steaming (baking time 20 min). The baked loaves were evaluated by objective methods and the volume of products (cm^3), a specific volume of products ($cm^3.100g^{-1}$), volume yield ($cm^3.100g^{-1}$ flour), cambering (the ratio between height and width) were determined.

RESULTS AND DISCUSSION

The function of hydrocolloids lies in their ability to modify dough and improve its rheological properties, thus contribute to the maintenance of its quality **(Yaseen et al., 2010)**. Another important function of the gums is their ability to enhance the absorption of water in the baked products **(Mandala et al., 2008)** resulting in effecting the rheological properties of the dough and the extended shelf life of the products as they prevent migration of water during the staling of bread **(Fanta et al., 1996; Collar et al., 1999, Kohajdová et al., 2009)**.

Table 1 shows the effect of the addition of guar and xanthan gum on farinograph characteristics of composite flour. The addition of 0.5% of guar gum reduced farinograph water absorption capacity and development of dough compared to samples without this additive. **Rodge et al., (2012)** found that the addition of guar gum worked on shortening the time of dough development and extending dough stability. The application of 0.16% of xanthan gum we have implemented within our experiments did not significantly affect the water absorption capacity of the mixture, but contributed to the prolongation of the dough development. We have found that the addition of guar and xanthan gum had a positive effect on the stability of the dough extension and on the farinographic quality number, which was significantly higher (by 30) than in the control sample. The fact that the xanthan gum in the recipe contributes to increase of farinographic water absorption capacity, extension of dough development and its stability was also confirmed by **Rosell et al., (2001)** and **Davari-Ketilateh et al., (2013)**.

Time of dough development depends on the amount and quality of gluten, granularity of flour and the level of grinding and is determined primarily by the process of gluten hydration **(Dodok et Szemes, 1998)**. Strong flour is according **Muchová (2007)** defined as one that will during processing bind large amount of water, will reach optimum rheological properties slowly and retains them for a long time.

The results presented in Figure 1, which expresses amylographic evaluation of samples, show that the addition of guar gum and xanthan gum in all observed samples caused a slight decrease in the initial gelatinization temperature in comparison to a control sample without the gums. Hydrocolloids according to **Mandala (2012)** impact the acceleration of gelatinization and slow down the retrogradation and our results are consistent with these findings. We also noted that the addition of xanthan gum did not affect the maximum temperature of gelatinization.

The optimum value of amylographic maximum should vary in wheat flours between 300 AU and 650 AU, which is a sign of optimum amylase activity and products from these flours are identified as technologically very good **(Dodok et Szemes, 1998; Šedivý et al., 2013)**. Very high curve values of amylographic maximum above 800 AU predict flours with low amylase activity, and thus we can assume that the products from them would have very dry crumb with potential cracks and bland taste.

The results presented in Figure 2 describing extensographic parameters of composite flour with the addition of spelt flour and hydrocolloids show that with increasing time of maturing the value of extensographic energy has increased. Optimal values according to **Šedivý et al., (2013)** are between 90 cm^2 and 300 cm^2. After thirty minutes of maturing the highest value of extensographic energy was in the sample with guar gum at 94 cm^2, then the sample with xanthan gum at 84 cm^2 and lowest in the control sample without hydrocolloids at 80 cm^2. After thirty minutes of maturing the highest value of extensographic energy was in the sample with guar gum at 94 cm^2, then the sample with xanthan gum at 84 cm^2 and lowest in the control sample without hydrocolloids at 80 cm^2. Based on our findings it can be concluded that the best value of extensographic energy was in samples with guar gum.

Table 1 Farinographic parameters of composite flour (70% T 650 and 30% spelt wheat flour with addition of hydrocolloids).

Flour sample	farinographic flour water absorption capacity %	dough development time min	dough stability min	farinographic quality number
70%T650 + 30% T. spelt flour control	62.0	5.3	7.7	101
70%T650 + 30% T. spelt flour +0,5% guar gum	51.0	3.8	9.6	131
70%T650 + 30% T. spelt flour +0,16% xanthan gum	62.1	7.2	10.6	131

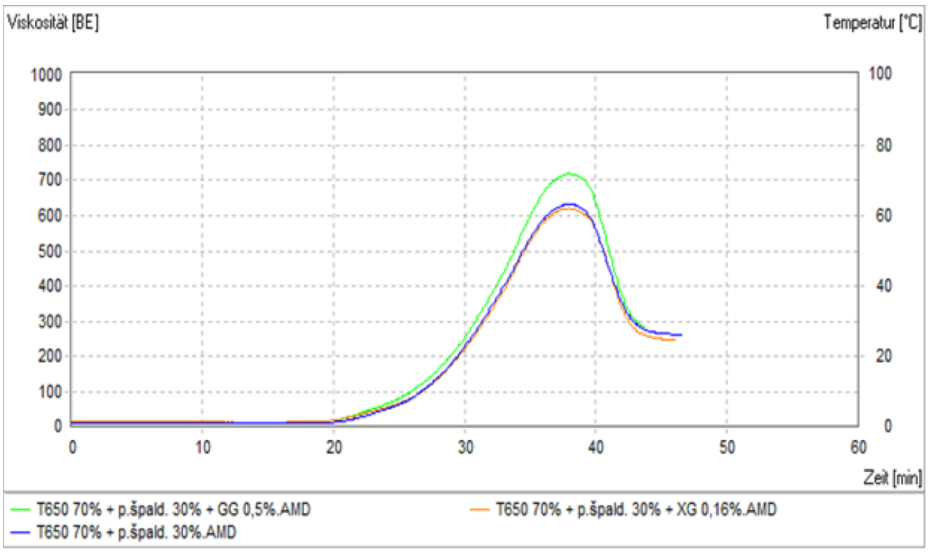


Figure 1 Amylographic parameters of composite flour with addition of spelt wheat and hydrocolloids.

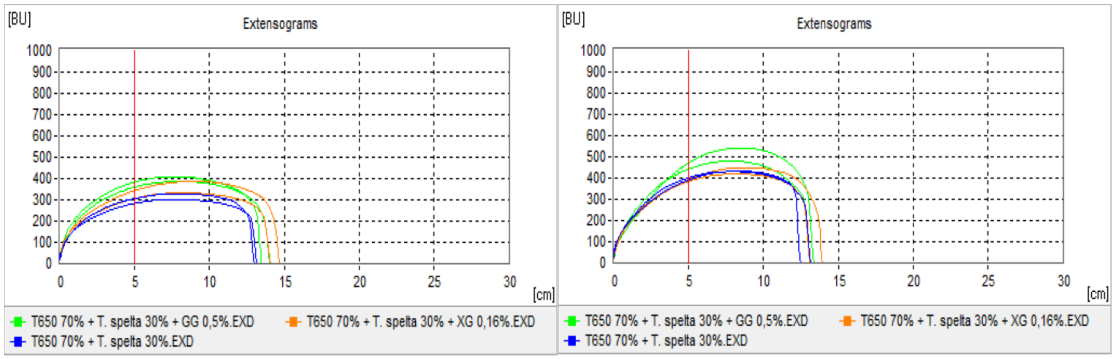
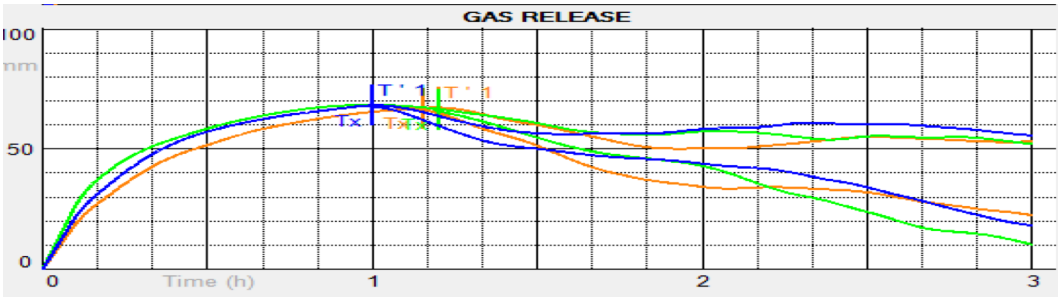


Figure 2 Extensographic parameters of composite flour with addition of spelt wheat and hydrocolloids after 15 and 30 minutes of maturing.



Picture 3 Reofermentometric parameters of composite flour with addition:

Spelt wheat ——, Guar gum ——, Xantan gum ——.

Higher value of extensographic resistance is a sign of strong gluten of good quality, which is mechanically resistant during manipulation with dough **(Příhoda et al., 2003)**. We found that with increasing time of maturing the dough resistance and extensographic maximum have increased which can be considered a positive result.

A comparison of the effect of hydrocolloids on extensographic maximum and dough resistance shows that guar gum had a significant impact on increasing of these values and it predicts products with a good volume.

For leavened bakery products the high elongation of dough is not desirable **(Dodok et Szemes, 1998)**. Optimal values for wheat flour are according to **Šedivý et al., (2013)** from 120 mm to 200 mm (±8 mm). With increasing of dough maturing time there was a slight decrease of elongation spotted in a control sample as well as in dough sample with the addition of hydrocolloids. In dough from composite flour with the addition of guar gum after fifteen and thirty minutes of maturing the elongation has increased in comparison to control.

Table 2 Results of bakery experiment with composite flour of 70% T 650 and 30% flour from spelt wheat with addition of hydrocolloids.

	70% T650+30% T. spelt flour control	*70% T650+30% T. spelt flour + 0,5% guar gum*	*70% T650+30% T. spelt flour + 0,16% xanthan gum*
Loaf volume cm^3	200.0	212.5	212.5
Specific loaf volume (cm^3.100g)	226.7	240.0	242.5
Volume recovery (cm^3.100g flour)	320.0	340.0	340.0
Recovery of product (%)	141.1	141.5	140.4
Baking loss %	14.6	13.8	14.6

Similar results describing that the addition of guar gum influenced the increasing of elongation were published by **Ribotta et al., (2004)**. Addition of hydrocolloids increased dough resistance during manipulation, which is from technological view point of significant importance.

Reofermentometric evaluation, the results of which are presented in Figure 3 showed that the addition of guar and xanthan gum does not significantly affect dough retention capacity. It can be regarded as positive that in dough with the addition of xanthan gum the breaking point occurred about nine minutes later, and in dough with guar gum even 12 minutes later than in the control dough without additives. Similar results in which the guar gum demonstrated itself as an additive delaying the time at which a break point occurs, and which increases retention were also found in the previous research with doughs made from flour of common wheat.

Results of experimental baking presented in Table 2 show that guar and xanthan gum positively influenced the total volume of products, the volume yield and specific volume compared with products without hydrocolloids. Similar results regarding the positive impact of hydrocolloids on baking properties were published by **Kohajdová et al., (2008a)**. Table 2 also shows that the addition of hydrocolloids did not influence the baking yield and baking loss during baking, since they were in all variants at a comparable level.

CONCLUSION

Summarizing and comparing the results of rheological evaluation of composite flour with the addition of spelt flour showed that the addition of guar gum extended dough stability time and increased dough farinograph number of quality compared to the control.

Significant improvement of the farinograph characteristics was observed in dough with the addition of xanthan gum, and this finding is very positive. Addition of guar gum in an amount of 0.5% positively influenced and improved extensographic properties of composite flour. Guar gum contributed to the increase of amylographic maximum, but in this case it can be considered as negative because an excessive increase of this value predicts reduced sensory quality of baked products.

The use of xanthan gum, in contrast to guar gum optimised amylographic properties, and thus an excellent quality of the crumb can be predicted. Evaluation of composite flours in reofermentometer showed that using of guar gum may slightly increase dough retention capacity, which is technologically positive finding, because maintaining of fermentation gas is a key factor to ensure sufficient volume of bakery products. Based on the evaluation of baking experiment it can be concluded that the loaves containing added guar and xanthan gum achieved compared to a sample without additives better indicators of bakery quality, which was reflected mainly by higher volume, specific volume and volume yield.

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Contact address:

doc. Ing. Tatiana Bojňanská, CSc. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storage and Processing of Plant Products, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, Tel. 037 6414 703, E-mail: tatiana.bojnanska@uniag.sk.

Ing. Jana Šmitalová, PhD. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storage and Processing of Plant Products, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: jana.smitalova@gmail.com.

prof. RNDr. Alena Vollmannová, PhD. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, Tel. 037 6414 374, E-mail: alena.vollmannova@uniag.sk.

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EFFECT OF DIFFERENT PHYTOGENIC ADDITIVES ON OXIDATION STABILITY OF CHICKEN MEAT

Marek Bobko, Peter Haščík, Martin Mellen, Alica Bobková, Jana Tkáčová, Peter Czako, Adriana Pavelková, Lenka Trembecká

ABSTRACT

The aim of the study was to evaluate the oxidative stability (TBARS method) of breast and thigh muscle after application of feed mixtures enriched by phytogenic additives. The experiment started with 150 pieces one-day-old chicks of Cobb 500 hybrid combination. They were divided into one control (C) and two experimental groups (1st EG and 2nd EG). Each group included 50 chicks. In experimental groups, feed additives were applied as followed: 100 mg.kg^{-1} Agolin Poultry (in the 1st EG) and 500 mg.kg^{-1} Agolin Tannin Plus (in the 2nd EG). Experimental broiler chickens were fed during 42 days by *ad libitum.* Chicken meat samples of breast and thigh muscle were analysed in the 1st day, 1st, 2nd, 3rd, 4th, 5th and 6th month of storage in frozen storage at -18 °C. We recorded positive influence on chicken meat oxidative stability in all experimental groups with application of phytogenic feed additives. Obtained results showed that applied phytogenic additives had positive influence on oxidative stability of breast and thigh muscles. At the end of frozen storage (in 6th month), we found higher malondialdehyde (MDA) values and lower oxidative stability (p <0.05) of breast muscle in control group (0.167 mg.kg^{-1}) compared to experimental groups (from 0.150 mg.kg^{-1} in 1. EG to 0.155 mg.kg^{-1} in 2. EG). In the thigh muscle, we found similar tendency of oxidative changes as in the breast muscle. At the end of frozen storage (in the 6th month), MDA average values of thigh muscle were higher (p <0.05) in control group (0.181 mg.kg^{-1}) compared to experimental groups (1. EG 0.164 mg.kg^{-1} and 2. EG 0.169 mg.kg^{-1}). Significant differences (p <0.05) between the control and experimental groups were found from the 5th month of storage in thigh and breast muscle. Obtained results indicate positive influence of phytogenic additives applied in chicken nutrition, namely on stabilization of fatty substance to degradation processes.

Keywords: phytogenic additives; chicken meat; oxidative stability

INTRODUCTION

Phytogenic feed additives (PFA) are commonly defined as plant-derived compounds incorporated into diets to improve the productivity of livestock through amelioration of feed properties, promotion of the animal's production performance, and improving quality of food derived from those animals. Although this definition is driven by purpose of use, other terms are commonly used to classify the vast variety of phytogenic compounds, mainly with respect to origin and processing, such asherbs (flowering, nonwoody, and nonpersistent plants), spices (herbs with intensive smell or taste commonly added to human food), essential oils (volatile lipophilic compounds derived by cold expression or by steam or alcohol distillation), or oleoresins (extract derived by nonaqueous solvents). Within phytogenic feed additives, the content of active substance in products may vary widely, depending on the plant part used (e.g. seeds, leaf, root, or bark), harvesting season, and geographical origin. The technique for processing (e.g. cold expression, steam distillation, extraction with nonaqueous solvents, etc.) modifies the active substances and associated compounds within the final product **(Windisch et al., 2008; Jacela et al., 2010)**. This is class of feed additives is at present used to a great extent as alternatives to the antibiotic growth promoters in poultry and swine nutrition **(Wati et al., 2015)**.

Aromatic plants, also known as herbs and spices, have been used in the Middle East since approximately 5000 BC for their preservative and medical properties, in addition to enhancing the aroma and flavour of foods **(Chang, 2000)**. Their use continues undiminished today and according to the World Health Organization (WHO) nearly 80% of the planet population, especially in developing countries still depends on plant produced medicines for their healthcare **(Grubik-Fakim, 2006)**. Currently, there is an increasing interest in using herbs and spices in animal nutrition, in order to replace the use of antibiotics and ionophore anticoccidials, especially after the ban of antibiotics feed additives within the European Union countries in 2006 and discussions to restrict their use outside Europe **(Greathead, 2003; Windisch et al., 2008; Hashemi and Davoodi, 2010; Yitbarek, 2015)**.

The nutritional properties of poultry meat are highly valued; it is a meat with low fat content and less saturated fatty acid than the most ruminant tissues **(Starčevič et al., 2015)**. At average broilers have from 3.5 to 5.0% of fatty tissuses. Poultry fat contain higher amount of polyunsatured fatty acids than fatty tissues other slaughtered animals. Exactly, polyunsatured fatty acids are the most sensible fractions to oxidation processes. Lipid oxidation oxidation in meat is one of the reasons for quality degradation during storage. This process is associated with the presence of free radicals that lead to

the production of aldehydes responsible for the development on rancid flavours and changes in the colour of meat **(Fasseas et al., 2007)**. The rate of oxidation increases in result of the following: (1) high intake of oxidized lipids and prooxidants; (2) deterioration of sensitive polyunsaturated fatty acids (polyunsatured fatty acids); and (3) low intake of antioxidative nutrients. In muscle foods, oxidative reactions continue postmortem and are a leading cause of quality deterioration during processing and storage. With a relatively high proportion of PUFA, poultry meat is more susceptible to oxidative processes, specifically lipid oxidation, than beef or pork **(Smet et al., 2008)**. Lipid oxidation is a major cause of meat quality deterioration which lowers the functional, sensory and nutritive values of meat and neat products; and therefore, consumer's acceptability **(Bou et al., 2004)**. Oxidative stability of poultry meat is influenced not only by bird genotype but also feeding, rearing practices and the degree of muscle tissue damages during preslaughter, e.g. physical damage, early post-mortem conditions, pH and carcass temperature **(Morissey et al., 1998; Zamora and Hildago, 2001)**. These factors could by manipulated by supplementing the animal diet with phytogenic compounds such as different essential oils and polyphenols to improve animal productivity and the quality of food derived from those animals **(Lee et al., 2003; Jang et al., 2004; Okuda, 2005)**.

Phytogenic feed additives are often applied into the feed mixtures, because they improve the taste and odour of feed and subsequently, body weight gain and feed intake are increased and feed conversion is improved, too **(Angelovičová et al., 2010)**. Phytogenic feed additives enhance productivity through the improvement of digestibility, nutrient absorption and elimination of pathogens residents in the animal gut **(Athanasiadou et al., 2007)**. Digestive stimulation by phytogenic additives is achieved through stimulation of salvia secretion, liver, pancreas and intestine enzymes activities, intestine function and morphohistology and metabolism **(Perič et al., 2010)**. Antioxidant effects of plant extracts may be used to slow or prevent the fat oxidation in food products **(Rababah et al., 2004)**. Application of oils and plant extracts in poultry nutrition is important for health state of animals and animal performance as well as for oxidative stability of produced meat **(Frankič et al., 2009)**. Antioxidant activity of plants and their extracts is directly correlated with phenols content **(Chrpová et al., 2010)**. Several studies about phytogenic additives in poultry nutrition were published, mainly about application of aromatic herbs like a cloves **(Isabel and Santos, 2009)**, a rosemary **(Šperňáková et al., 2007)**, a cinnamon **(Ciftci et al., 2010)**, an anise **(Al-Kassie, 2008)**, an oregano **(Fiková et al., 2009)** and a salvia **(Hernandez et al., 2004)**.

The aim of the experiment was to determine the oxidative stability in the most valuable parts of chicken carcasses (Cobb 500 hybrid combination) during the frozen storage (6 months) after application of phytogenic feed additives Agolin Poultry, Agolin Tannin Plus, in their diet.

MATERIAL AND METHODOLOGY

Animals and diets

The experiment was undertaken in poultry test station Zamostie Company. The experiment started with 150 pieces of one-day-old hybrid chicks Cobb 500, which were divided into 3 groups (n = 50): control (C) and 2 experimental groups (1st EG and 2nd EG).

Experimental broiler chickens were fed during 42 days by *ad libitum* system with feed mixtures: BR1 starter feed mixture (until the 10th day of age), BR2 growth feed mixture (from 11th to 20th day of age), BR3 growth feed mixture (from 21st to 35th day of age) and BR4 final feed mixture (from 36th to 42nd day of age). Feed mixtures were produced with coccidiostats in powder form.

Nutritional value (Table 1) of feed mixture was 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 a dose of 100 $mg.kg^{-1}$ (1st EG); Agolin Tannin Plus at a dose of 500 $mg.kg^{-1}$ (2nd EG).

Sample analysis

At the end of feeding (day 42th) from each group were selected 10 pieces of chicken for slaughter analysis. Slaughtering and cutting of chickens were undertaken in the Department of animal products evaluation and processing. To determine changes in lipid degradation (determination of thiobarbiturates numbers, TBA) the samples of chickens were boned and thigh and breast muscle packed into polyethylene bags and stored for 6 months at -18 °C.

TBARS analysis

TBA value expressed in number of malondialdehyde (MDA) was measured in the process of first storage day of 1st, 2nd, 3rd, 4th, 5th and 6th months. TBA number was determined according to **Marcinčák et al., (2006)**. Absorbance of samples was measured at a wavelength of 532 nm on UV-VIS spectrophotometer T80 (PG Limeted Instruments, UK). Results were calculated as the amount of MDA in 1 kg of sample.

RESULTS AND DISCUSSION

The lipids in poultry exhibit a higher degree of unsaturation compared with red meat, because of a relatively high content of phospholipids. The degree of unsaturation of phospholipids in subcellular membranes is an important factor in the determination of oxidative stability of meats. The oxidative potential increases as the degree of unsaturation of lipids in meat increases **(Coetzee and Hoffman, 2001)**. The oxidation of lipids is influenced by the addition of antioxidant substances. The practical application of antioxidants can be difficult from the point of view of hygiene and technology. It is much better when natural antioxidants are incorporated in feed mixes **(Kušev et al., 1996)**.

Table 1 Composition of the diets.

Ingredients (%)	Starter (1st to 10th day of age)	Grower I (11th to 20th day of age)	Grower II (21st to 35th day of age)	Finisher (36th to 42nd day of age)
Maize	46.33	48.50	50.05	50.91
Wheat	14.00	15.00	15.00	15.00
Soybean meal (45% CP[1])	30.00	26.60	28.00	26.70
Fish meal (72% CP[1])	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	-
Analysed 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
$^{2}ME_N$ (MJ kg^{-1})	12.30	12.75	13.15	13.15

Legend: ^{1}CP – Crude protein, $^{2}ME_N$ – Metabolizable energy.

Table 2 Effect of frozen storage (-18 ºC) on the concentration of MDA (mg.kg^{-1}) in breast muscle (mean ±*SD*).

Time of storage	Group		
	Control	1. EG	2. EG
Day – 1	0.108 ±0.009^{a}	0.101 ±0.010^{a}	0.098 ±0.008^{a}
Month – 1	0.119 ±0.009^{a}	0.117 ±0.0009^{a}	0.117 ±0.009^{a}
Month – 2	0.127 ±0.009^{a}	0.124 ±0.010^{a}	0.126 ±0.009^{a}
Month – 3	0.137 ±0.015^{a}	0.131 ±0.006^{a}	0.131 ±0.008^{a}
Month – 4	0.143 ±0.006^{a}	0.139 ±0.012^{ab}	0.137 ±0.010^{b}
Month – 5	0.155 ±0.006^{a}	0.144 ±0.006^{ab}	0.147 ±0.013^{b}
Month – 6	0.167 ±0.010^{a}	0.150 ±0.018^{b}	0.155 ±0.011^{b}

The results of the oxidation stability determined in breast muscle of chickens COBB 500 during 6 months storage at -18 °C are shown in Table 2. Immediately after slaughtering and processing of poultry samples we recorded low values of MDA. Obtained results indicate that addition of antioxidants had effect on reducing of oxidation processes in meat. Process of production of meat products (cutting, grinding, and mixing) causes degradation of muscle membrane system and has a strong influence on oxidation of intracellular fat, primarly phospolipids **(Bystrický and Dičáková, 1998)**. During freeze storage of the breast muscles (6 months) were detected increased content of MDA in comparison to the first day of storage. During whole period of freeze storage were higher values of MDA determined in control group compare to experimental groups. The higher average MDA value determined in breast muscles of broiler chicken hybrid combination COBB 500 was in samples of control group (0.167 mg.kg^{-1}) compared to experimental groups E2 (0.155 mg.kg^{-1}) and E1 (0.150 mg.kg^{-1}) after 6-month of freezing storage. Significantly higher values of MDA were determined in control group compare to experimental group from fifth month to the end of storage. Reached results oxidation stability breast muscle during freeze storage are in accordance with **Ahadi et al., (2010)**; **Marcinčák et al., (2010)**.

Table 3 Effect of frozen storage (-18 ºC) on the concentration of MDA (mg.kg^{-1}) in thigh muscle (mean ±*SD*).

Time of storage	Group		
	Control	1.EG	2.EG
Day – 1	0.129 ±0.013^{a}	0.125 ±0.011^{a}	0.120 ±0.008^{a}
Month – 1	0.132 ±0.009^{a}	0.129 ±0.005^{a}	0.128 ±0.009^{a}
Month – 2	0.139 ±0.004^{a}	0.135 ±0.005^{a}	0.136 ±0.010^{a}
Month – 3	0.148 ±0.011^{a}	0.143 ±0.011^{a}	0.146 ±0.015^{a}
Month – 4	0.160 ±0.012^{a}	0.151 ±0.012^{ab}	0.156 ±0.015^{b}
Month – 5	0.171 ±0.011^{a}	0.159 ±0.014^{ab}	0.163 ±0.008^{b}
Month – 6	0.181 ±0.021^{a}	0.164 ±0.013^{b}	0.169 ±0.009^{b}

Trend of thigh muscle oxidation stability of chicken hybrid combination COBB 500 was during 6 months of freeze storage similar than in breast muscle. The results of the oxidation stability determined in thigh muscle of chickens COBB 500 during 6 months storage at -18 °C are shown in Table 3. The higher average MDA value determined in thigh muscles was in samples of control group (0.181 mg.kg^{-1}) compared to experimental groups E1 (0.164 mg.kg^{-1}) and E2 (0.169 mg.kg^{-1}) after 6-month of frozen storage. Significantly higher values of MDA were determined in control group compare to experimental groups from fifth month to the end of storage. Higher amount of MDA in thigh muscle compare to breast muscle is due to by higher amount of fat occurred in thigh muscle **Botsoglou et al., (2002)**.

Reached results of oxidation stability determined in chicken meat of hybrid combination COBB 500 after phytogenic additives addition in their diet are in accordance with **Imik et al., (2010)** and **Rahimi et al., (2011)**. The possibilities of using alternative feed supplements containing various antioxidant active substances for poultry which increase the oxidation stability of the meat during its period of freeze storage are showen in works of **Skřivan et al., (2010)**; **Karaalp and Genc (2013)**.

Botsoglou et al., (2007) reported that a higher concentration of antioxidants in poultry meat has the effect of reducing lipid oxidation, i.e. there is a reduction in MDA values during chilling and refrigeration storage, which was confirmed by our findings. Also **Samouru et al., (2007)** and **Ramos Avila et al., (2013)** state that the degradation pathways of fatty substances play one of the main causes of foods deterioration and unpleasant odours. This factor is also responsible for the loss of flavour, texture, appearance, nutritional value of food, increases the drop losses, pigment, polyunsaturated fatty acids, fat-soluble vitamins, reduces the quality of meat intended for human consumption and ultimately reduces its stability, shelf life and safety.

CONCLUSION

Results achieved in the experiment show that the addition of different phytogenic feed additives (Agolin Poultry and Agolin Tannin Plus) in feed mixture for broiler chickens had a significantly ($p \leq 0.05$) positive impact on the reduction of oxidative processes in the breast and thigh muscles during 6 months freeze storage at -18°C.

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Contact address:

Marek Bobko, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and *Processing* of *Animal* Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marek.bobko@uniag.sk.

Peter Haščík, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and *Processing* of *Animal* Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.hascik@uniag.sk.

Martin Mellen, Hydina Slovensko s.r.o., Nová Ľubovňa 505, 065 11 Nová Ľubovňa, Slovakia, E-mail: martin.mellen@gmail.com.

Alica Bobková, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: alica.bobkova@uniag.sk.

Jana Tkáčová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.tkacova@uniag.sk.

Peter Czako, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Plant Processing and Storage, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.czako@uniag.sk,

Adriana Pavelkova, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and *Processing* of *Animal* Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: adriana.pavelkova@uniag.sk.

Lenka Trembecká, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: lenka.trembecka@uniag.sk.

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IDENTIFICATION OF DIFFERENCES IN CHEMICAL COMPOSITION AMONG WHOLE STICK AND SLICED NITRAN SALAMIS TROUGH PRINCIPAL COMPONENT ANALYSIS

Tomáš Fekete, Marek Šnirc, Ľubomír Belej, Jozef Golian, Peter Zajác, Jozef Čapla

ABSTRACT

The subject of this work was to examine differences in chemical composition of sliced and whole stick Nitran salamis, purchased from various manufacturers. Nitran salamis are traditional dry fermented meat products of Slovak origin. Taking into account variations in raw materials, production process and potential adulteration, differences in chemical composition within one brand of salami from different manufacturers might be expected. Ten salamis were determined for basic chemical composition attributes and Principal Component Analysis was applied on data matrix to identify anomalous ones. It has been shown that six attributes, namely: protein without collagen of total protein, total protein, total meat, total fat, collagen of total protein and NaCl, were the most important for salamis as first two Principal Components together explained 70.16% of variance among them. Nitran D was found to be the most anomalous salami, as had the lowest value of protein without collagen of total protein (14.14% ±0.26%), total protein (17.42% ±0.44%), total meat (120.29% ±0.98%) and the highest one of total fat (50.85% ±0.95%), collagen of total protein (18.83% ±0.50%) and NaCl (9.55% ±1.93%), when compared to its whole stick variant Nitran C and other samples. In addition to collagen of total protein content, Nitran D together with Nitran A, F and H did not satisfied the legislatively determined criterion, which is ≤16%. This suggested that extra connective tissues were added to intermediate products, which resulted in high variability and inferior quality of final products. It is a common practice in the meat industry to increase the protein content or water binding properties of meat products.

Keywords: PCA; Nitran salami; quality; protein; collagen

INTRODUCTION

Salamis are dry fermented meat products that are popular across most of European countries **(Fabbri and Cevoli, 2015)**. Such countries or their geographic regions produce characteristic salamis trough traditional manufacturing processes. In brief, meat (pork and beef) and fat are minced and mixed with salt, curing agents (nitrate and nitrite), spices, herbs, sugar, starter cultures and other additives such as non-meat proteins **(Fongaro et al., 2015; Cevoli et al., 2014)**. The mixture is stuffed into natural or artificial casing and then subjected to fermentation and drying (ripening) stage **(Fongaro et al., 2015)**. During these phases, physical, chemical and microbiological transformations take place in salami **(Jerković et al., 2010; Martín-Sanchéz et al., 2011)**, gradually giving a product with characteristic colour, flavour, taste and texture **(Papavergou et al., 2012)**. The degree of changes and the final quality of salami depend on product formulation, the variations in raw meat used, the starter culture and processing conditions **(Marino et al., 2015; Van Schalkwyk et al., 2011, Zajác et al., 2015)**.

After ripening, when the desired characteristics are reached, the product can leave the ripening room and is ready to be placed in market **(Fongaro et al., 2015)**. However, the physical and biochemical activities inside the salamis are not stopped at this phase and proceed at a rate depending on several factors, mainly temperature. In particular, further water lost can be avoided by using of modified packaging atmosphere **(Tabanelli et al., 2013)**. Taking into account variations in raw materials, production process and potential adulteration, differences in chemical composition within one brand of salami from different manufacturers might be expected.

The subject of study was to identify the differences in chemical composition of traditional Slovak Nitran salamis in relation to the manufacturer and variant (i.e. either whole sticks or slices packaged in modified atmosphere) using Principal Component Analysis (PCA).

The PCA is multivariate statistical method used for the identification of the most important directions of variability in a multivariate data matrix and presenting the results graphically. This technique has already been used by **Bianchi et al., (2007)** who discriminated between the two kinds of Italian salamis according to profile of volatile compounds. **Herranz et al., (2008)** applied the PCA on the fatty acid profile in order to separate Milano-type salamis into different groups. **Van Schalkwyk et al., (2011)** performed PCA on chemical composition of salamis from game meat, in order to examine differences and consumer acceptability. **Corral et al., (2013)** used this technique to examine the relationship among reduction of salt content and textural parameters, chemical composition and physical properties of Italian salamis.

MATERIAL AND METHODOLOGY

Samples

Ten Nitran salamis from five different manufacturers were purchased from local supermarkets in Nitra, Slovakia. From each manufacturer, Nitran salami was purchased as whole stick and slices packaged in modified atmosphere. Salamis were labelled and assigned by codes according to variant (W = whole stick, S = slices) and manufacturer (number 1 – 5) (Table 1).

Table 1 Codes for salamis.

Salamilabel	Variant	Manufacturer
Nitran A	W	1
Nitran B	S	1
Nitran C	W	2
Nitran D	S	2
Nitran E	W	3
Nitran F	S	3
Nitran G	W	4
Nitran H	S	4
Nitran I	W	5
Nitran J	S	5

Chemical analysis

Analysis of chemical composition was accomplished at Department of Food Hygiene and Safety, SUA, Nitra, Slovakia. Determined attributes were as follows: the content of water (W), ash (A) and NaCl according to ISO 1442:1997, ISO 937:1998 and ISO 1841-1:1996, respectively; content total fat (TF) by acid hydrolysis and ether extraction according to AOAC 991.36; content of total protein (TP) by Kjeldahl method according to AOAC 2011.11 (content of nitrogen multiplied by factor 6.25) and content of hydroxyproline (H) according to ISO 3496:1994. The content of collagen (C) was calculated by multiplying of H with factor 8. The TP was used to calculate the protein without collagen as percentage of total protein (P-CTP), the collagen as percentage of total protein (CTP). Apparent total meat content (TM) was calculated according to **McLean (1999)**. Each determination was performed in triplicate and results represent mean values with standard deviations (SDs).

Statistical analysis

The means and SDs of numeric data were computed using Microsoft Office Excel 2010. The PCA analysis was then performed on mean values of numeric data for 9 attributes (without H) using the TANAGRA 1.4.50 software. In order to enchance the interpretation of principal components (PCs), both the CTR coefficients (contributions of points to dimensions) and the correlation coefficients among attributes were calculated within the PCA.

RESULTS AND DISCUSSION

Table 2 summarises the means and SDs of the measurements.

The correlation coefficients among attributes are shown in Table 3. There existed several strong correlations among some attributes. Besides the positive and moderate correlation with P-CTP, TM and A, TP correlated with 3 attributes (NaCl, CTP and TF) negatively and 2 attributes (C and W) slightly. The weak correlation among TP-related attributes and W was expected, as salamis are basically dried and should have low content of water after drying and ripening stage **(Corral et al., (2013)**.

Table 2 Means and standard deviations of measurements for salamis attributes.

Sample	NaCl (%)	A (%)	W (%)	TF (%)	H (%)	C (%)	TP (%)	P-CTP (%)	CTP (%)	TM (%)
Nitran A	3.99 ±1.19	5.32 ±0.04	35.06 ±0.55	36.62 ±1.33	0.52 ±0.08	4.16 ±0.97	23.19 ±0.18	19.03 ±0.21	17.94 ±0.44	148.12 ±2.49
Nitran B	3.44 ±0.75	4.39 ±0.18	31.33 ±0.91	43.12 ±1.13	0.37 ±0.05	2.96 ±0.17	20.84 ±0.10	17.88 ±0.97	14.20 ±0.97	142.31 ±1.78
Nitran C	1.19 ±0.58	5.02 ±0.27	31.82 ±1.07	39.29 ±0.82	0.31 ±0.02	2.48 ±0.22	23.10 ±0.58	20.62 ±0.58	10.74 ±0.82	149.05 ±1.85
Nitran D	9.55 ±1.93	4.68 ±0.05	28.09 ±0.51	50.85 ±0.95	0.41 ±0.12	3.28 ±0.41	17.42 ±0.44	14.14 ±0.26	18.83 ±0.50	120.29 ±0.98
Nitran E	3.30 ±1.33	4.88 ±0.17	34.87 ±0.16	38.19 ±0.42	0.44 ±0.10	3.52 ±0.24	24.94 ±0.15	21.40 ±0.74	14.13 ±1.21	159.51 ±1.59
Nitran F	3.61 ±1.80	4.40 ±0.11	35.44 ±1.27	36.46 ±0.74	0.48 ±0.06	3.84 ±0.45	22.12 ±0.62	18.28 ±0.23	17.36 ±1.32	141.13 ±0.71
Nitran G	1.25 ±0.36	4.89 ±0.20	33.82 ±0.46	39.52 ±0.60	0.41 ±0.22	3.28 ±0.22	21.75 ±0.34	18.47 ±0.34	15.08 ±0.71	131.26 ±0.57
Nitran H	3.85 ±1.17	4.70 ±0.30	22.80 ±1.50	49.79 ±0.31	0.51 ±0.03	4.08 ±0.70	24.62 ±0.83	20.54 ±0.63	16.57 ±0.94	162.01 ±1.14
Nitran I	3.65 ±1.05	4.58 ±0.09	33.37 ±0.46	39.44 ±1.04	0.38 ±0.02	3.04 ±0.56	20.81 ±0.70	17.77 ±0.50	14.61 ±1.24	133.00 ±1.82
Nitran J	4.42 ±0.86	5.45 ±0.13	23.29 ±0.38	47.95 ±0.42	0.44 ±0.05	3.48 ±0.28	22.25 ±0.51	18.77 ±0.22	15.64 ±0.35	144.50 ±0.66

Table 3 Correlation coefficients among attributes of chemical composition of Nitran salamis.

	P-CTP	TP	NaCl	TM	CTP	TF	C	W	A
P-CTP	1.00								
TP	0.97	1.00							
NaCl	-0.75	-0.65	1.00						
TM	0.90	0.93	-0.46	1.00					
CTP	-0.57	-0.35	0.71	-0.31	1.00				
TF	-0.39	-0.35	0.62	-0.14	0.31	1.00			
C	0.12	0.36	0.22	0.34	0.75	0.04	1.00		
W	0.07	0.04	-0.35	-0.14	-0.14	-0.91	-0.11	1.00	
A	0.29	0.32	-0.11	0.23	-0.05	0.02	0.16	-0.22	1.00

Table 4 Results from the PCA analysis for the first five PCs.

Principal component	Eigen value	Proportion of variance explained (%)	Cumulative variance explained (%)
1	4.02	44.91	44.91
2	2.27	25.25	70.16
3	1.55	17.24	87.40
4	0.84	9.36	96.76
5	0.24	2.75	99.51

The results of the PCA analysis are presented in Table 4. Four PCs were extracted that accounted for 96.76% of the total variation. The first 3 of these PCs explain together 87.40% of total variation. In other words, these PCs are the most important, because 87.40% of total variance for Nitran salamis, in the 9 considered attributes, can be condensed into three new attributes (PCs). The eigen value of PC correspond with its importance.

For example, when **Bianchi et al., (2007)** performed the PCA on the class of aldehydes, the first two PCs accounting for the 68.00% of the variance, allowed to group the salamis according to their kind. **Herranz et al., (2008)** analysed nutritional indices in Milano salamis using 4 attributes and found that first two PCs for salamis explained 76.50% of the total variation. **Van Schalkwyk et al., (2011)** found the first two PCs analysing variables of sensory, microbiological, textural and physicochemical, from matured game salamis explained 86.74% of the total variability of those measurements. In Italian salamis, **Corral et al., (2013)** reported that 57.87% of total variation is explained by the first two PCs with measurements using number of parameters including fat, protein and water content.

Table 5 shows that all attributes of salamis had similar proportion (correlation value) in the 1st PC except for C, W and A. After P-CTP, the most important attributes for the 1st PC were TP, TM, TF, CTP and NaCl. So, the 1st PC is mainly defined by these attributes, while the 2nd one is mainly described by C, TF, TM and W. The 3rd PC the best describes differences in TF, CTP, W and C among the samples. The 4th PC is predominantly defined by A, as that had little importance in the previous PCs. Ultimately, the last 5th PC explains the smallest proportional variance among attributes.

Figure 1, Figure 2 and Figure 3 display the correlation scatterplot of attributes on first four PCs. The attributes are interpreted according to the correlations among each other (Table 3) and each PC (Table 5). Thus, attributes close to each other are positively correlated, attributes separated 180° are negatively correlated, whereas if they are separated by 90° they are independent.

The Figure 1 displays that P-CTP is the most positively correlated with TP and TM. On the other hand, this attribute group is negatively correlated with TF, CTP and NaCl, which are, by contrary, positively correlated to each other. The 2nd PC is the best characterized by C and W because they are placed farthest from its origin. The 3rd PC shows that TF is in the highest negative correlation with C (Figure 2). The Figure 3 indicates the independence of A from other attributes.

The most valuable asset of the CTR coefficients (Table 6) to the PCA consists in their utility, when finding the samples that contributed to the particular PC markedly is

Table 5 Correlation coefficients in the eigen vectors (loadings) for the five first PCs, with percent and total percent contributions to explained variance.

Attribute	PC 1		PC 2		PC 3		PC 4		PC 5	
	ρ	% (tot.%)	ρ	% (tot.%)	ρ	% (tot.%)	ρ	% (tot.%)	ρ	% (tot.%)
P-CTP	0.96	93 (93)	0.22	5 (98)	0.07	1 (99)	-0.07	1 (99)	0.04	0 (99)
TP	0.91	83 (83)	0.38	15 (98)	-0.09	1 (99)	-0.08	1 (100)	0.01	0 (100)
NaCl	-0.86	74 (74)	0.30	9 (84)	-0.08	1 (84)	-0.01	0 (84)	0.39	16 (100)
TM	0.79	63 (63)	0.50	26 (89)	0.02	0 (89)	-0.24	6 (95)	0.19	4 (99)
CTP	-0.64	41 (41)	0.46	22 (63)	-0.60	37 (99)	0.01	0 (99)	-0.07	1 (100)
TF	-0.58	34 (34)	0.56	32 (66)	0.54	30 (96)	-0.15	3 (99)	-0.02	0 (99)
C	0.01	0 (0)	0.71	52 (52)	-0.68	46 (98)	-0.05	0 (98)	-0.12	2 (100)
W	0.28	8 (8)	-0.70	50 (58)	-0.61	38 (96)	0.08	1 (97)	0.15	3 (99)
A	0.26	7 (7)	0.41	18 (24)	0.13	2 (26)	0.85	73 (100)	0.04	0 (100)
Variation explained	4.04	45 (45)	2.27	25 (70)	1.55	17 (87)	0.84	9 (97)	0.24	3 (100)

Table 6 CTR coefficients of samples to each PC.

Salami	CTR to PC1	CTR to PC2	CTR to PC3	CTR to PC4	CTR to PC5
Nitran A	1.37	4.02	29.53	22.63	1.27
Nitran B	0.54	6.57	3.34	14.07	0.00
Nitran C	13.68	9.97	23.87	2.54	3.60
Nitran D	66.15	0.05	0.37	0.24	10.97
Nitran E	15.51	0.23	1.84	0.89	33.41
Nitran F	0.02	1.93	25.84	9.15	2.34
Nitran G	0.23	6.90	0.28	4.65	41.04
Nitran H	1.40	41.73	1.85	25.59	4.84
Nitran I	0.78	12.16	0.01	0.45	0.23
Nitran J	0.33	16.45	13.06	19.78	2.31

needed, i.e. to uncover the anomalous parameters of the samples in which they differ in each other (Table 5).

According to the CTR coefficients for 1st PC it can be noted that variance in P-CTP, TP, TM, TF, CTP and NaCl is mainly given by opposition between Nitran D (CTR = 66.15) and remaining samples of Nitran salamis (CTR <16.00) (Table 6). Numeric data confirm this, as Nitran D had the lowest content of P-CTP, TP and TM (14.14%, 17.42% and 120.29%, respectively) and the highest one of TF, CTP and NaCl (50.85%, 18.83% and 9.55%, respectively).

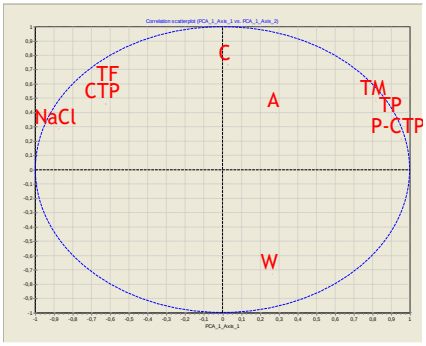

Figure 1 Correlation scatterplot – PC1 vs. PC2.

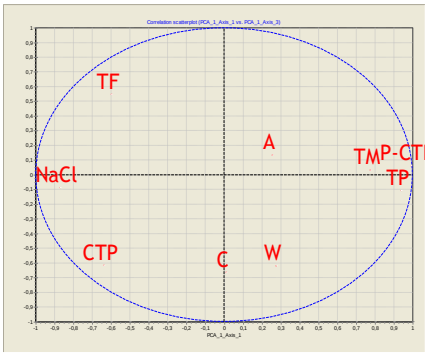

Figure 2 Correlation scatterplot – PC1 vs. PC3.

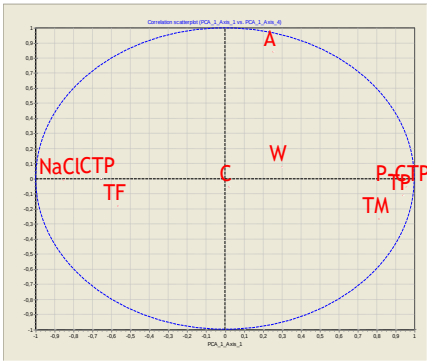

Figure 3 Correlation scatterplot – PC1 vs. PC4.

Collagen content is used as an index of the quality for fermented and dried meat products **(da Silva et al., 2015)**. However, the total content is limited by regulatory agencies **(Sentandreu and Sentandreu, 2014)**. According to **Decree of the Ministry of Agriculture of the Slovak Republic and the Ministry of Health of the Slovak Republic no. 1895/2004-100 establishing a chapter of the Food Codex of the Slovak Republic regulating meat products (2005)**, fermented and dried meat products have to contain maximally 16% CTP. Thus, the values above this limit indicate extra addition of collagen or its hydrolysates, which is a common practice in the meat industry to increase the protein content or water binding properties of meat products **(Sentandreu and Sentandreu, 2014)**. Nitran D was not the most in accordance with this criterion among the salamis (CTP = 18.83%). On the contrary, Nitran E, C and H belonged to group of the TP-rich salamis, when compared to Nitran D, though Nitran H did not satisfy CTP content.

The positions of labels on the loading plot also correspond with this observation (Figure 4). Nitran E, C, H and A are clustered together on the right side of the scatterpplot, because of similarities in attributes explained by 1st PC. However, Nitran D, due to its unlikeness, is separated from other salamis, on the left opposite side of the scatterplot.

The CTR coefficients indicated that Nitran H, J, I and C also marcantly contributed to variance in C, TF, TM and W, described by 2nd PC. Nitran H and A were those with the highest amount of C (4.08% and 4.16%, respectively), whereas Nitran C contained the lowest one (2.48%). But in turn, Nitran H and J had the lowest content of W (22.80% and 23.29%, respectively) and the highest one of TF (49.79% and 47.95%, respectively). Salami is one of the meat products that contain high fat content, usually up to 30% **(Pramualkijja et al., 2015)**.

The CTR values for 3rd PC showed that Nitran A was the poorest in TF (36.62%), but on the other hand the richest in C and CTP (4.16% and 17.94%, respectively) (Figure 5).

Nitran H, A, J and B contributed to variance in A, which was explained by 4th PC. Nitran H and B belonged to the group of low content of A, while Nitran A and J belonged to that one with the highest one (5.32% and 5.45%, respectively) (Figure 6).

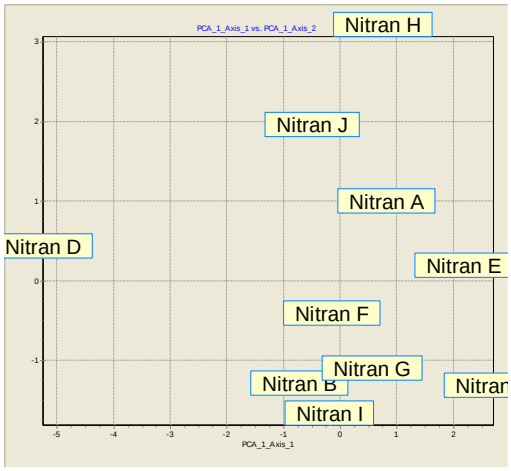

Figure 4 Loading plot - PC1 vs. PC2.

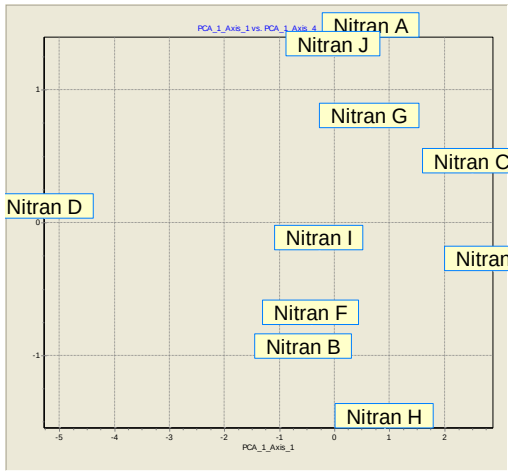

Figure 5 Loading plot - PC1 vs. PC3.

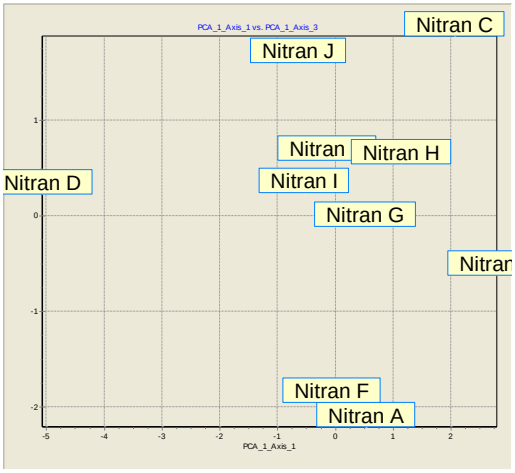

Figure 6 Loading plot - PC1 vs. PC4.

CONCLUSION

It can be concluded that the PCA has shown how chemical attributes of salamis are grouped in the independent sets. In both the 1st and the 2nd PCs, the P-CTP, TP, TM, TF, CTP and NaCl attributes had the highest loadings. In other words, these attributes explained the large part of observed variation in chemical composition among Nitran salamis, which make these attributes as a main predictor of salamis quality. The most distinct differences in these attributes were observed within a pair of Nitran salamis from manufacturer 2 (Nitran C and D). The sliced variant (Nitran D) had the lowest value of TP-associated attributes and the highest ones of TF, CTP and NaCl, even within all the salamis. Besides Nitran D, Nitran A, F and H also did not satisfy CTP content specified in the Decree of MASR and MHSR no. 1895/2004-100, which might indicate extra addition of connective tissue. The CTP content of Nitran B, C, E, G, I and J was in accordance with the decree, whereas Nitran C was that sample with the lowest one (10.74%). Some differences in other attributes were also observed within and among the all couples of salamis, which confirmed the uniqueness of each one. These differences were notable, but not so relevant compared to those described by 1st PC.

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Contact address:

Ing. Tomáš Fekete, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xfeketet@is.uniag.sk.

Ing. Marek Šnirc, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xsnirc@is.uniag.sk.

Ing. Ľubomír Belej, PhD., Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: lubomir.belej@uniag.sk.

prof. Ing. Jozef Golian, Dr., Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jozef.golian@uniag.sk.

Ing. Peter Zajác, PhD., Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.zajac@uniag.sk.

Ing. Jozef Čapla, PhD., Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jozef.capla@uniag.sk.

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EVALUATION OF THE NUTRITIONAL QUALITY OF VEAL SUPPLEMENTED WITH ORGANIC SELENIUM AND ITS EFFECT ON SELENIUM STATUS OF PEOPLE

Klára Vavrišínová, Jana Mrázová, Ondřej Bučko, Petra Lenártová, Jana Moravčíková

ABSTRACT

In the first stage of our research we found out a higher content of selenium in the meat of calves of experimental group (with added the organic form of selenium to the feed mixture) compared to control group (fed without organic form of selenium). In the second stage of our research we focused on monitoring the impact of selenium enriched veal meat and on selenium concentration in blood serum and the selected biochemical parameters of lipid spectrum of the experimental group of volunteers. Ten people who were participating in the research were at the age range between 29 – 56 years. All the volunteers consumed veal meat enriched with organic selenium for 4 weeks. Before starting the experiment we took venous blood of the volunteers and this blood was considered as a control sample of selenium in blood serum of the experimental group. Selenium concentration in blood serum of the examined group was determined by an average of 58.31 ±5.36 μg.L^{-1} and none of them reached the optimal level of selenium. Consequently, we carried out the additional blood sampling after 2 and 4 weeks of the consumption of veal meat. There was registered a slight increasing of selenium status, whereas after the finishing the consumption, we determined the average selenium concentration in blood serum of the experimental group 60.73 ±4.05 μg.L^{-1}. The evaluation of lipid profile of the experimental group showed (after input blood sampling) higher values of total cholesterol level and lower levels of HDL cholesterol. This fact shows the higher risk of starting the cardiovascular diseases. Reported research results didn't show statistically significant changes of blood lipid spectrum of the experimental group. We concluded that the consumption of supplemented veal meat can positively affect the level of selenium in our body and thereby increase it can increase the protective effect against the influence of free radicals.

Keywords: organic selenium; supplementation of veal; veal quality; selenium status; lipid profile of poeple

INTRODUCTION

The importance of selenium was proved in 1957, when the presence of selenium was found in so-called factor 3 which is the prevention against necrosis in the liver of the rats. In 1976 many experiments showed obvious necessity of selenium for people, in spite of that, it was pointed on its negative effects in the 1940s of last century. Nowadays, an interest about selenium and its role and significance in food considerably increased because many researches point out the importance of this element for the health of people. In the past, we only knew its toxic effect on the organism but present studies focus on, that a lack of selenium can cause cardiovascular diseases and also oncogenous diseases **(Hegedus et al., 2007)**. Selenium belongs to the important antioxidants which improve defensive power of the organism and these antioxidants also protect some elements of food, mainly vitamins and food fats against unwilling oxidation. It is important essential mineral element which is important for the health of people and animals. The selenium together with the vitamin E positive influence on the technological characteristics (properties) of meat because of its antioxidants features **(Pavlata et al., 2002**). In food of animal origin the concentration of selenium is given by the nourishment of the animal or its content in feedstuff **(Lyons et al., 2007)**. According to several publications, the use of organic selenium in animal nutrition and consumption the products of these animals are accessible source of selenium in the human diet **(Fisinin et al., 2009)**. Mainly at red meat in this regard refers **Wiliams (2007)**, however probably its concentration is of the heavily influenced by nutrition. **Marounek et al., (2006)** in the experiment found out differences in the content of selenium in veal. In group experimental group (with selenium yeast) was content Se higher compared to the control group. In the different parts of world there are also different intake of selenium in people and animals. It is also regarded according to the selenium status, it means saturation of the organism by this microelement and its combinations. Selenium status depends on different factors such as absorption, food intake, excretion according to biological accessibility **(Ermidou-Pollete et al., 2005)**. The concentrations of selenium in blood plasma/serum of people in the European countries are in the scale between 60 – 111 μg.L^{-1}. Selenium status in Slovak population is in the low limits of this scale **(Combs, 2001)**. **Thomson (2004)** confirmed starting concentration of selenium in blood serum for protective effect against the influence of free radicals 100 - 200 μg.L^{-1}. Selenium has a strong antioxidant activity and participates in the system of conversion of aggressive oxidant products, transforms intracellular free radicals into less reactive or neutral elements **(Elasal et al., 2014)**. Increased intake of selenium decreases the risk of starting the cancer and

softens the progress of other pathological processes causing oxidative stress and an irritation **(Lukáč, 2007)**. Sufficient supplementation with selenium of the animals is important not only because of good health state and utility of the animals, but it can be increased in human population by higher content of selenium in the products. The features of organic form of selenium allow an effective transfer in foodweb. It is used in the world practise in modern approach of the production of so-called functional articles of food. The animal products can belong to this category and they are enriched by organic selenium **(Lagin et al., 2009)**.

The aim of work was evaluation physical and chemical patrameters of veal enriched of organic selenium and its effect on selenium status of people.

MATERIAL AND METHODOLOGY

Two groups of calves (10 +10 heads) to 150 kg of the body weight (at the same time, the same age and rearing condition) were reared for the purposes of the experiment. The difference between the groups was in the feeding after weaning to the end of experiment. The organic form of selenium was added to the feed mixture in the experimental group (selenium content per 1 kg of mixture was: E8 form 1.12 mg and 3b8.10 form 0.8 mg). The control group was fed without organic form of selenium.

There was analysed an effect of supplementation of veal meat on selenium status and we also examined chosen biochemical parameters of lipid spectrum of the consumers. In the experiment was selected group of people. People who participated in the research were represented by 5 women and 5 men at the age scale between 29 – 56 years, with the average age of the experimental group which was 46.3 ±8.34 years. The experimental group consisted of the healthy volunteers, without any healthy problems and pathologic changes in basic biochemical parameters in blood. All the members of the experimental group didn't use supplements before starting the clinical study, and not also during the realization of the research.

Referring to our experiment which was focused on monitoring the impact of supplementation of selenium to the feeding mixture for the calves, we gain meat from the MLT in the experimental groups of the animals. Meat which was enriched by selenium was canned in 1% salt brine in the airtight cans and sterilised them in the thermostatic pot.

Meat was sterilised for 3 hours and the temperature was 100 °C. The consumption of veal meat was done three times a week during 28 days individually. The amount of meat was 130 g.

The biochemical examination of blood tests before starting the consumption was carried out. The 1st blood sampling was determined total cholesterol level, a level of HDL-cholesterol, LDL-cholesterol level, triglycerides and concentration of selenium in blood of the experimental group of the volunteers. We did the 2nd (repeated) blood taking after two weeks of consumption of supplemented veal meat and the last (the 3rd) blood taking was done immediately after finishing the consumption. The samples of blood serum were stored in the fridge on the temperature 80 °C after their separation and consequently after de-freezing them we specified (estimated) biochemical parameters of blood serum of the experimental group.

Biochemical parameters of blood were defined by the estimation analyser (estimation device) Biolis 24i premium (Tokyo Boeki Medisys, Japan). Total cholesterol, triglycerides were determined by calorimetric method fy Randox CHOD-PAP and HDL cholesterol level, LDL cholesterol level were defined by direct method clearance fy Randox.

The atomic absorptive spectrometer made by the company Perkin-Elmer 4100ZL (Norwalk, CT, USA) was used for defining the concentration of selenium. This spectrometer has cross heating electrothermic atomizer (THGA, Part No.B050-4033). And this spectrometer was also used in connection with automatic feeder machine with the samples AS-70. We used corrector of Zemanovsky for the correction of the background. EDL (System 2) for Se (Perkin-Elmer) was used as a source of radiation, which was working in 260 mA. The wave length was 196.0 nm and width of the gap (crack) was 2.0 nm.

We evaluated gained data from the experiments by adequate biostatistical methods using applicative programmes. Statistical data processing was realised by algorithms which were found in the applications SAS in 9 and also by statistical functions in MS Excel. Data processing was also done by one factor analysis of dispersion using ANOVA.

RESULTS AND DISCUSSION

The Table 1 showes the higher content of selenium in the meat of calves of experimental group compared to control group. There was not found significant differences in the chemical composition between groups. Ing the meat of the control group was higher decrease in pH levels at 24 hours after slaughter. **Marounek et al., (2006)** in the experiment found out higher Se content also in the control of the experimental group (with the addition selenium yeast) and also in the control group, compared with our results.

By the determined significant differences in selenium

Table 1 The physical and chemical patrameters and selenium content of *m. longissimus thoracis et lumborum* (MLT).

Parameters	Control group $\bar{x}$ ±SD	Experimental group $\bar{x}$ ±SD	Significance
Se content (mg.kg^{-1})	0.064 ±0.003	0.101 ±0.006	+++
Protein content (g.100g^{-1})	22.510 ±0.467	22.880 ±0.798	-
IMF content (g.100g^{-1})	1.703 ±0.358	1.893 ±0.148	-
Water content (g.100g^{-1})	74.707 ±0.682	74.420 ±0.349	-
pH_1	6.313 ±0.152	6.377 ±0.037	-
pH_{24}	6.033 ±0.029	6.173 ±0.065	+

content between the control and the experimental group we can perform the second stage of the experiment. The veal from the experimental group was used in the second stage.

The impact of supplementation of veal meat with organic selenium on the concentration of selenium in blood of the experimental group

In the second stage of our research was focused on monitoring of veal meat enriched with organic selenium and its influence on the concentration of selenium in blood serum in the experimental group and we also examined chosen biochemical parameters of lipid spectrum of consumers.

The volunteers in the nutritional protocols where they wrote what kind of food they ate during the whole day. The nutritional software Alimenta version 4.3e was used to intake of selenium from food of the volunteers (the experimental group) during the days when they didn't eat veal meat enriched by selenium. Average daily taking of selenium in the group of ten people we registered an amount 131.34 μg.day^{-1}. The selenium intake of veal meat supplemented was 10 μg.100g^{-1}. According to The World Health Organization taking selenium per day moves between 50 to 200 μg.day^{-1} **(Rayman, 2012)**.

The first blood sampling in the experimental group before starting our research and we noticed that average concentration of selenium was 58.31 ±5.36 μg.L^{-1}, and we can say that none of the volunteers had an optimal level of the concentration of selenium in blood. The second blood sampling we did after two weeks of consumption of supplemented veal meat with organic selenium and we marked slight increasing of selenium in blood serum in average 59.99 ±4.16 μg.L^{-1}. The last blood taking was done after finishing the consumption of veal meat enriched by selenium. It was after four weeks and was also noticed increasing of the concentration of selenium in blood in average 60.73 ±4.05 μg.L^{-1}. The results didn't show evidentiary changes in concentration of selenium in blood serum of the experimental group. The results were made after short time of consuming of veal meat with selenium content. The particular concentrations of selenium in blood are shown in the figure 1 and table 2.

According to determined concentrations of selenium in blood is probably that the interval of the concentrations in monitored group, which is 49.6 – 67.8 μg.L^{-1}, is comparable with the results of the last study which was done in Slovakia by **Maďarič and Karabová (1998)**.

They determined concentrations of selenium in blood plasma of 1056 chosen people who were examined and they were from different parts of Slovakia. This concentration was in the range 46 – 77 μg.L^{-1}. Another similar research was done in Czech Republic by **Střitecká et al. (2009)**. In this study was experimental group of 386 healthy people and the concentration of selenium in their blood was in the range 52.9 – 73.43 μg.L^{-1}. This study also approved slight deficiency of selenium concentration similarly than it was shown in our experiment. Low levels of selenium in blood which were shown in above mentioned studies are connected with low saturation of selenium in soil.

The influence of supplementation of veal meat enriched with organic selenium on lipid profile of the experimental group

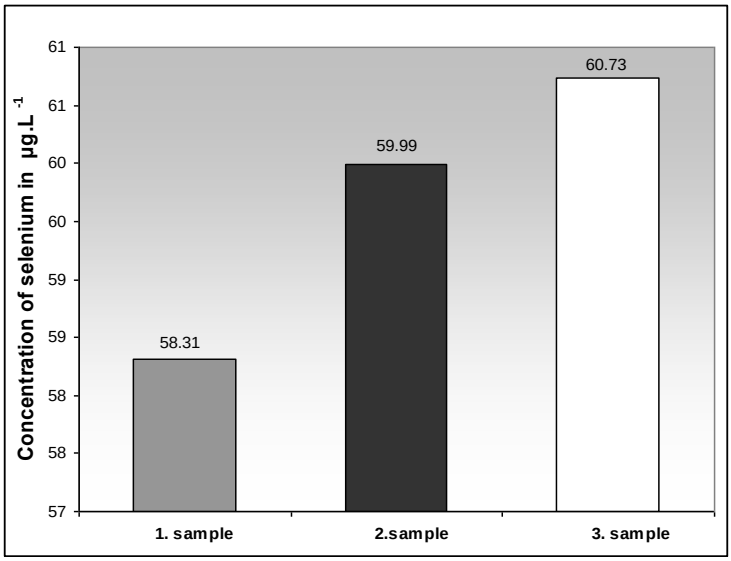

Figure 1 Comparing the concentration of selenium in blood serum of the experimental group during the realisation of the research.

Table 2 Selenium concentration in human blood serum.

Sex	1.sample μg.L^{-1}			2. sample μg.L^{-1}			3. sample μg.L^{-1}		
	$\bar{x}$	s	min-max	$\bar{x}$	s	min-max	$\bar{x}$	s	min-max
Men	61.28	3.72	58.8 – 67.8	62.30	2.75	58.8-65.4	60.88	3.91	56.7 – 66.9
Women	55.34	5.37	49.6 – 62.2	57.68	4.25	54.5-63.4	60.58	4.64	55.4 – 65.5
Total	58.31	5.36	49.6 – 67.8	59.99	4.16	54.5-65.4	60.73	4.05	55.4 – 66.9

significance $p \geq 0.05$.

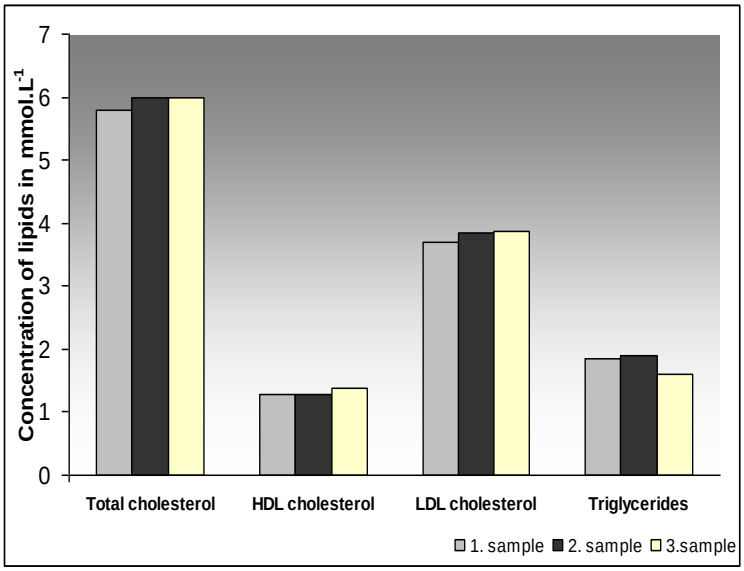

Figure 2 Comparing the concentration of lipids in blood of the experimental group.

Table 3 The lipid profile in human blood serum.

PARAMETERS	**1. SAMPLE** $\bar{x}$ **±SD**	**2. SAMPLE** $\bar{x}$ **±SD**	**3. SAMPLE** $\bar{x}$ **±SD**
Total cholesterol (mmol.L^{-1})	5.80 ±1.34	5.98 ±1.28	5.99 ±1.53
LDL cholesterol (mmol.L^{-1})	3.70 ±1.02	3.84 ±1.05	3.87 ±1.18
HDL cholesterol (mmol.L^{-1})	1.27 ±0.29	1.29 ±0.26	1.39 ±0.34
Triacylglycerols (mmol.L^{-1})	1.86 ±0.69	1.91 ±0.48	1.60 ±0.56

significance $p \geq 0.05$.

In evaluation of lipid profile of the volunteers we recorded increased values of total cholesterol which were seen in entry blood taking and these values were in average 5.80 ±1.34 mmol.L^{-1} and was found out low levels of HDL cholesterol in average 1.27 ±0.29 mmol.L^{-1} which in 30% of the volunteers points on higher risk of cardiovascular diseases. **Ferenčík et al., (2002)** states that the following parameters belong to the most important effects of supplementation of selenium: they are - decreasing of the risk of starting arteriosclerosis and cardiovascular diseases, stimulation of immune system, preventive effect against inflammatory diseases, decreasing of virulence of some viruses.

Opposite of results of **Ferenčík et al., (2002)** we didn´t found out a positive effect on concentration of total cholesterol in blood in the experimental group during the experiment, we can say that after short time of the consumption of veal meat enriched with organic selenium we found out that metabolism of lipids was better whereby the concentration of HDL cholesterol increased and the level of triglycerides slightly decreased. The results of our research didn't show statistically significant changes in lipid spectrum of the volunteers in the experimental group. The average values of the parameters of lipid profile are shown in the Figure 2 and Table 3.

The impact of selenium on lipid profile was examined on the experimental group of the animals. It was shown that the supplementation of selenium decreased the value of total cholesterol and also LDL cholesterol level and increased the value of HDL cholesterol level whereas the lack of selenium had an opposite effect.

It was found that an inactivation of synthesis of selenoproteins of the mice causes increasing concentration of cholesterol in plasma, increasing an amount of apolipoprotein E, improves gene expression for biosynthesis of cholesterol and decreases gene expression which is responsible for metabolism and transport of cholesterol. Relevancy of these studies connected with people is a questionable. It is supposed that the association between selenium status and the risk of starting the cardiovascular diseases depends on selenium status of monitored population **(Rayman, 2011)**.

CONCLUSION

According to our results was concluded that the application of organic selenium to feed mixture in the fattening process of the calves has the significance for effective transfer / transmission of essential microelement selenium to the foodweb. Integration of veal meat enriched with selenium to food of people leads to the increasing of selenium status of consumers and it can also lead to the protection of the cells of immune system against the damage during oxidative stress.

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Contact address:

Klára Vavrišínová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Husbandry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: klara.vavrisinova@uniag.sk.

Jana Mrázová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Human Nutrition, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.mrazova@uniag.sk.

Ondřej Bučko, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, of Animal Husbandry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: ondrej.bucko@uniag.sk.

Petra Lenártová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: petra.lenartova@uniag.sk.

Jana Moravčíková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Husbandry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: janamoravcikova@hotmail.com.

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INFLUENCE OF HARVEST DAY ON CHANGES IN MECHANICAL PROPERTIES OF GRAPE BERRIES

Šárka Nedomová, Vojtěch Kumbár, Pavel Pavloušek, Roman Pytel, Jaroslav Začal, Jaroslav Buchar

ABSTRACT

Changes in the composition, physical and mechanical properties occur in grape berries during the ripening process, but the heterogeneity of the grapes harvested at different ripening stages affects the reliability of the results obtained. The characterization of the mechanical properties of grape berries seems to be an important parameter for understanding grape ripening. In this work, these changes were studied in seven grapevine varieties (*Riesling, Blaufränkisch, Pinot Noir, Cerason, Malverina, Laurot,* and *Hibernal*) harvested during six consecutive weeks. Mechanical behaviour was measured using compression and puncture tests using of TIRATEST 27025 testing machine. Skin mechanical properties were evaluated using a puncture test carried out on the equatorial side. The dependence of these properties on the chemical composition has been evaluated. These parameters of force/time curves were studied by puncture test: the berry skin break force, the needle displacement at the skin break and the berry skin break energy. The crushing force, the plate displacement at the crushing strength and the berry crushing energy were studied from force/time curves by compression test. Results of the puncture test shows that there the skin break strength and the acidity content are monotonic functions of the time. A comparison of different varieties from the point of the value of the crushing force was obtained by vertical and transversal loading. The crushing force is monotonically decreasing function of the harvesting time like the break force evaluated at the puncture test. The correlation between the skin break strength and the sugar content is significant namely for the varieties: *Hibernal, Riesling, Malverina,* and *Cerason*.

Keywords: grapes; acidity; sugars; texture; rupture

INTRODUCTION

Wine grapes undergo numerous physiological and biochemical changes during ripening inducing colour and texture changes **(Ribereau-Gayon et al., 2006; Coombe and McCarthy, 2000; Letaief et al., 2013; Le Moigne, 2008)**. During ripening, changes in the composition and structure of the cell wall as well as in the structure of the tissue, may determine the mechanical resistance and the texture of the fruit **(Abbott, 2004; Brummell et al., 2004; Hertog et al., 2004; Brummell et al., 2006; Deytieux-Belleau et al., 2008; Rolle et al., 2011)**. Grapes with low level of mechanical properties and damaged may be contaminated by fungi (e.g. *Penicillium expansum*) **(Tančinová et al., 2016)**. From this point of view the characterization of the mechanical properties of grape berries seems to be an important parameter for understanding grape ripening **(Doumouya et al., 2014; Carbajal-Ida et al., 2016; Fava et al., 2011)**. Previous studies applied the puncture test to characterize and compare the crunch texture of different table grapes **(Sato et al., 1997; Sato and Yamada, 2003)** and to follow the ripening process of white wine grapes such as Chardonnay and Riesling **(Lee and Bourne, 1980)**. These last authors showed that the mechanical properties of grape skin evolved during ripening and were significantly correlated with the °Brix for most grapes. Further work showed differences in mechanical properties of red wine grape varieties at a chosen harvest maturity level **(Letaief et al., 2008)** and differences in grape skin hardness **(Río Segade et al., 2008)**. However, there is no published work addressing the assessment of a mechanical method designed to monitor wine grapes ripening.

Preliminary research on the grape texture change showed that compression measurements were able to recognize veraison (a marker stage of berry development) earlier than a visual identification performed in the field, which is of particular importance for white grapes for which the colour change is slight **(Robin et al., 1997; Grotte et al., 2001)**. **Bernstein and Lustig (1985)** measured grape firmness and showed the relationship between turgor pressure and firmness. **Zouid et al., (2013)** show that the instrumental texture analysis can be very useful for to study the impact of the grapes heterogeneity according to sugar level on the physical and mechanical properties of *Cabernet Franc* grapes and to select the best instrumental parameters of the whole berries or of the skin linked with anthocyanins extractability. The next information on the instrumental texture analysis is presented by **Rolle et al., (2012)**.

The aim of this study was to define the best conditions to describe grape texture during ripening in order to obtain additional parameters that could be of benefit to ascertain

the quality of ripening grape berries, in addition to the physiological parameters commonly used such as the acidity and sugar content.

MATERIAL AND METHODOLOGY

All grapevine varieties under study were grown in the experimental vineyard of the aforementioned faculty. This vineyard is situated in the vineyard site called "V Mendeleu" (In Mendeleum) in the wine village Lednice (region South Moravia, Czech Republic). The spacing of plants was 2.2 x 1.0 m and the plants were trained using Guyot pruning with 10 eyes per vine. This vineyard was established in 1993 and all varieties were grafted on the rootstock 5C.

Within the framework of this study altogether 3 cultivars of *Vitis vinifera* L. – *Riesling, Blaufränkisch* and *Pinot Noir* were evaluated together with 4 interspecific varieties: *Cerason*, *Malverina*, *Laurot*, and *Hibernal*. These varieties are maintained and evaluated within the framework of a collection of genetic resources of grapevine. Berries were sampled using the method described by **Iland et al., (2004)**.

Berries were randomly picked once per week, during the maturation period (from September to October in 2015). For compression test has been chosen six different dates: September 4 (week 1), September 13 (week 2), September 22 (week 3), September 30 (week 4), October 7 (week 5), and October 13 (week 6). For puncture test has been chosen the same dates without September 4 (week 1).

Each day of the harvest the following parameters were evaluated total acids in grapes. Total acid was calculated as all acids determined by HPLC method and expressed as tartaric acid. Total sugar was the sum of glucose and fructose **(Katalinic et al., 2013)**. The detail description of this method of analysis is described in **Pavloušek and Kumšta (2011)** briefly.

Mechanical behaviour was measured using compression and puncture tests. These tests were performed using of TIRATEST 27025 (TIRA Maschinenbau GmbH, Germany) testing machine. Skin mechanical properties were evaluated using a puncture test carried out on the equatorial side. Tests were performed with a cylindrical needle probe of 0.56 mm in diameter at speed test of 10 mm·s^{-1}. Force/time curves were analyzed and three parameters were studied: the berry skin break force F_{sk} in Newton, the needle displacement p_{sk} [mm]at the skin break and the berry skin break energy W_{sk} [J = N·mm], see Eq. (1). These tests have been conduced on the lateral side of the berry, positioned on the base of the texture analyser **(Brummell et al., 2004)**.

$$W_{sk} = \int_0^{p_{sk}} F_{sk}\, dp. \qquad (1)$$

Whole berry mechanical properties were assessed using a compression test. Berries were compressed both in the equatorial position (perpendicular to berry height *L*, [mm]) and vertically along of the berry symmetry axis. The compression velocity was also 10 mm·s^{-1}. The following mechanical parameters have been measured: crushing force F_c [N], the plate displacement at the crushing strength p_c [mm] and the berry crushing energy W_c [J = N·mm], see Eq. (2). The crushing force is the compression force that is necessary to cause the skin break when the first grape juice is coming out **(Brummell et al., 2004)**.

$$W_c = \int_0^{p_c} F_c\, dp. \qquad (2)$$

The results obtained were statistically analysed using the statistical toolbox of software MATLAB version 7.12.0.635 (R2011a) (The MathWorks, MA, USA). Evaluated were the means and standard deviations using ANOVA with subsequent Tukey's test at significance levels of $p < 0.05$.

RESULTS AND DISCUSSION

In the Figure 1 an example of the experimental record force *F* vs displacement *p* is shown. The same qualitative features exhibited all experimental records. The force increases up to some maximum value corresponding to the skin break force F_{sk}. The force is non-linear function of the displacement. This is slightly different result than that obtained e.g. by **Maury et al., (2009)** and/or **Río Segade et al., (2011)**. In these papers the considered dependence was linear.

The berry skin break force F_{sk} for different wine varieties is displayed in the Figure 2. This force decreases with the time of the harvesting. It means this force exhibits a good correlation with the content of total acids, see Figure 3. This dependence is different for the different wine varieties. It means the value of this force cannot be used for the identification of single varieties.

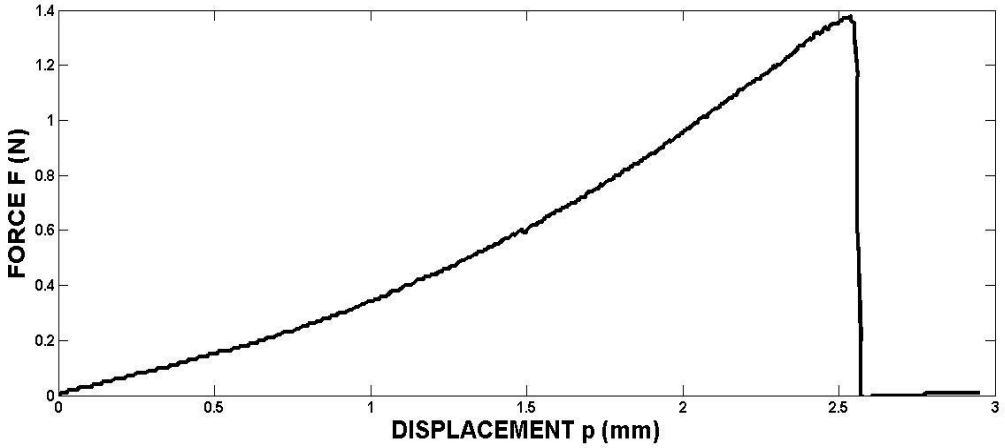

Figure 1 Example of the experimental record break force – displacement during the puncture test.

The dependence of the break force F_{sk} on the sugar content can be considered as a linear. The best correlation, i.e. higher than 0.85 have been observed for the following varieties: *Hibernal*, *Riesling*, *Malverina*, and *Cerason*. For the remaining grapevines the correlation coefficient was between 0.73 and 0.82. Nearly no correlation has been found between displacement at the skin break p_{sk} and the total sugars content. Very good correlation has been also found between the berry skin break energy W_{sk} and total content both of sugars and acids. Development of this energy during the harvest period is displayed in the Figure 4.

In the Figure 5 an example of the experimental record of the force F_c – displacement during the compression test is displayed. The qualitative features of this record are the same like in the case of the puncture test, see Figure 1. This conclusion is valid for both transversal and vertical tests and for all winegrape varieties.

The average values of the crushing force F_c for different wine varieties are displayed in the Figure 6.

This force F_c decreases with the time of the harvesting. Qualitatively the same dependence exhibits crushing force

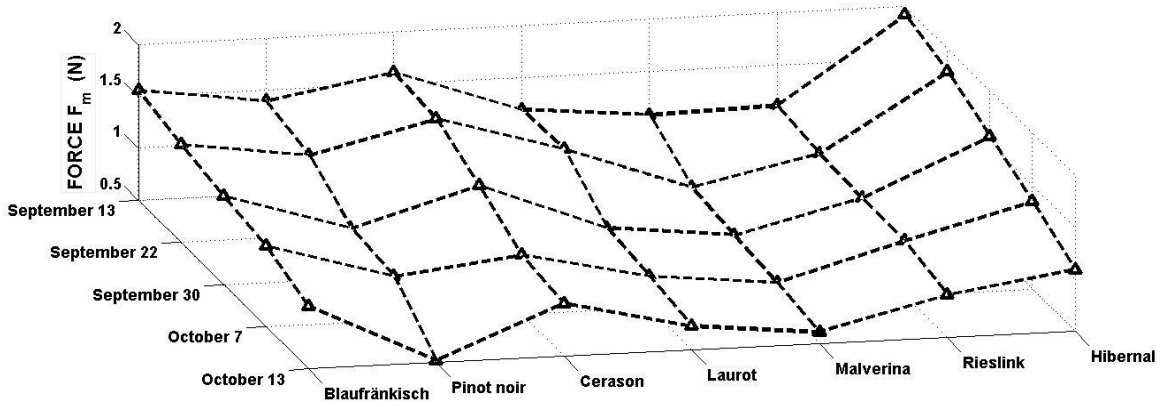

Figure 2 Skin break force evaluated from the puncture test.

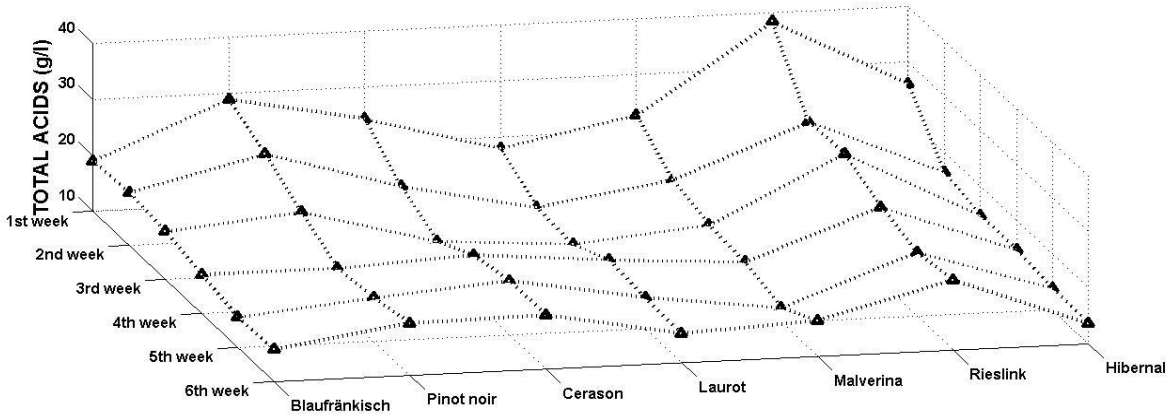

Figure 3 Content of total acids in grapes of tested varieties.

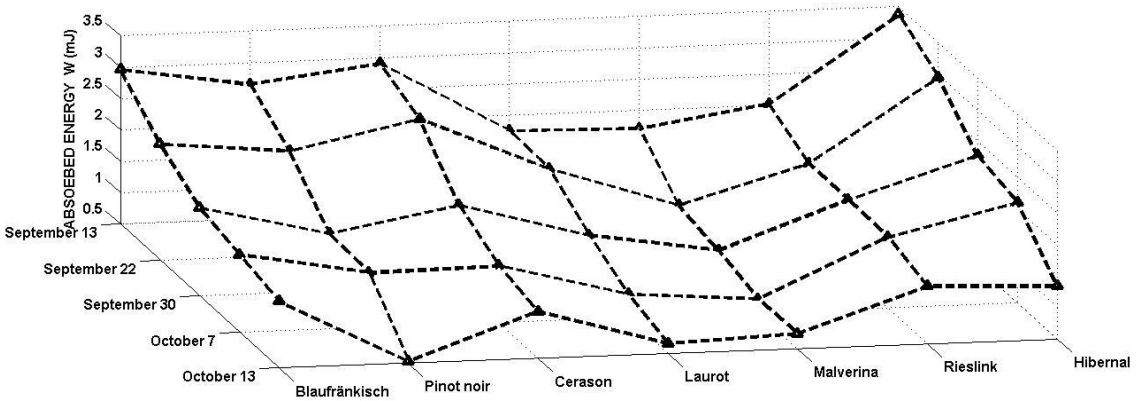

Figure 4 Berry skin break energy at the different days of the harvest.

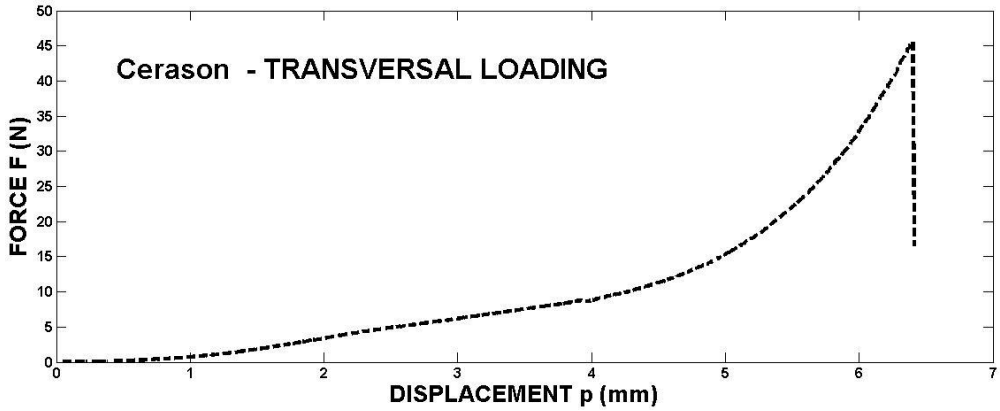

Figure 5 Example of the experimental record of the wine berry compression.

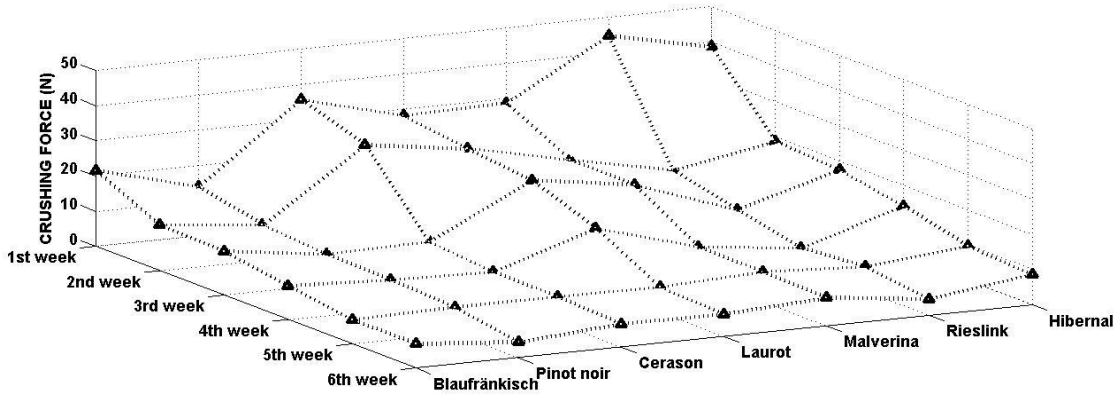

Figure 6 Average values of the crushing force during harvesting period – transversal compression.

obtained at the vertical loading. The differences between values of these force is described in the Table 1. In this Table 1 corresponds to the situation when the crushing force obtained during the transversal compression is higher than that obtained at the vertical compression. Zero corresponds to the opposite case.

It is evident that the crushing force corresponding to *Pinot Noir, Blaufränkisch* varieties evaluated at the lateral compression is higher than that evaluated at the vertical compression. The crushing force of remaining varieties does not exhibit this tendency. The crushing force is monotonically decreasing function of the harvesting time like the break force evaluated at the puncture test. If we perform a comparison of different varieties from the point of the value of the crushing force we obtain an arrangement given in the Table 2.

The minimum value of the crushing force exhibits *Pinot Noir* grapevine variety. The order of remaining varieties is different at different days of the harvesting. The arrangement made according to the crushing force evaluated at the lateral compression is different from that given in the Table 2, see Table 3.

The same arrangement according to the values of the break force evaluated at the puncture test is given in the Table 4.

The results are different from those obtained at the compression test. Qualitatively the same conclusions can be deduced from the values of the absorbed energy and from the values of the displacements at the crushing force. One can see that the critical values of the forces which describe the strength of the berry skin (puncture test) and the whole berry (compression test) gives a different order of grapevine varieties at different days of harvesting.

As it has been mentioned in the introduction, grape

Table 1 Comparison of crushing force for transversal and vertical compression.

Week	*Hibernal*	*Riesling*	*Malverina*	*Laurot*	*Cerason*	*Pinot Noir*	*Blaufränkisch*
1st	1	0	0	0	0	1	1
2nd	1	0	0	0	0	1	1
3rd	1	0	0	0	1	1	1
4th	1	1	1	0	1	1	1
5th	1	0	1	1	1	1	1
6th	0	1	0	1	1	1	1

Table 2 Order of grapevine varieties at the single data of their harvesting – vertical loading.

1st week	2nd week	3rd week	4th week	5th week	6th week
September 4	September 13	September 22	September 30	October 7	October 13
Riesling	*Riesling*	*Riesling*	*Riesling*	*Riesling*	*Riesling*
Malverina	*Malverina*	*Laurot*	*Laurot*	*Cerason*	*Hibernal*
Laurot	*Laurot*	*Malverina*	*Malverina*	*Malverina*	*Laurot*
Cerason	*Cerason*	*Pinot Noir*	*Pinot Noir*	*Laurot*	*Cerason*
Blaufränkisch	*Blaufränkisch*	*Blaufränkisch*	*Blaufränkisch*	*Pinot Noir*	*Malverina*
Hibernal	*Pinot Noir*	*Cerason*	*Cerason*	*Blaufränkisch*	*Pinot Noir*
Pinot Noir	*Hibernal*	*Hibernal*	*Hibernal*	*Hibernal*	*Blaufränkisch*

Table 3 Order of grapevine varieties at the single data of their harvesting – transversal loading.

1st week	2nd week	3rd week	4th week	5th week	6th week
September 4	September 13	September 22	September 30	October 7	October 13
Pinot Noir	*Pinot Noir*	*Pinot Noir*	*Riesling*	*Blaufränkisch*	*Pinot Noir*
Blaufränkisch	*Blaufränkisch*	*Cerason*	*Cerason*	*Riesling*	*Riesling*
Laurot	*Riesling*	*Riesling*	*Pinot Noir*	*Laurot*	*Laurot*
Malverina	*Hibernal*	*Blaufränkisch*	*Malverina*	*Cerason*	*Cerason*
Cerason	*Malverina*	*Hibernal*	*Blaufränkisch*	*Pinot Noir*	*Blaufränkisch*
Hibernal	*Laurot*	*Malverina*	*Hibernal*	*Malverina*	*Malverina*
Riesling	*Cerason*	*Laurot*	*Laurot*	*Hibernal*	*Hibernal*

Table 4 Order of grapevine varieties at the single data of their harvesting – puncture test.

2nd week	3rd week	4th week	5th week	6th week
September 13	September 22	September 30	October 7	October 13
Malverina	*Malverina*	*Malverina*	*Malverina*	*Pinot Noir*
Riesling	*Riesling*	*Laurot*	*Laurot*	*Malverina*
Laurot	*Laurot*	*Pinot Noir*	*Pinot Noir*	*Laurot*
Pinot Noir	*Pinot Noir*	*Riesling*	*Riesling*	*Riesling*
Blaufränkisch	*Blaufränkisch*	*Cerason*	*Cerason*	*Cerason*
Cerason	*Cerason*	*Blaufränkisch*	*Blaufränkisch*	*Hibernal*
Hibernal	*Hibernal*	*Hibernal*	*Hibernal*	*Blaufränkisch*

maturity is associated with changes in the composition and structure of the cell wall of skin and pulp as well as in the structure of the tissue. Therefore, the test conducted on whole berry, which assess the parameters such as crushing strength etc., is actually the best test to monitoring the ripeness, although the values of parameters measured can be affected by rainfalls **(Malheiro et al., 2011; Bonada et al. 2015)**. In this type of test, pulp and skin data are aggregate. On the contrary, by puncture test conduced with thin rounded probe only skin characteristics can be defined. Actually, the break skin force F_{sk} could be considered an important parameter to be monitored for the assessment of the anthocyanins extractability. It means both tests must be used for the evaluation of the berry softening during the maturation.

CONCLUSION

A detail study of the mechanical characteristics of seven winegrape varieties during ripening has been performed. Results of the puncture test shows that there the skin break strength and the acidity content are monotonic functions of the time. The correlation between the skin break strength and the sugar content is significant namely for the varieties: *Hibernal*, *Riesling*, *Malverina*, and *Cerason*. The correlation for the remaining varieties is weaker. Very similar results are valid for the parameters of the compression test. Results of these tests are dependent on the loading orientation. The effect of this parameter is different at different stage of the ripening. Generally the results obtained in this work approved some previous hypothesis that mechanical texture parameters were able to show differences between grapes having different ripening level. In order to support results performed up to now it is necessary to perform some additional experiments with different values of compression velocities and with different diameters of the cylindrical needle probe.

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Contact address:

doc. Ing. Šárka Nedomová, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: snedomov@mendelu.cz.

Ing. Vojtěch Kumbár, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Technology and Automobile Transport, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: vojtech.kumbar@mendelu.cz.

prof. Ing. Pavel Pavloušek, Ph.D., Mendel University in Brno, Department of Viticulture and Oenology, Faculty of Horticulture, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: pavel.pavlousek@mendelu.cz.

Ing. Roman Pytel, Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: roman.pytel@mendelu.cz.

Ing. Jaroslav Začal, Mendel University in Brno, Faculty of AgriSciences, Department of Technology and Automobile Transport, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: jaroslav.zacal@mendelu.cz.

prof. Ing. Jaroslav Buchar, DrSc., Mendel University in Brno, Faculty of AgriSciences, Department of Technology and Automobile Transport, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: buchar@mendelu.cz.

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MicroRNA (miRNA) IN FOOD RESOURCES AND MEDICINAL PLANT

Katarína Ražná, Milan Bežo, Lucia Hlavačková, Jana Žiarovská, Marián Miko, Ján Gažo, Miroslav Habán

ABSTRACT

MicroRNAs (miRNAs) are a class of 19 – 24 nucleotide long non-coding RNAs derived from hairpin precursors, regulating various biological, metabolic and developmental processes at the post-transcriptional level. Many of the known miRNAs are evolutionary conserved across diverse plant species and function in the regulatory control of fundamentally important biological processes. It is known that exogenous plant miRNAs specifically target approximately 30% of protein-coding genes in mammals. The research was focused to analyze the occurrence of selected families of miRNAs (miR156, miR168 and miR171) in less used species but nutritionally important plant food resources (flax and medlar) and medicinal plant (milk thistle). The analyses were done by two individual approaches, by (a) miRNA-based molecular markers - as a novel type of functional markers and (b) qualitative Real-Time PCR. The expression pattern of selected miRNAs was analyzed depending on various plant tissues and developmental stages. Results have confirmed the significance and reliability of novel type of markers based on miRNA molecules as well as the species-specific and tissues-specific expression patterns of plants miRNAs. Significant polymorphism profile of miR156b was detected in various flax tissues of genotypes varying in the content of alpha-linolenic acid. Conclusions indicate that the variable behavior of the miRNA molecules, depending on various factors, may reflect the variability of the gene expression regulation of the human genome. The exploitation of the background of miRNA functioning within different species and plant tissues will help us to understand the molecular machinery as well as the regulatory mechanisms involved in the expression of miRNAs in plants and consequently in human genome.

Keywords: miRNA; human nutrition; functional food; medicinal plant

INTRODUCTION

Recent findings show that genetic material in plant foods may survive digestion, circulate through our bodies and modulate our gene expression **(Hirschi, 2012)**. Exogenous plant microRNAs that are primarily acquired orally, through food intake, are present in the sera and tissue of various animals **(Zhang et al., 2012)**. Microvesicles (MVs) may encapsulate these miRNAs, because these small vesicles are shed from almost all cell types. Stable microRNAs in mammalian serum and plasma are actively secreted from tissues and cells and can serve as a novel class of biomarkers for diseases, and act as signaling molecules in intercellular communication **(Zhang et al., 2012)**. MicroRNAs (miRNAs) are small RNAs that can regulate target mRNAs by binding to their 3′-UTRs **(Singh et al., 2008)**, leading to either translation delay or mRNA degradation **(Erson-Bensan, 2014)**. A single miRNA can regulate many mRNA targets, and several miRNAs can regulate a single mRNA. All miRNAs have similar secondary hairpin structures, many of these are evolutionary conserved **(Zhang et al., 2006)**. The high conservation of miRNA sequences provides an opportunity to develop a novel type of molecular markers **(Fu et al., 2013; Yadav et al., 2014; Mondal and Ganie 2014; Ganie, Mondal, 2015)**.

miRNAs have been implicated in a number of diseases, and both miRNA inhibition and activation show great promise in the treatment of various types of cancer, and viral and metabolic diseases **(Singh et al., 2008)**.

Plants miRNAs play important roles in plant development and physiology, as well as tolerance to abiotic and biotic stresses **(Taylor et al., 2014)**. Expression of miRNAs in plants involves transcription from *MIRNA* loci by RNA polymerase II, multi-step processing of the primary transcripts, pri-miRNAs by the Dicer-like complex in plants and Drosha and Dicer in animals into precursors, pre-miRNAs with a characteristic hairpin structure **(Xie et al., 2010; Zhang et al., 2006)**. Then, pre-miRNA is further cleaved to a miRNA duplex (miRNA: miRNA*), a short double-stranded RNA (dsRNA) and a mature miRNA. Finally, mature miRNAs are predominantly incorporated in the in the RNA-induced silencing complex (RICS) **(Bartel, 2004)**.

The findings of **Zhang et al., (2012)** have demonstrated that exogenous plant miRNAs in food can regulate the expression of target genes in mammals. miR156a and miR168a are abundant in rice and the miR168a is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. Functional studies demonstrated that MIR168a could decrease low-density lipoprotein (LDL) removal from mouse plasma.

Lukasik and Zielenkiewicz (2014) by *in silico* approach identified in mammalian breast milk exosomes the highest abundance levels yielded the ath-miR166a (ath, *Arabidopsis thaliana*), while in the porcine breast milk exosomes, the zma-miR168a, zma-miR156a (zma, *Zea mays*) and ath- miR166a.

Several miRNA families have multiple members within the same plant species. For instance, miR395 has 18 members in rice. Although they are conserved as mature miRNA sequences, the other parts of miRNA precursor differ widely, suggesting that the different members of the same miRNA family may evolve at different rates within the same plant species **(Zhang et al., 2006)**.

As the link between metabolism and major disease processes becomes more well-defined, the identification of key molecular targets is leading to new therapeutic strategies **(Palmer et al., 2014)**. Dietary interventions have been used to change metabolism and to potentially alter disease progression. Since microRNAs may fine tune many molecular processes, it is reasonable to assume that dietary alterations that induce miRNA changes will modulate these pathways. Many microRNA families have already been associated with various nutrient interventions. MiRNA represent a link between nutrient intake, obesity and insulin resistance, and disease **(Ali et al., 2011)**.

Within our research we are focused on the exploitation of microRNA as molecular markers of plant genome characterization and mapping their activity in different plant species of nutritional and pharmaceutical importance (*Linum usitatissimum, Messpilus germanica, Silybum marianum, Hedera helix* and *Ginkgo biloba,*), plant organs and tissues (flower buds, flowers, bolls, leaves, seeds) and developmental stages (flowers development, flowering, seed development). The abundance of mature miRNAs, which is linked to the expression of *MIRNA* genes, varies greatly among different miRNAs, tissue types or developmental stages, indicating the spatially and temporally regulated expression patterns of plant miRNAs **(Xie et al., 2010)**.

Understanding the function of miRNAs in the complex molecular network regulating the development and function of various cells and tissues will increases our knowledge about the potential role of miRNAs and their involvement in gene regulation **(Singh et al., 2008)**.

MATERIAL AND METHODOLOGY

Based on the type of plant biological material was the total genomic DNA extracted either commercial isolation kit or different isolation protocols **(Saghai-Maroof et al., 1984; Padmalantha and Prasad, 2006)**. The extracted DNA was quantified by the Implen NanoPhotometer®, and diluted to 70 ng.µl^{-1}. The primers for the miRNA-based markers were designed according to the mature miRNAs sequences, which are part of the miRNA precursors (pre-miRNA), originating from the miRNA database (http://www.mirbase.org/). The single forward primers and the universal miRNA reverse primer **(Kulcheski et al., 2010; Chen et al., 2005)** were combined to perform a marker assays. The effectiveness and species transferability of used primers was confirmed in previous studies **(Hlavačková et al., 2015; Ražná et al., 2015)**.

miRNA-marker assay

Polymorphism analyzes were applied for three flax genotypes of different alpha-linolenic acid content, genotype Amon (less than 3%), Raciol (30%) and Libra (more than 57%), 5 genotypes of milk thistle of various origins, 6 genotypes of medlar and one genotype of ivy.

The modification of miRNA-based markers assay was perfomed based on methodologies **Fu et al., (2013)** and **Yadav et al., (2014)**. PCR amplifications were perfomed in a 20-µl reaction mixture containing 70 ng of genomic DNA, 10 pmol.dm^{-3} of each primer, 2 units of DreamTaq

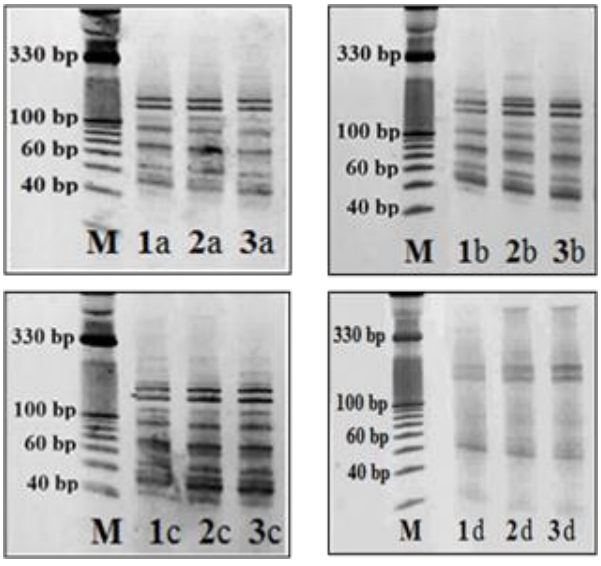

Figure 1 PCR amplification profiles generated with lus-miR168-F/miR-R markers across tissues of buds (a), flower petals (b), bolls (c), leaves (d) of flax genotypes. Legend: M- 10 bp DNA Ladder, genotypes: 1- Amon, 2- Libra, 3- Raciol.

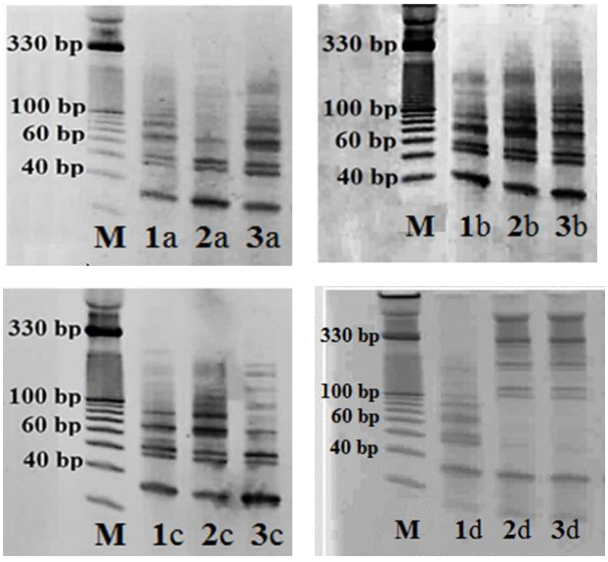

Figure 2 PCR amplification profiles generated with gm-miR156b-F/miR-R markers across tissues of buds (a), flower petals (b), bolls (c), leaves (d) of flax genotypes. Legend: M- 10 bp DNA Ladder, genotypes: 1- Amon, 2- Libra, 3- Raciol.

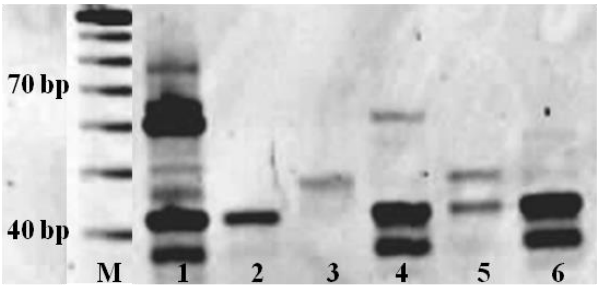

Figure 3 PCR amplification profiles generated with markers gm-miR156b/gm-miR171a of *Mespilus germanica* genotypes. Legend: M - 10 bp DNA Ladder, genotypes: 1 - Sz. Rozsa, 2 - Holandská Veľkoplodá, 3 - GR1, 4 - GR2, 5 - GR3, 6 - GR4.

DNA polymerase, 0.8 mmol.dm^{-3} dNTPs (Bioline) and 1 × DreamTaq Buffer (KCl, $(NH_4)_2SO_4$, 20 mmol.dm^{-3} $MgCl_2$). The PCR amplification programme used the 'touchdown' method as follows: initial denaturation at 94 °C for 5 min; 5 cycles of 30 s at 94 °C, 45 s at 64 °C (with a 1 °C decrease in annealing temperature per cycle), and 60 s at 72 °C; 30 cycles of 30 s at 94 °C, 45 s at 60 °C, and 60 s at 72 °C; and the final extension at 72 °C for 10 min. The samples were subsequently stored at 8 °C.

The PCR products were separated using 15% TBE-PAGE gels, running in 1 × TBE Running Buffer at a constant power 90 V, 25 mA for 120 min. The polyacrylamide gels were stained with the GelRed™ Nucleic Acid Gel stain and were visualized in the G-Box Syngene electrophoresis documentation system. For the recording of loci number and unique identification of fragments, the gels were analyzed by the GeneTools software (Syngene).

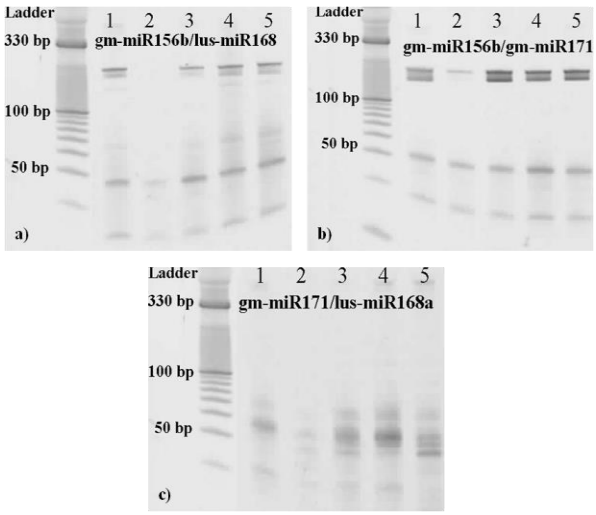

Figure 4 PCR amplification profiles generated with combination of markers: a) gm-miR156b/lus-miR168, b) gm-miR156b/gm-miR171a and c) gm-miR171a/lus-miR168 of *Silybum marianum* samples.
Legend: M - 10 bp DNA Ladder, genotypes: 1 - Silyb 1, 2 - Silyb 2, 3 - Mirel, 4 - Silma, 5 - sample of unknown origin.

miRNA expression analysis by qRT-PCR

The methodology of qRT-PCR analysis of miRNA was done based on **Barvkar et al., (2013)** and **Neutelings et al., (2012)** approach. For qRT-PCR analysis were used three genotypes of flax differing content of alpha-linolenic acid (Amon, raciol, Libra). From the 10-days old *in vitro* seedlings was isolated miRNA by PureLink miRNA Isolation Kit (Life Technologies). Consequently was miRNA diluted in 10 mM Tris-HCl, pH 7.0 in ratio 1:1 and quantified by NanoPhotometr (Implen). By means of the kit NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR (Invitrogen) was done miRNA polyadenylation and cDNA synthesis. qRT-PCR reactions were performed by SYBR® GreenER qPCR SuperMix Universal (Invitrogen) based on manufacturer instructions. cDNA was diluted in ratio 1:10. Two types of miRNA, gm-miR156b and lus-miR168 were analyzed. As a reference gene *UBE2* (Ubiquitin-conjugating enzymes E2) was selected **(Barvkar et al., 2013)**.

The conditions of qRT-PCR were as followed: 2 min incubation at 50 °C, 95 °C 10 min, 40 cycles of 95 °C 15 sec, 57 °C 60 sec and 95 °C 10 sec. Fluorescence reading of the PCR product took place after the analysis phase of the amplification and melting points were read for 30 seconds and the temperature rise of 0.5 °C. Analyzes were performed by CFX96 Real-Time detection system. Reactions were done in triplicates.

On the basis of the average value of threshold cycle of miRNA and reference gene *UBE2* value $2^{-\delta CT}$ **(Shi and Chiang, 2005)** was calculated.

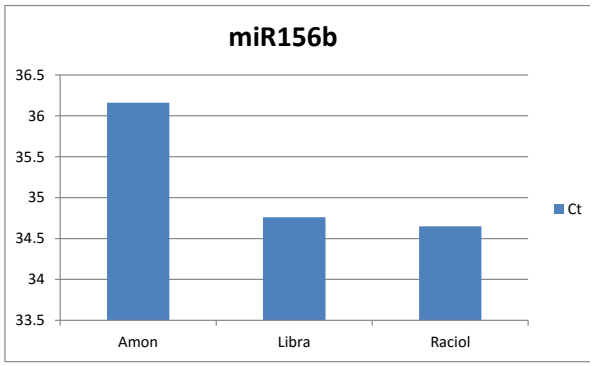

Figure 5 Comparison of gm-miR156b expression based on values of threshold cycle (C_T) in flax genotypes of different alpha-linolenic acid content.

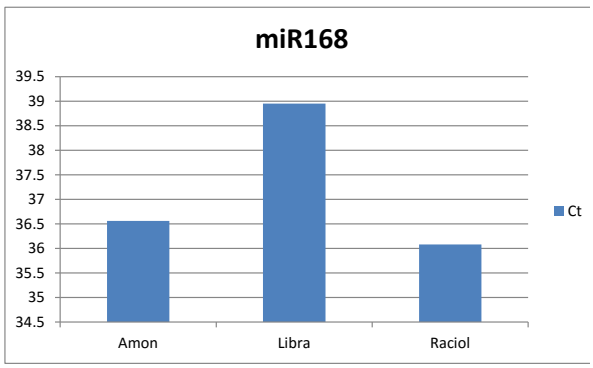

Figure 6 Comparison of lus-miR168 expression based on values of threshold cycle (C_T) in flax genotypes of different alpha-linolenic acid content.

RESULTS AND DISCUSSION

The aim of our research was mapping the abundance, polymorphism and activity of several conservative (miR156, miR168 and miR171) miRNAs in plants genome.

One of the extensively reviewed miRNA networks in plants includes the conservative miR156 family, which consists of 10 miRNAs (miR156a-j), and miRNA156a-f have identical nucleotide sequences (miRBase) **(Bari et al., 2013)**. miR156 family members are predicted to be associated with the mRNAs of genes encoding the DNa-binding proteins - squamosa promoter binding protein (SBP), transcription factor in monocot and dicots and F-box protein sequences **(Barvkar et al., 2013; Xie et al., 2010)**. SBS transcription factors regulated many developmental processes of plants. miR156 regulates processes at post-germinative stages, which is important for the transition to autotrophic growth, it regulates transition phase from the juvenile to adult stage **(Nonogaki, 2010)** and also play a critical role in reproductive phases such as shoot maturation **(Shikata et al., 2009)**. The study of **Kulcheski et al., (2010)** provided evidence that the expression stability of miR156b was the highest across the soybean tissue and applied stress conditions.

One of the target sequences of miR168 family are sequences of cytochromeP450 which is involved in a wide range of biosynthetic reactions, including fatty acid biosynthesis. The miR168 is also considered as the biomarker of plant stress response **(Bej and Basak 2014)**.

Target sequences of the miR171a are represented by HAMs genes **(Bari et al., 2013)** which belong to the GRAS transcription factor family. These genes play crucial roles in diverse fundamental processes of plant growth and development **(Huang et al., 2015)**.

Of the following figures (Figure 1, 2, 3 and 4) it is observed that molecular markers based on microRNA represent polymorphic and significant type of molecular markers. It is more apparent that the expression profile of miR156b, miR168 and miR171a is species specific and even tissue specific as confirmed by several studies (**Barvkar et al., 2013; Neutelings et al., 2012**). Tissue-specific expression of miRNA (Figure 1 and 2) also points to the different levels of miRNA activity in various stages of development of the plant organism. The same miRNA can be found in different abundance among tissue types or developmental stages, indicating the spatially and temporally regulated expression patterns of plants miRNAs **(Jones-Rhoades et al., 2006; Xie et al., 2010)**. From this point of view is quite significant polymorphism profile of miR156b in various flax tissues of genotypes varying in the content of alpha-linolenic acid (ALA). It can be observed visible difference among individual genotypes in regard to miRNA profile. Interesting is distinguished pattern of intermediate type of flax genotype Amon (less than 3% content of ALA) and other two oily genotypes with higher ALA content (Raciol 30%, Libra more than 57%). Detected polymorphism by miRNA-based molecular markers may indicate sequence changes in the miRNA loci, which consequently may change the regulation pattern of targeted genes **(Htwe et al., 2015; Fu et al., 2013)**.

Considering the indirect correlation between the abundance of miRNAs and the expression level of their target sequences **(Barvkar et al., 2013; Neutelings et al., 2012)** we can assume the spatially and temporally machinery of metabolic processes regulation as well as the expression patterns of plant miRNAs.

The effectiveness and reliability of miRNA molecular markers has been confirmed for medlar genotypes (Figure 3). Medlar as a source of new valuable compounds and their pharmacological properties, has gained a value in human consumption and commercial importance in recent years **(Rop et al., 2011)**. By the combination of miRNAs markers, miR156b and miR171a, was possible to distinguish almost all six genetic resources collected on the territory of Slovak Republic. This confirms the status that miRNA-based molecular markers comprise a novel functional molecular marker **(Yadav et al., 2014; Fu et al., 2013)**. miR171 potentially targets a beta-1,3 glycanase-like transcript. The corresponding enzyme is implicated in developmental as well as biotic and abiotic stress processes **(Roy Choudhury et al., 2010)**.

Silybum marianum (L.) Gaertn. or milk thistle is a medicinal plant of unique pharmaceutical properties. It is the most cultivated medicinal plant in Slovakia. In the years 2014-2015 it exceeded the growing area of 1000 hectares **(Habán et al., 2015)**.

Although monomorphic but miRNA-type specific microRNA profile can be observed in milk thistle genotypes of different origine (Silyb 1- Malanta, Slovak Republic, Silyb 2 - Šumperk, Czech Republic, Mirel - Brno, Czech Republic, Silma – Poland and sample of unknown origin) in two miRNA markers combination (Figure 4, a and b). The fingerprint profile amplified by primer pairs combinations ranged from 4 (gm-miR156b/gm-miR171a) to 7 (gm-miR156b/lus-miR168) miRNA loci per genotype. The another marker combination (Figure 4, c) has provided polymorphic miRNA loci pattern, even genotype specific. In comparison with previous two species, namely the flax genotypes, it can be stated that the abundance of analyzed types of miRNAs in milk thistle genome is not so significant although the studied miRNAs families represent conservative types of miRNA families. It seems that for the mapping of this genome will be required the application of species-specific miRNAs.

It is apparent that the research of food resources includes various approaches based on application of different types of molecular markers or molecular analyses **(Balážová et al., 2016; Gálová et al., 2015; Žiarovská et al., 2015)**.

Results based on qRT-PCR and evaluation of $2^{-\delta CT}$ value suggest significant difference in miR156b activity of Amon genotype (low content of ALA) in comparison to other two genotypes with medium and high content of alpha-linolenic acid (Figure 5). Within the miR168 expression analysis was the difference recorded between genotype Libra (high content of ALA) and other two genotypes Amon and Raciol (Figure 6).

The most of miRNA families, including miR156 and miR168, are characterized by negative correlation between miRNA expression and expression of their target sequences. It means, that if the expression of a specific miRNA increased, the activity of target sequences

regulated by this miRNA will be suppressed and vice versa.

The miR168 expression profile, from the above point of view, can indicate two possible explanations. As we mentioned before, most of the miRNA families have several target sequences, not excluding these two types of miRNAs. Significantly higher expression of miR168 in Libra genotype (57% ALA) points out downregulation of the target sequences, one of which is the cytochrome P450 involved in a wide range of biosynthetic reactions. It seems that the genome of this genotype mediates the production of miRNA168 increasingly over other genotypes. . It should be recalled that for the analysis were used 10-days old seedling *in vitro*. There is another explanation connected to reaction of the flax genome to stress factor presented by cultivation under *in vitro* conditions. miR168 as a stress biomarker molecule may indicate greater sensitivity of genotypes with high content of fatty acid to abiotic stress.

From the Figures 2d and Figure 5 can be observed similar pattern of miRNA expression in leaves tissues. miRNA loci profile generated by miR156b-F/miR-R markers and expression profile generated by qRT-PCR seems to show different behavior of miR156 in genotype Amon in comparison to oily genotypes Libra and Raciol. The answer might be found in the character of the major group of target sequences of miR156, what does mean the SBS transcription factors. It seems that in oily genotypes (Libra and Raciol) are, due to downregulation of miR156, its target sequences more active than in intermediate genotype (Amon), which may be associated with a plant structure of oily genotypes or indirectly with higher metabolism of fatty acids in those two genotypes. These results are confirmed by the research of **Nonogaki (2010)**. As the consequence of a decrease expression in miRNA levels is the increased accumulation of some of SPS transcripts (and proteins) which are necessary for the juvenile to adult transition in *Arabidopsis* seedlings.

The aim of the research was to highlight the broad spectrum of miRNA molecules behavior in various food resources, functional foods and medicinal plant. As was observed, different plants miRNAs accumulate at different levels depending on developmental stage or the plant tissues. It can be presumed that their regulation pattern of gene expression in human genome may be influenced also by several aspects of human metabolism and health conditions.

CONCLUSION

The aim of the research was to highlight the broad spectrum of regulatory impact activities of miRNA molecules in different plant species of nutritional and pharmaceutical uses. As has been recorded, the polymorphism and expression of analyzed gm-miR156, lus-miR168 and gm-miR171a is not only species- but also tissue- and developmentally-specific. It points out the fact that, depending on the type of food of plant origin (species, state of maturity, bio products or traditional agriculture), miRNA molecules can regulate the expression of genes of the human genome in many ways.

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Contact address:
Katarína Ražná, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: katarina.razna@uniag.sk.

Milan Bežo, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: milan.bezo@uniag.sk.

Lucia Hlavačková, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: lucia.hlavackova@uniag.sk.

Jana Žiarovská, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.ziarovska@uniag.sk.

Marián Miko, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marian.miko@uniag.sk.

Ján Gažo, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jan.gazo@uniag.sk.

Miroslav Habán, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Sustainable Agriculture and Herbology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslav.haban@uniag.sk.

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EFFECTS OF CROSS-LINKING MODIFICATION WITH PHOSPHORYL CHLORIDE ($POCL_3$) ON PYSIOCHEMICAL PROPERTIES OF BARLEY STARCH

Zahra Malekpour, Mohammad Hojjatoleslamy, Hooman Molavi, Javad Keramat, Amirpouya Ghandehari Yazdi, Mohammad Ali Shariati

ABSTRACT

Chemical methods are one of the comon method in starch modification. This study aimed at investigating of cross-link affection of phosphoryl chloride with two different levels 0.5 and 1g.kg^{-1} in order to enhance funciotnal proeprties and physiochemical changes on extracted starch from barley variety Bahman which cultivates in Chahr-Mahal Bakhtiari Province of Iran. Obtained results indicated that cross-linking leads to reduce sweeling power of strach granuls compred to natural starch and the amount of reduciton increase via the substitituin level. Powerfull cross-linkingnetween starch chains casue more resistance of granules to seweeling which is increased by means of cross-linking dgree. Additioally, investigationresults from synersis revealed that releasing water percentage in cross-linked starches increase in comparison to natural starches and this amount depends onthe amount of cross-link surface with a significantly difference in (α <0.05). Gelatinization temperature in both levels negligibly increased by modification where in low level of cross-linking was more. Furthermoe evaluating gelation temperatures of both natural and cross-linked modified starches showed that addition of phosphate groups in starch and creating extra coovalent bonds make granues more compressed reulting in slight increase of To, Tp, Tcin barley starch. Icreasing of temperature observed more in less concentration of cross-links. Evaluation of viscosity changes also revealed that this modification depending on increasing the amount of Phosphoryl Chloride led to increasing peak temperature, diminish peak and setback viscosity. Result also exhibited that in morphological level, cross-link causes to incidence changes in particles' diameter size. The comparison of diameter average and frequency between natural starch and cross-links starch exhibited that in cross-linkd treatment with 0.5% phosphoryl chloride, increase in frequency of granules with diameter of 6 – 10μm and >20 μm obersced. While frequency of granules with diamater size of 2 – 6 μm and 10 – 20 μm has been reduced to 0 which create bigger granules.

Keywords: Barley; starch; modification; cross-linking; physiochemical properties

INTRODUCTION

Barley belongs to Poaceae and Hordeum species **(Sullivan et al., 2013)** uses more in malting, feeding animals, production of starch and ethanol as well **(Myllärinen et al., 1998)**. Starch is composed of two main constituents including amylose and amylopectin (72 – 87%). Starch is used as thickener, stabilizer, and gelling agent in food industries **(Dubois et al., 2001)**, but due to some restricting factors such as low thermal and cutting resistance **(Singh and Singh, 2005)** high tendency to staling and high synersis (**Yosif et al., 2012)** its application has limited in industries; however application can widen through modification **(Singh and Singh, 2005)**. First time, starch modification operated in year 1800 **(Kaur, Singh and Singh, 2006)**. Several targets define for development of functional properties such as strengthening the bonds, increase of thermal resistance, and increase of water binding capacity, emulsion stability and economic benefits **(Light, 1989)**. Cross-linking or intertwined starch is one of the conventional chemical modifications **(Zhao et al., 2012)**. Cross-linking factors include Sodium triphosphate (STM), Epihydrochlorine (EPI), phosphoril chloride (POCl3), and mixture of adipic acid, anhydride acetic and vinyl chloride **(Singh et al., 2007; Zhao et al., 2012)**. In this method, reaction factors react with starch hydroxyl groups **(Miyazaki et al., 2006)** which enhance through covalent or hydrogen bond inter and among granule molecules **(Singh et al., 2007; Ackar et al., 2010)**. Cross-linked starch strengthens versus heat, acid and cutting in comparison with natural starch **(Hung and Morita, 2005; Polnaya et al., 2013; Raina et al., 2007; Xiao et al, 2012)**. The target of this study is to investigate barley starch properties. Base on **Jun et al., (2003)** barley and corn starches use to microencapsulation of volatile compounds of flavor in meat industry **(Abbas et al., 2010)**.

MATERIALS AND METHODS

In present study, starch has extracted from barley Bahman variety which cultivated in Lordegan region, Chaharmahale Bakhtiari province of Iran. Initially 100 g of barley flour weighed with balance model Mark Sartoris AC 120 s, Germany and 0.0001 accuracy, mixed with 500 mL sodium hydroxide solution (0.005 – 0.025 M) and stirred at 25 °C for 30 min. Obtained mixture centrifuged with 1400 g (centrifuge Tehtnika, model 322-A, Slovenia), then sedimentation filter through a screen with mesh size 270 (50 μm). Permeated suspension neutralized with chloridric acid 1 M and recentrifuged, and over layer of starch separated with spatula remained sedimentation

dissolved in the water again and dissolving continues to reach the minimum amount of creamy layer on it (3 times). Final sedimentation dried in oven (model Mark Memmert UNB-400, Germany) at 40 °C for 24 hr **(Lim et al., 1992)**.

PRODUCTION OF CROSSLINKED STARCH

Regarding production of cross-linked starch, **Kaur et al., (2012)** method used. In this method, 15 g of starch weighed with balance model Marksaritus AC 120S, Germany AND 0.0001 accuracy, then mixed with 24ml water and 0.3 g sodium sulfate added to it, pH of obtained mixture(pH meter model Mark metrum 827, Switzerland used to measured pH) adjusted by sodium hydroxide solution (0.5M) at 25 °C. Phosphoryl chloride (0.5 and 1 g per starch kilo) added by micro-syringe and immediately container sealed. pH adjusted by chloridric acid (0.1 M) on 5.5 after 1hr. sedimentation washed by distilled water and filtered by vacuum filter and finally dried in oven Markmemmert model UNB-400, Germany) at 40 °C **(Kaur et al., 2012).**

DETERMINATION OF SWEELING POWER

Lich et al., (1959) method used to determine swelling power. Initially, 0.1 g sample base on dried weight weighed in lidded test tube and 10 mL water added to it. Tubes placed and shook in water bath (Mark hak model SWB-20, Germany and equipped with shaker with constant race) at 95 °C for 30 min, then cooled to ambient temperature and centrifuged in 2500 x g for 10 min. Supernatant accurately removed and tube containing sedimentation reweighed. Regarding equation 1 swelling power measured **(Leach et al., 1959)**.

Equation (1):

SP – Sweeling percent

$$SP = \frac{\textbf{final weight} - \textbf{weight of empty pipe}}{\textbf{starch weight}} \times \mathbf{100}$$

DETERMINATION OF SYNERSIS PERCENT

To determine synersis percent, **Gioti et al., (2006)** method was used. Starch suspension 5% w/w prepared and 30 min mixed in water bath (model Markmemert w3 B10), heated at 90 – 95 °C, and then quickly cooled to ambient temperature in cooling bath. Starch paste placed at 4 °C for 24 hr, centrifuged at 2700 x g for 15 min and measured released water reported as synersis percent **(Jyothi, Moorthy and Rajasekharan, 2006)**.

INVESTIGATION OF VISCOSITY CHANGES

To determine viscosity changes, Initially, a 8% w/w suspension of starch prepared in pH = 5, then viscosity changes measured by viscometer model Brookfield DV III, America in temperature range 40 – 93 °C, keeping at 93 °C and then reduces it from 93 °C to 40 °C **(Das et al., 2010)**. Determination of substitution degree of cross-link

Substitution degree defines as the number of hydroxyl group in each anhydrous glucose having the ability of derivation with replacing groups **(Yosif et al., 2012)**. To investigate of this factor in cross-link starch, measures based on **Ackar et al., (2010)** method, viscosity data and equation 2 **(Hung and Morita, 2005)**.

Equation (2):

DS – Degree of substitution

$$DS = \frac{\textit{viscosity peak of natural starch} - \textit{viscosity peak of modified starch}}{\textit{viscosity peak of natural starch}} \times \mathbf{100}$$

INVESTIGATION OF THERMAL PROPERTIES

To investigate thermal properties of barley starch **Bello-Perez et al., (2010)** method used. Differential scanning calorimeter used to conduct thermal parameters of starch. In this method, 2 mg starch base on dry weight weighed in aluminum container and 7 mL deionized water added to it, then container sealed, and placed in ambient temperature in order to uniformly distribution of water and homogenization of sample . Sample placed in DSC model F3-200 and heated with race of 10 °C.min^{-1} from 20 °C – 120 °C, and automatically present data including Onset (To), peak (Tp), conclusive (Tc) and ΔH **(Bello-Perez, et al., 2010)**.

INVESTIGATION OF MORPHOLOGICAL PROPERTIES

Electronic microscope model Markzayef used take images and **Blupers et al., (2010)** method with a few changes. Samples fix on a conductive stick and cover with a gold layer **(Bello-Perez, et al., 2010)**. Image proplus software used to analyze images.

Statistical analysis

Complete random design and Duncan test using software SPSS ver. 21th used to conduct statistical analysis of data (α <0.05).

RESULTS AND DISCUSSION

DEGREE OF CROSS LINKING

Table 1 shows that increase of cross linking, cross-linking degree increased. Obtained results were in agreement with **Xiao et al., (2012)**. on investigation of different concentration of epi hydrochlorine on rice starch where cross linking degree increased within increase of epihydrochlorine concentration (16) **Co et al., (2010)**. Reported that an increase 0 – 10% of cross-linking concentration on corn starch cross-linking degree increased **(Koo et al., 2010)**.

SWELL POWER

Comparison of obtained results of swelling power of control and modified samples have summarized in Table 2 revealed a significant difference. Creation of cross-links reduces swelling power of granules with respect to natural starch and this reduction increase by the amount of substitution level. These results were in agreement with **Kim and Yoo (2010)** about using POCl3 on sweet potato and **Majzoobi et al., (2012)** in investigation of wheat starch phosphorlization. It is thought that reduction of swelling power attributed to creation of intermolecular bridges by remained phosphorus after cross linking reaction **(Majzoobi et al., 2012)**.

Cross-linking develops hydrogen bonds among granules and restricts swelling during gelatinization **(Kim et al., 2010)** due to high concentration of cross-linking degree in

Table 1 degree of cross-linking related to control starch and modfied starches.

Treatment	Degree of cross-link percent (%)
Control	0
Cross-linked starch (0.5%)	32.30
Cross-linked starch (1%)	46.15

Table 2 the average of sweeling power related to related to control starch and modfied starches.

Control	Cross-linked starch (0.5%)	Cross-linked starch (1%)
$14.21^{b} \pm 0.66$	$10.05^{a} \pm 2.02$	$7.27^{a} \pm 0.27$

Table 3 the amount of synersis in control and cross-linked starches.

Control	Cross-linked starch (0.5%)	Cross-linked starch (1%)
$65.10^{a} \pm 2.90$	$74.80^{b} \pm 2.34$	$78.94^{b} \pm 6.92$

Table 4 viscosity measurement of control and cross-linked starch.

Starch type	Setback viscosity	Breaking viscosity	Peak viscosity	Pasting Temp. (°C)	peak Temp (°C)
Control starch	5.4	0.96	2.6	63.60	69.55
Cross-linked starch (0.5%)	0.16	1.72	1.76	65.38	72.55
Cross-linked starch (1%)	0.08	1.36	1.4	66.98	76.2

Table 5 Affection of modification amount of thermal properties of barley starch.

Treatment	T_0	T_P	T_C	T_C- T_0	H(J/g)Δ
Control starch	59.8	65.4	73.2	13.4	-0.3412
Cross-linked starch (0.5%)	61.8	66.4	73.8	12	-0.2822
Cross-linked starch (1%)	60.7	65.9	73.4	12.7	-0.2403

presence of more concentrations of cross-linking factor **(Kaur et al., 2012)**.

Choi and Ker (2004) believe that cross-linked starch granules have more resistance to time and temperature of heating. Strong links between starch chains leads to increase of granules' resistance to swelling i.e. by increasing cross-linking degree, resistance increases **(Yosif et al., 2012)**.

AFFECTION OF CROSS-LINKING ON SYNERSIS PERCENT

Comparison of data average in Table 3 shows a significant difference between the amount of released water in natural starch and modified starch. The amount of released water in intertwined starch has increased in comparison with natural starch, furthermore increased by increasing of cross-link factor. These results are in agreement with **Mirmoghtadaei et al., (2009)** on oat starch.

VISCOSITY

Table 4 depicts that heating in 40 – 93°C causes to increase of viscosity gradually. When starch heats in high amount of eater, granules swell, some parts of it dissolves and in form of suspension distributed in surrounded medium (continuous phase) and maximum of viscosity occurs in this point. Continuously, due to dispersion of starch molecule when temperature is constant at 93 °C viscosity decreased, then in temperature reduction from 93 °C to 43 °Conce again viscosity increases. It is thought that arrangement of amylose linear chains (those formerly dissolved as a result of heating and keeping in constant temperature) causes to create lots of cross-links within gel forming process **(Bello-Perez et al., 2010)**.

Investigation of obtained results exhibited that viscosity peak in cross-linked starch has reduced while temperature of viscosity peak increased. It is thought that increase of strong intermolecular bonds due to cross-linking process which results in swelling and decrease of viscosity peak. Besides by increasing of the cross-linking surface, viscosity peak showed more reduction and Peak temperature more increase.

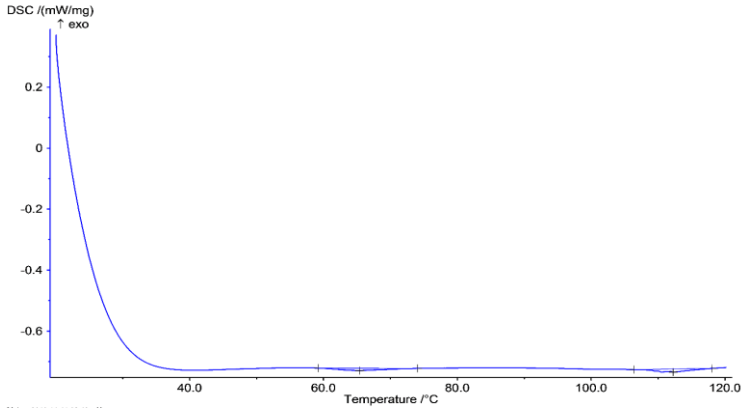

Figure 1 Thermal analysis curve of control starch.

THERMAL PROPERTIES

Analysis of obtained results of Table 5 including analysis of thermal curve related to natural barley starch (Figure 1), curves related to thermal analysis of cross-linked starch with 0.5% (Figure 2) and Figure 3 which shows cross-linked starch with 1% revealed that onset temperature (T0), peak temperature (Tp) and conclusive temperature (Tc) were 59.8 °C, 65.4 °C and 73.2 °C respectively. Obtained temperature were in the range measured by **Gujeral et al., (2013)** where the range of onset, peak and conclusive temperatures were 59.08 – 62 °C, 63.56 – 68.3 °C and 68.56 – 74.71 °C investigated respectively **(Gujral et al., 2013)**. Investigation of natural starch and cross-linked starch in present study suggested that this modification has increased negligibly To, Tp, and Tc of barley starch. These results were in agreement with **Majzoobi et al., (2012)** on wheat starch. Phosphates groups bond with starch molecules through covalent bonds and thus starch granules become more compressed, consequently followed by less molecule motivation and therefore gelatinization occurs in higher temperatures **(Carmona-Garcia et al., 2009)**.

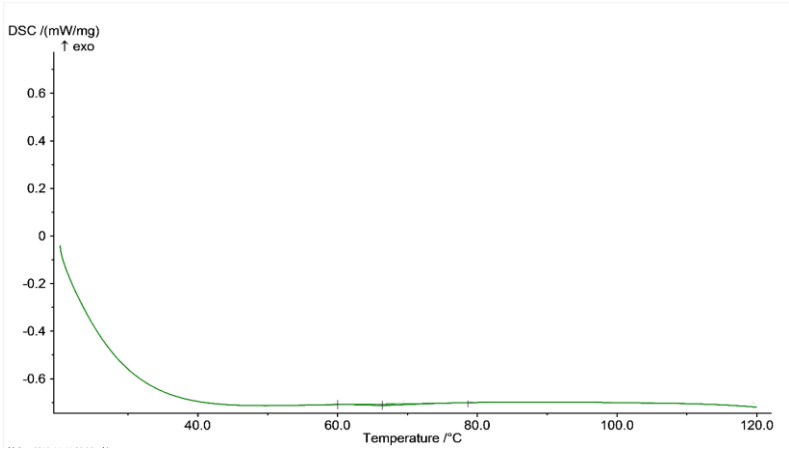

Figure 2 Thermal analysis curve of cross-linked starch (0.5%).

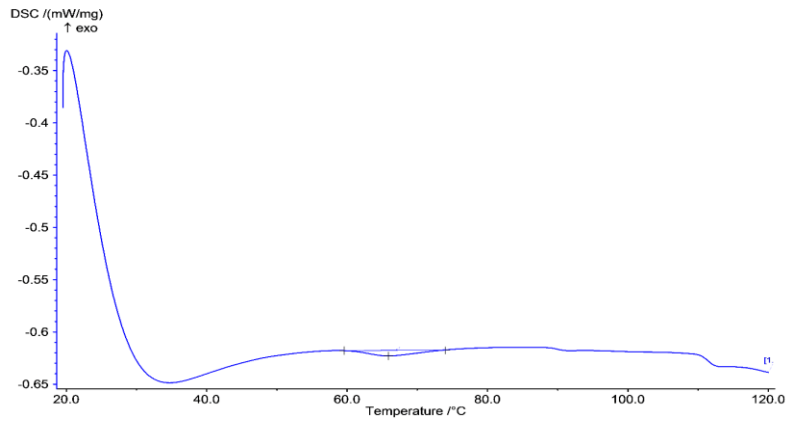

Figure 3 Thermal analysis curve of cross-linked starch (1%).

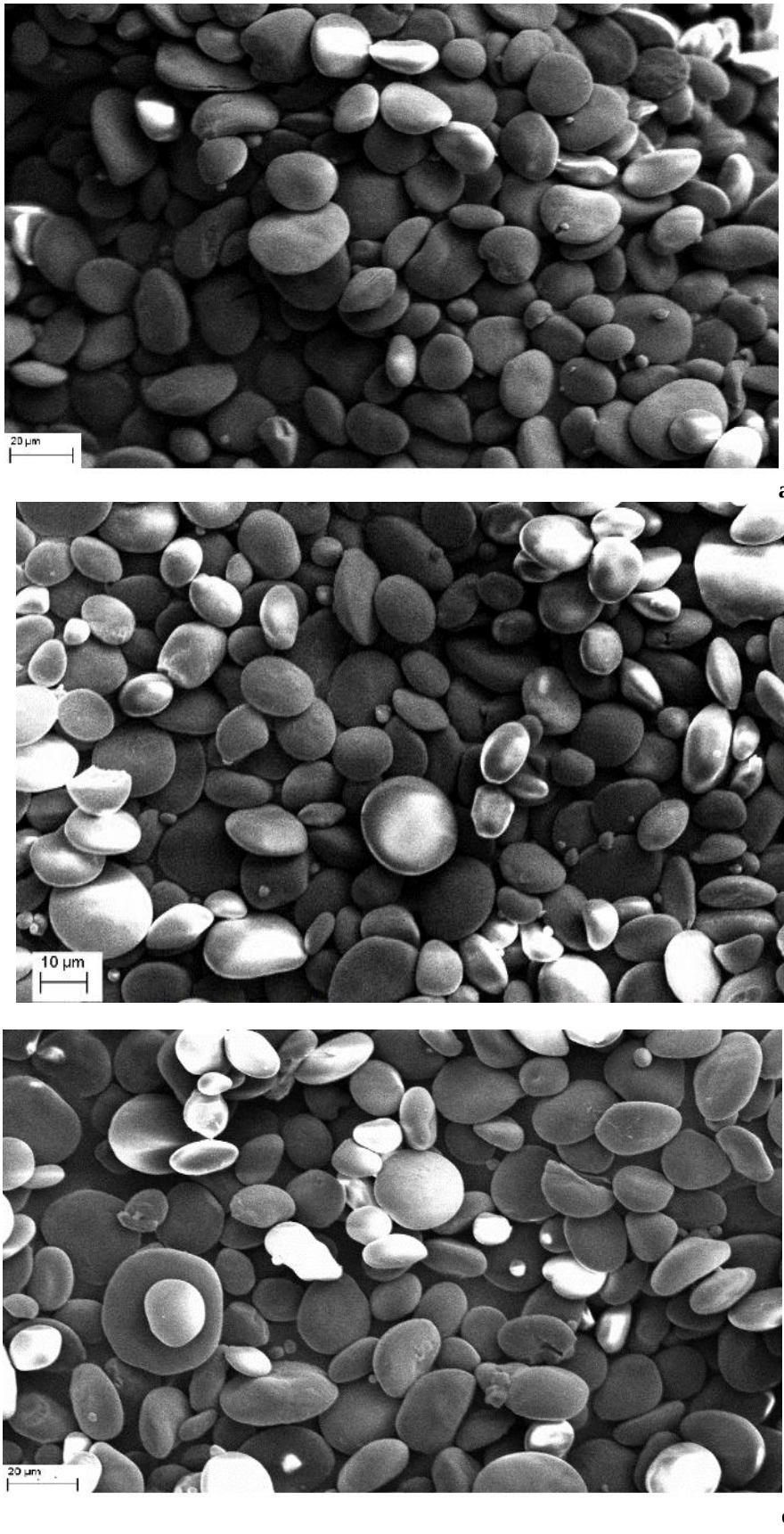

Figure 4 images of electronic microscope: a) control starch b) cross-linked starch (0.5%) c) cross-linked starch (1%) with zoom of 1.5 KX.

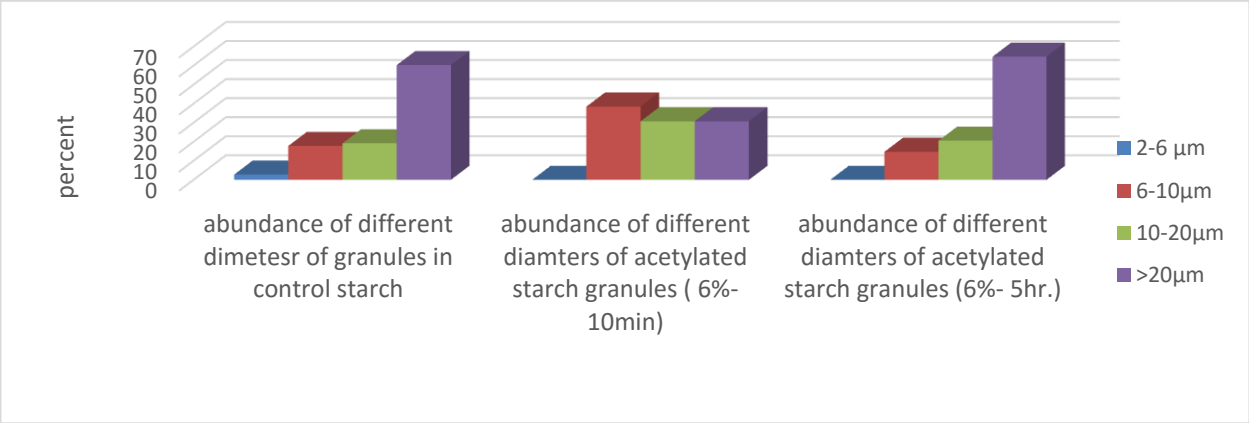

Figure 5 Investigations of abundant percent of granules' thickness in control and cross-linked starches.

Kim and Yoo (2010) found that in sweet potato, cross-linking with POCL3 creates no change in onset and conclusive temperatures. As an appropriate parameter of being crystal, gelatinization enthalpy point at damaging to molecule discipline due to breaking of hydrogen bonds **(Alvani et al., 2011)** after gelatinization. Low enthalpy introduces low stability of crystal structure **(Sing et al., 2006)**.

MORPHOLOGICAL PROEPRTIES

Comparison average of diameter and frequency (Figure 5) between image processing of natural starch and cross-linked starch (Figure 4) suggested that in crossed-link starch with 0.5% phosphoryl chloride, more granules with diameter of 20 µm and 6 – 10 µm observed.

While frequency of granules with diameter of 2 – 6 µm and 10 – 20 µm diminished. It is thought that aggregation of smaller granules and creation of larger granules is the reason of disappear of some size of granules. In cross-linked starch with 1% phosphoryl chloride, more reduction in granules with diameter of 20 µm observed, in addition that frequency of granules with 6 – 20 µm increased.

CONCLUSION

Starch modification creates novel properties in starch. Cross linking causes to increase of synersis and reduce of swelling so that has direct proportional with cross-linking factor (chloride phosphoryl). In comparison with control, Gelatinization temperature of modified starch increased. However no proportional trend observed through increase of cross linking. Furthermore the viscosity of cross-linked starch decreases with respect to control.

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CONTACT ADDRESS:

Zahra Malekpour, Department of Food Science and Technology, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran, E-mail: zahra.malekpour56@yahoo.com

Mohammad Hojatoleslamy, Department of Food Science and Technology, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran, E-mail: mohojjat@iaushk.ac.ir

Hooman Molavi, Department of Food Science and Technology, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran, E-mail: hmolavi2010@yahoo.com

Javad Keramat, Department of Food Science and Technology, Isfahan University of Technology, Esfahan, Iran, E-mail: keramat@cc.iut.ac.ir

Amirpouya Ghandehari Yazdi, Department Agriculture Faculty, Tarbiat modares, Tehran, Iran, E-mail: foodtechnology43@yahoo.com.

Mohammad Ali Shariati, Orel State Agrarian University, Russia, E-mail: Shariatymohammadali@gmail.com

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POSSIBILITIES OF MICROSCOPIC DETECTION OF ISOLATED PORCINE PROTEINS IN MODEL MEAT PRODUCTS

Michaela Petrášová, Eliška Zichová, Matej Pospiech, Bohuslava Tremlová, Zdeňka Javůrková

ABSTRACT

In recent years, various protein additives intended for manufacture of meat products have increasing importance in the food industry. These ingredients include both, plant-origin as well as animal-origin proteins. Among animal proteins, blood plasma, milk protein or collagen are used most commonly. Collagen is obtained from pork, beef, and poultry or fish skin. Collagen does not contain all the essential amino acids, thus it is not a full protein in terms of essential amino acids supply for one's organism. However, it is rather rich in amino acids of glycine, hydroxyproline and proline which are almost absent in other proteins and their synthesis is very energy intensive. Collagen, which is added to the soft and small meat products in the form of isolated porcine protein, significantly affects the organoleptic properties of these products. This work focused on detection of isolated porcine protein in model meat products where detection of isolated porcine protein was verified by histological staining and light microscopy. Seven model meat products from poultry meat and 7 model meat products from beef and pork in the ratio of 1:1, which contained 2.5% concentration of various commercially produced isolated porcine proteins, were examined. These model meat products were histologically processed by means of cryosections and stained with hematoxylin-eosin staining, toluidine blue staining and Calleja. For the validation phase, Calleja was utilized. To determine the sensitivity and specificity, five model meat products containing the addition of isolated porcine protein and five model meat products free of it were used. The sensitivity was determined for isolated porcine protein at 1.00 and specificity was determined at 1.00. The detection limit of the method was at the level of 0.001% addition. Repeatability of the method was carried out using products with addition as well as without addition of isolated porcine protein and detection was repeated 10 times. Repeatability in both, positive and negative samples, for isolated porcine protein was determined at 100%. The results show that the histological processing of cryosections stained using Calleja is suitable for detecting isolated porcine protein in meat products.

Keywords: meat; collagen; isolated protein; light microscopy

INTRODUCTION

In recent years, development of a variety of functional ingredients for the most possible efficient optimization of the quality and texture of meat products is increasing in the meat industry. Functional raw-materials of animal origin include different animal derivatives of meat, skin and blood, as well as milk and egg products (collagen, gelatin, whey protein, casein, albumin, dehydrated beef protein, and other protein isolates) **(Petracci et al., 2013)**. Proteins play an important role in the functional and sensory properties in meat products. Vegetable proteins often give the product an atypical flavor, reduce the meaty taste, and many are representatives of allergens. Therefore, animal protein isolates as functional ingredients in meat industry are rising again **(Khiari et al., 2014)**. The basic reasons to use protein additives include: increasing nutritional value of meat products, improving technological properties of processed raw materials, improving sensory characteristics of finished products, and last but not least, there are also economic reasons **(Lat et al., 1984)**. In their study, **Prabhu (2004)** reports that isolated porcine protein containing 85% protein and 12% fat obtained from fresh pork trimmings by a technology at low temperature is able to attach as much water as up to four times its weight.

The nutritional value of proteins is then judged mainly according to the amino acids contained, in particular by the essential amino acids contained. Nutritional value of animal proteins is high, while value of plant proteins is considerably lower because they lack some essential amino acid (AAs) (lysine or sulfur-free AAs). Proteinaceous ingredients are produced with a protein content of 50 – 90% **(Straka and Malota, 2006)**. The most natural alternative to meat proteins are therefore animal proteins which are widely used in meat production. For some types of meat products, addition of animal proteins is necessary, e.g. gelatin applied in cooked meat products and specialties. The most common protein used in the production of ground meat products are blood proteins, milk proteins, egg proteins, and collagen proteins **(Budig and Mathauser, 2007)**. Collagen is a group of insoluble fibrous proteins found in all multicellular organisms. It is the most abundant protein in mammals,

amounting approximately to 25 – 30% of the total body protein. Collagen is a major component of skin, bones, cartilages, tendons, blood vessels, basement membranes and teeth **(Tarté, 2010)**. Native collagen consists of alpha 1 and alpha 2 chains, which differ in their order of amino acids. These chains form a triple helix called tropocollagen. It is approximately 256 nm long and it represents the basic unit of collagen. Secondary structure of collagen is then formed by a left-handed helix of an elongated type with thread pitch of 0.95 nm and individual peptides in 0.286 nm distance from each other. The tertiary structure of collagen consists of three chains that are intertwined and have a central axis. The resulting rod-shaped configuration is about 290 nm long with a diameter of 1.4 nm and it is called tropocollagen **(Khoshnoodi et al., 2006)**. For meat industry, collagen sources include skin, bones, entrails and skeletal muscle **(Bailey and Light, 1989)**. Collagen may be added to meat products as an ingredient (meat raw materials rich in collagen: skeletal muscle with connective tissue, pork skin) or in concentrated form as a direct additive. This can be produced from bones (bone collagen extract), pork skin, and skeletal muscle with connective tissue **(Gillett, 1987)**. Collagen of skeletal muscle can be concentrated mechanically or extracted by low temperature rendering followed by extrusion, dehydration, grinding, flaking, milling **(Gillett, 1987)**. These forms of collagen can significantly affect the processability and sensory properties of meat products.

MATERIAL AND METHODOLOGY

Manufacture of Model Meat Products

Model meat products were manufactured with the addition of various types of isolated porcine proteins. The used proteins included blood protein: pork plasma P; collagen-type proteins: PF, pork gel, Scancure DI 100, Scancure 95; combination of collagen-type proteins and pure muscle proteins: Scanpork D 80, Scanpork D 90 (Scanflavour, Denmark). The first set of model products was made of poultry meat with additions of 7 types of various isolated porcine proteins at concentration of 2.5%. The second set of model products was made of pork and beef in the ratio 1:1 with additions of 7 types of various proteins at concentration of 2.5%. Other ingredients to all model products included salt at a final concentration of 1.5% and 10 mL of water. Model samples were ground in a blender, then shaped and pressed in a ham mould in which they were boiled for 10 minutes after reaching 70 °C in its core, subsequently they were cooled down, cut into cubes and placed in a freezer. Next, cryosections were cut at the HM 550 cryostat (Germany, Microm). The sample was attached to a metal stip of the freezing microtome using Killik freezing medium. The stip was left in the freezer bar of the cryostat to solidify. The sample was thus ready for cutting, so it was attached to the holder and slicing started. 16 μm thick sections were transferred to specific Thermo Superfrost slides (Germany, Thermo scientific). 42 sections on 21 slides were made from each sample – each slide contained two sections. After adhering, the sections were stored at a cooling temperature until further processing.

Histological Staining

The sections were stained using differential and targeted staining. For each staining 3 slides containing 2 sections each were used. From differential stainings, hematoxylin-eosin and toluidine blue staining and targeted Calleja were applied. The prepared samples were examined using Leica DM 3000 microscope (Germany, Leica). Images were created using Leica DFC 295 camera (Germany, Leica) connected to a computer, by means of Xn Wiew software. Based on the results of this part of the experiment, the most suitable method to detect isolated porcine proteins was selected and pork protein called Scancure 95 by Scanflavour (Denmark) was chosen to be used for the production of model meat products in the second phase of the work, which was aimed at determining the detection limit of this method.

Samples for Determination of the Detection Limit

The following ingredients were selected for the second experiment: pork and beef in the ratio of 1:1 and isolated pork protein called Scancure 95 by Scanflavour. The following concentrations of samples were produced: 0.001%; 0.01%; 0.10%; 1.00%; 2.50%; 5.00%. Other ingredients to all model products included salt at a final concentration of 1.5% and 10 mL of water. Model samples were processed according to the procedure in the previous chapter. And they were stained by targeted Calleja.

RESULTS AND DISCUSSION

In the first step of the protocols, model meat products were manufactured from available commercially produced isolated porcine proteins. A combination of poultry, pork and beef meat was used as the basis of individual meat products in order to find the most suitable combination for the production of model meat products. Model meat products were manufactured with the addition of isolated porcine protein in concentration of 2.5%. This step resulted in the selection of the best detectable isolated porcine protein. Based on processing and examining samples of poultry meat and the combination of pork and beef, it was found that the raw material itself has no effect on the microscopic detection of isolated porcine protein. The raw material in particular affects processing of the samples and primarily it's cutting and staining. For this reason, the combination of pork and beef was used in the next phase.

The most widely used collagen protein (in terms of production of ground meat products) is collagen protein powder of pork origin obtained from pig skins. These are used primarily where there is a need to increase elasticity, improve slicability and cohesion. Stabilization of the product is achieved by a combination of collagen protein and other proteins of animal origin, e.g. blood plasma. Another property is a significant contribution to the reduction of syneresis in the finished meat products packaged in a vacuum or a modified atmosphere. Pork skins in the form of skin emulsion are used most commonly **(Budig and Mathauser, 2007)**. Functional properties of the added collagen used in meat products depend on the species and age of animal, anatomical sources, and extraction conditions. The potential use of collagen as an additive in meat products dates back to

approximately 1960 **(Tarté, 2010)**. From the created staining range applicable to detect collagen in practice, it was necessary to determine which ones are suitable to identify isolated porcine protein. They were verified by 2 differential and one targeted staining. From among differential staining, haematoxylin - eosin and toluidine blue stainings were used and, from among the targeted ones, Calleja was used (Figure 1). Targeted staining procedures were focused on the detection of collagen which should be distinguished from other structures in the product in each staining. All stainings applied are primarily intended for paraffin sections. However, our experiment showed that they are also suitable for cryosections. Cryosections were used because their examination is financially less demanding as well as less time consuming.

The first differential staining was hematoxylin - eosin. This staining is not too demanding on chemicals and time, but it does not belong among the simplest ones. It is highly effective in distinguishing, all the structures in the sample can be identified, even though they are only in various shades of red and blue. Isolated porcine protein is dark pink, sometimes up to crimson. An experienced examiner can recognize it because of its characteristic structure. Collagen protein has a net-like structure with cores forming different circular shapes in the margins. No sections were lost in this process due to floating away.

The second differential staining was toluidine blue. This method is neither demanding in terms of chemicals nor of time. Of all the applied stainings, this one is the easiest and quickest. It is very effective in terms of substances identification. Collagen protein is slightly purple and it is characterized by its typical structure. No cryosections were lost.

The targeted staining was performed according to Calleja. Calleja staining is relatively time-efficient. It is very effective in differentiation. There is a clear distinction in structures within the meat product. Collagen protein is stained kerosene or bluish-green and it is clearly distinguishable from other structures that are predominantly green (Figure 2). Another advantage is limited losses of sections. For these reasons, this staining method was selected as the most suitable for the detection of isolated porcine protein in meat products.

Method Validation

A graded series was produced in order to determine the detection limit. A combination of pork and beef in the ratio of 1:1 and isolated pork protein of Scancure 95 (Scanflavour, Denmark) were utilized. This isolated pork protein has a high content of collagen protein in its composition and thus it was the most suitable material for the microscopic detection. Scancure 95 is composed of 98% collagen protein and 1 – 2% pure muscle proteins. It is intended as an addition to smoked meat. The following concentrations were created: 0.001%; 0.01%; 0.10%; 1.00%; 2.5%; 5%. Of the above staining methods, Calleja was selected as the most suitable for the detection of isolated porcine protein because of the best color contrast between different structures in the meat product. Therefore, the following parameters were also established for this staining in order to validate this method: sensitivity and specificity, and repeatability. The method was validated as a qualitative method, i.e. a method to detect whether the analyte is present or absent. These methods are common in routine laboratory testing in particular as the first step for subsequent determination of the concentration of the investigated substance. They can therefore be ranked among screening methods. The advantage of its use is the reduction of costs as well as time **(Trullols et al., 2004)**. As with quantitative methods, user must be confident that the results of the qualitative method applied are suitable for the aimed purposes, which means that the method must be validated **(European Commission, 2002)**. Commonly selected methods for validation are based on quantitative methods, and there are also many validation procedures which are accepted by the supervisory authorities and professional community working in the field **(Trullols et al., 2004)**.

For detection of isolated porcine protein, the detection limit was determined at 0.001 % addition. Using isolated porcine proteins in meat products can be expected at about 10 % as suggested by a study **(Doerscher, et al., 2003)**. With regard to the optimum concentration applicable in meat production, the method is suitable for detection of isolated porcine proteins in meat products.

The evaluation criteria for qualitative methods inlcude also sensitivity and specificity **(European Food Safety Authority, 2004)**. For qualitative methods, sensitivity means the ability of a method to detect truly positive

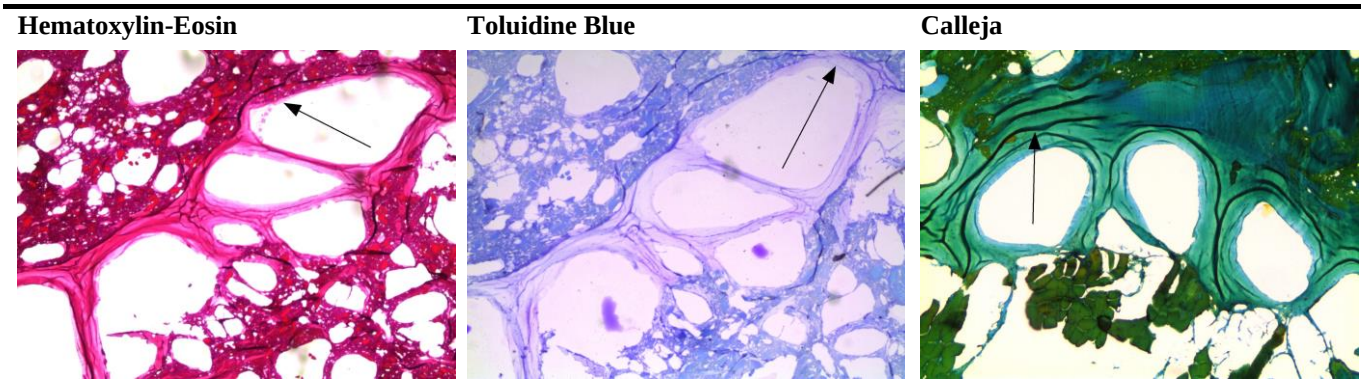

Figure 1 Isolated porcine protein in a model meat product (25 x magnification).

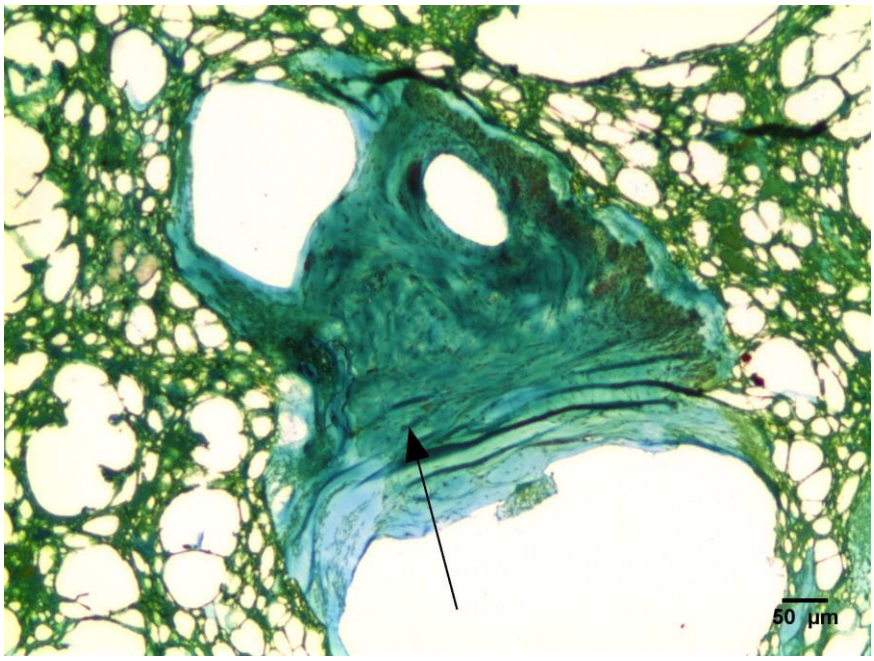

Figure 2 Isolated porcine protein (bluish green) in a model meat product (25 x magnification).

Table 1 Detection of sensitivity and specificity of the Calleja method.

Sample no.	1/2	1/3	1/4	1/5	1/6	2/2	2/3	2/4	2/5	2/6
Declaration	N	N	N	N	N	P	P	P	P	P
Examiner A	N	N	N	N	N	P	P	P	P	P
Examiner B	N	N	N	N	N	P	P	P	P	P

Note: Declaration – N (negative), P (positive)-addition of the isolated porcine protein in model sample.
Sample no: 1/2- 1/6: model sample without the isolated porcine protein.
Sample no: 2/2- 2/6: model sample with the isolated porcine protein.

samples as positive **(O'Rangers and Condon, 2000)**. The sensitivity rate is thus the probability for a given concentration, that the method will classify the test sample as positive **(Massart et al., 1997)**. In contrast, specificity is defined as the ability of a method to detect truly negative samples as negative **(O'Rangers and Condon, 2000)**. The specificity rate is thus the probability, for a given concentration, that the method will classify the test sample as negative **(Massart et al., 1997)**. For Calleja staining method, sensitivity was determined at 1.00 for isolated pork protein. Specificity was determined at 1.00 according to the protocols **(Trullols et al., 2004)** (Table 1). An optional parameter for qualitative methods is also method repeatability. For histological methods, this parameter is particularly suitable because cutting the samples, sample processing and staining can take place on different days. This parameter is recommended to exclude the role of the environment. Repeatability of the method was carried out using the product with addition as well as without addition of isolated porcine protein and it was performed in harmony with the protocols **(Suchánek, 1999)**. Evaluation was performed by two examiners and the measurement was repeated 10 times. There was a 100% match.

CONCLUSION

Besides dairy products and bread, meat and meat products belong to the basic foodstuffs needed for good human nutrition. Above all, however, they are consumed due to their organoleptic properties. Nevertheless, with regard to the pressure on meat products buying prices and special offers in retail chains, industrial manufacturers have to substitute a technologically substantial portion of binding meat either by cheaper meat raw materials of inferior quality or by suitable ingredients and additives in order to stabilize the product, which applies in particular to ground meat products. To achieve the desired properties in the finished meat product, manufacturers use a considerably cheaper source, namely collagen proteins in the form of isolated porcine proteins. Due to their very specific functional properties, collagen proteins are used in particular for products where it is necessary to increase their elasticity, improve slicability and cohesion. Application of collagen protein significantly contributes to the reduction of syneresis in finished meat products

packaged in a vacuum or a modified atmosphere. In combination with other animal proteins, such as blood plasma, collagen proteins are also used as ingredients that facilitate emulsification or stabilization of the ground meat products. The aim of this work was to detect isolated pork protein in model meat products using histological staining of cryosections and light microscopy. In the first part of this work, 7 model meat products from poultry meat and 7 model meat products from beef and pork in the ratio of 1:1, which contained 2.5% concentration of various commercially produced isolated porcine proteins, were examined. These model meat products were histologically processed by means of cryosections and stained with hematoxylin-eosin staining, toluidine blue staining and Calleja staining. The second part focused on validation of Calleja method, where model meat products with the addition of isolated porcine protein of Scancure 95 containing 98% of collagen protein and 1 – 2% of pure muscle protein were used. The sensitivity was determined for isolated porcine protein at 1.00 and specificity was determined at 1.00. The detection limit of the method was determined at 0.001% addition. Repeatability in both, positive and negative samples, for isolated porcine protein was determined at 100%. The results show that the histological processing of cryosections stained using Calleja is suitable for detecting isolated porcine protein in meat products.

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Contact Address:

Mgr. Michaela Petrášová, Ph.D. University of Veterinary and pharmaceutical sciences, Brno, FVHE, Palackého tř. 1946/1, 612 42 Brno, Czech Republic, E-mail: petrasovam@vfu.cz.

Bc. Eliška Zichová, University of Veterinary and pharmaceutical sciences Brno, FVHE, Palackého tř. 1946/1, 612 42 Brno, Czech Republic, E-mail: eliska.zichova@seznam.cz.

MVDr. Matej Pospiech, Ph.D., University of Veterinary and pharmaceutical sciences Brno, FVHE, Palackého tř. 1946/1, 612 42 Brno, Czech Republic, E-mail: pospiechm@vfu.cz.

Doc. MVDr. Bohuslava Tremlová, Ph.D., University of Veterinary and pharmaceutical sciences Brno, FVHE, Palackého tř. 1946/1, 612 42 Brno, Czech Republic, E-mail: tremlovab@vfu.cz.

Mgr. Zdeňka Javůrková, Ph.D. University of Veterinary and pharmaceutical sciences Brno, FVHE, Palackého tř. 1946/1, 612 42 Brno, Czech Republic, E-mail: javurkovaz@vfu.cz.

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THE EFFECT OF STORAGE ON QUALITY OF HERBS GENUS *ORIGANUM*

Lenka Kouřimská, Kateřina Ešlerová, Yunus Khatri

ABSTRACT

Herbs of *Origanum* genus are rich in essential oils and contain large amounts of phenols, lipids, fatty acids, flavonoids and anthocyanins. Antioxidant activity of these herbs depends on many factors, including the type herbs, post-harvest processing and subsequent processing. The aim of this study was therefore to confirm the hypothesis that the composition of oils of these two herbs of the *Origanum* genus depends on the post-harvest treatment of herbs and that the dried herb antioxidant activity is higher for fresh than that of frozen herbs. *Lamiaceae* family herbs: oregano (*Origanum vulgare* L.) and Greek oregano (*Origanum heracleoticum* L.) were planted and analyzed. Herb samples were extracted by hot demineralised water. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method was used for antioxidant activity assessment. The total phenolic content was determined spectrophotometrically by using Folin-Ciocalteu reagent. Steam distillation of essential oils was carried out via Clevenger Apparatus. The obtained essential oils were analysed by GC-MS technique. Results of tested fresh, dried and frozen herbs showed a considerable potential for quenching the free DPPH radical. Significantly higher antioxidant activity was found in dried herbs comparing to fresh and frozen, but only in case of values calculated per 100 g of the sample. However, the differences were not statistically significant after recalculation when expressed on dry matter content. There was no difference between fresh and frozen samples. The content of total phenols was significantly higher in dried than in frozen herbs in values recalculated per 100 g of sample. A strong correlation between the results of DPPH and TPC was found again only for values expressed per 100 g of the sample. Post-harvest treatment of herbs affects the composition of their essential oils. The dominant essential oil component of Greek oregano is carvacrol with a proportion of 60% or more. On the contrary, there is no such dominating component in oregano essential oil but there are more components with a share of 10 to 20%.

Keywords: Oregano; Greek oregano; DPPH method; total phenolics contents; essential oils composition

INTRODUCTION

Herbs of the *Origanum* genus are found mainly in the Mediterranean region and Asia. This genus is rich in essential oils where the quantity and quality vary considerably **(Asensio et al., 2015)**. Herbs of *Origanum* genus contain large amounts of phenols, lipids, fatty acids, flavonoids and anthocyanins **(Kintzios, 2002)**.

Oregano is a perennial plant of *Lamiaceae* family groving in Asia, Europe and North Africa. This aromatic herb is widely used in many world cuisines **(Teixeira et al., 2013)**, and in traditional medicine **(Kintzios, 2002)**. There are many studies describing the effect of oregano for food preservation **(Chorianopoulos et al., 2004; Chouliara et al., 2007; Carmo et al., 2008)**, as well as its antioxidant activity **(Sahin et al., 2004; Capecka et al., 2005; Ličina et al., 2013)**. Biologically active substances of oregano include phenols, phenolic acids, flavonoids, glycosides and their esters and steroids. According to **Crocoll et al., (2010)**, the dominant components of essential oils of this herb are monoterpenes and sesquiterpenes showing significant antioxidant activity **(Kinzitos et al., 2002; Grassmann, 2005; Beena et al., 2013)**. The main compounds with antifungal properties include thymol and carvacrol **(Rao et al., 2011)**.

Origanum vulgare L. has been used in Persian traditional medicine for its anti-inflamantory **(Javadian et al., 2016)** and other effects such as diuretics, stomachics, antineuralgics, antitussives and expectorans **(Afsharypour et al., 1997)**.

Greek oregano is widely used in gastronomy. Its typical flavour is produced by the presence of essential oils **(Stamenic et al., 2014)**. Antimicrobial **(De Martino et al., 2009; Govaris et al., 2010; Stamenic et al. 2014)**, antifungal **(Adam et al., 1998)** and antioxidant **(Kulisic et al., 2004; Zheng et al., 2009)** effects were described in this plant. **Charles (2013)** indicates that the most significant secondary metabolites in Greek oregano are tannins, resins, flavonoids, bitter substances, sterols, phenols, and essential oils. **Kikuzaki et al., (1989)** and **Koukoulitsa et al., (2006)** found a variety of phenolic compounds with antioxidant activity in Greek oregano such as rosmarinic acid and its derivatives, caffeic acid, protocatechuic acid and phenyl glucoside. In a report by **Zheng et al., (2009)**, the essential oil of Greek oregano consists of 78.28% of phenolic compounds carvacrol and thymol followed by γ-terpinene (5.54%) and p-cymene (7.35%). The antioxidant activity of essential oil of Greek

oregano and its use in the food industry was also discussed by **Kulisic et al., (2004)**.

Antioxidant activity of herbs depends on many factors, including the type herbs, the methods and conditions of cultivation, harvest, post-harvest processing and subsequent processing. The extraction method, the manner of extraction and the type of solvent used also affect the level of antioxidant activity **(Škrovánková et al., 2012)**. The effect of different growing conditions on the chemical composition and biological activity of the essential oil of oregano was studied **De Falco et al., (2013)**. **Kouřimská et al., (2014)** investigated the antioxidant activity of plants of the *Lamiaceae* family grown under organic and conventional conditions. **Ozkan et al., (2010) and Baranauskienė et al., (2013)** focused on the impact of harvest on essential oil composition and antioxidant activity of oregano. They found the highest antioxidant activity was found in herbs harvested during their flowering.

The water content in fresh *Lamiaceae* herbs is typically in the range of 75 – 80%. For the preservation of the herbs, it is necessary to reduce the amount of water to less than 15% **(Diaz-Maroto et al., 2002)**. Lowering the water activity inactivates the enzymes, which in its active form may be cause degradation of antioxidant ingredients of fresh herbs **(Hossain et al., 2010)**. Water activity may be reduced via different methods. The most commonly used method is drying. This method results in an increase in the content of some substances. Cell tissues of herbs are damaged during the drying process which leads to the release of phenolic compounds and increase of antioxidant activity. Changes in appearance and flavour are due to loss or development of volatile compounds because of oxidation and esterification reactions **(Hossain et al., 2010)**.

Air drying is the simplest and cheapest method of drying. Low temperature prevents the degradation of the active ingredients of herbs during this type of conservation method, but the drying is relatively slow and thus the metabolic processes may continue and cause changes in quality of herbs **(Keinänen and Julkunen-Tiitto, 1996)**. Slow loss of water can act as a stressor and the defensive mechanism of most plants is the production of phenolic compounds **(Dixon and Paiva, 1995; Hossain et al., 2010)** which may contribute to a higher antioxidant activity of dried herbs. **Hossain et al., (2010)** found significantly higher amounts of rosmarinic acid in the extracts of herbs dried at room temperature than in fresh samples. Higher amounts of carvacrol can be obtained during drying at lower temperatures (below 40 °C) **(Novák et al., 2011)**.

Freezing is another method to reduce the activity of water. Crystals are formed during freezing which causes destruction of plant cells enabling better extraction of the active substances **(Keinänen and Julkunen-Tiitto, 1996)**. **Tomsone and Kruma (2014)** considered this phenomenon as a possible explanation of higher phenol content of frozen herbs. **Chan et al., (2014)** argues that the effect of freezing and other methods on the antioxidant activity varies depending on the particular herb. **Tomsone and Kruma (2014)** investigated the effect of drying and freezing on the phenol content and antioxidant activity of lovage and horseradish. Both these parameters were highest in frozen herbs and therefore the authors evaluated this processing method as the most suitable for preserving the antioxidant activity and the content of phenols.

Different extraction methods can be employed for the isolation of antioxidants from herbs. Extraction from the solid phase into the liquid and steam distillation are frequently used methods. Extraction using non-toxic solvents, such as supercritical fluid extraction with carbon dioxide and subcritical water extraction are increasingly applied **(Rodríguez-Meizoso et al., 2006)**. **Škrovánková et al., (2012)** reported that polar solvents (ethanol, methanol, water, etc.) and non-polar (hexane, etc.) as the most commonly used extraction liquids. Different solvents for the isolation of antioxidative components were used for Greek oregano by **Tsimogiannis et al., (2006)**. The highest antioxidant activity determined by DPPH was found in diethyl ether and ethanol extract, the lowest in petroleum extract.

Although there is a lot of scientific literature focussed on the effects of drying or freezing on the antioxidant activity and composition of certain medicinal and aromatic herbs, there is still lack of a comprehensive study comparing these two conservation practices both at the same time in the case of oregano and Greek oregano. The aim of this study was therefore to confirm the hypothesis that the composition of oils of these two herbs of the *Origanum* genus depends on the post-harvest treatment of herbs and that the dried herb antioxidant activity is higher for fresh than that of frozen herbs.

MATERIAL AND METHODOLOGY

Herbs

Lamiaceae family herbs: oregano (*Origanum vulgare L.*) and Greek oregano (*Origanum heracleoticum L.*) were planted and analyzed. The seeds were sown on 20th April to the sunny, unfertilized plot of sandy loam medium soil in the Jirny locality (50° 6' 56'' N, 14° 41' 57'' E, district Prague-East). The seeds were purchased from Kiepenkerl company. Plant parts were harvested before flowering on 30th July and divided into three parts. One part was spread on papers in the laboratory and dried at 25 °C for one week. Another part was placed in plastic bags and frozen at -18 °C. The last third was analysed immediately.

Chemicals

All chemicals, methanol (Lachner, CR), sodium carbonate anhydrous (Lachner, CR), DPPH 2,2-difenyl-1-pikrylhydrazyl (Sigma Aldrich, USA), Folin & Ciocalteu´s phenol reagent (Merck, Germany), ascorbic acid (Penta, CR), Gallic acid (Sigma Aldrich, USA), n-hexane (Lachner, CR) and sodium sulphate anhydrous (Lachema, CR) were of analytical grade purity.

Determination of dry matter content

Balances with infrared dryer, Precisa HA 300 (Precisa Instruments, Swirzerland) were used for dry mater content determination. Samples of herbs (1 g) were ground and spread on aluminium foil and dried at a maximum temperature 105 °C to the constant weight (the weight difference less than 2 mg for 30 s). All samples were measured in triplicate and the average was calculated.

Herb extraction

Fresh herbs (6 g) or the equivalent amount of dried herbs (calculated from total dry matter of individual herbs) were taken for the preparation of water extracts. Herb samples were extracted twice by 50 mL of hot demineralised water in the ultrasonic bath for 10 min. Samples were then filtered into 100 mL volumetric flasks and filled up to the mark after cooling. The extracts were analysed on the same day.

Determination of antioxidant activity by the DPPH method

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method taken from **Adámková et al., (2015)** and **Chrpová et al., (2010)** was used for antioxidant activity assessment. The intensity of the violet DPPH radical solution was measured at 522 nm. As the reaction equilibrium is usually reached after two hours for most compounds, the absorbance of samples was measured after 1, 2 and 3 hours and its minimum was used for the antioxidant activity calculation. The method was calibrated with ascorbic acid and the results were expressed as equivalents of ascorbic acid per unit mass of sample.

Determination of total phenolic compounds (TPC)

The total phenolic content was determined spectrophotometrically (spectrophotometer UV-2900, Tsingtao Unicom-Optics Instruments Co., Ltd., China) at 760 nm by using Folin-Ciocalteu reagent. The method was previously reported by **Dorman et al., (2003)** and **Stratil et al., (2008)**. Results were expressed as the content of Gallic acid per unit mass of the sample.

Steam distillation of essential oils

Steam distillation of essential oils was carried out via Clevenger Apparatus (Wilmad-LabGlass, USA) for 4 hours **(Memarzadeh et al., 2015)**. The apparatus was also used for the determination of extracted volume of essential oil.

GC analysis of essential oils

The obtained essential oils (10 μL) were placed into the vials with 500 μL of n-hexane (with a few crystals of anhydrous sodium sulphate) and analysed by GC-MS using Agilent 7890A GC coupled to Agilent 5975C single-quadrupole mass detector equipped with a HP-5MS column (30 m × 0.25 mm ID, 0.25 μm film). The sample volume of 1 μL was injected in split mode (ratio 12:1) into the injector heated to 250 °C. The initial oven temperature was 60 °C (hold 3 min), ramp to 250 °C at 3 °C . min^{-1} (hold 10 min). Helium was used as carrier gas with the flow rate of 1 $mL.min^{-1}$. The MS analysis was carried out in full scan mode, the electron ionization energy was set at 70 eV. The analytes were identified according to their relative retention times and by the comparison of their mass spectra with the National Institute of Standards and Technology Library (NIST, USA). The results were calculated by area normalisation method.

Statistical evaluation

The data obtained were analysed using statistical software Statistica 12.0 (StatSoft Inc.). Analysis of variance (one-way ANOVA) was performed and the significant differences in the means were separated using the Scheffé's test. The data were expressed as an average of triplicates ± standard deviation. For all statistical tests, a 5% level of significance was used.

RESULTS AND DISCUSSION

Comparison of dry matter content, antioxidant activity and phenolics content

Dry matter content results of analysed herbs are presented in Table 1. The results correspond with the works of other authors, for example **Kouřimská et al., (2014) and Adámková et al., (2015)**. It can be seen that there are considerable losses of water content during drying compared to freezing.

Results of antioxidant activity of tested herbs analysed by DPPH method expressed in mg of ascorbic acid (AA) per 100 g of sample or per 100 g of dry matter are shown in

Table 1 Dry matter (DM) content of tested herbs in % (w/w).

Herb	DM (% w/w)		
	fresh	dried	frozen
Oregano (*Origanum vulgare L.*)	32.73 ±0.97	85.27 ±0.34	26.73 ±1.12
Greek oregano (*Origanum heracleoticum L.*)	24.04 ±1.41	83.62 ±0.12	21.35 ±0.96

Table 2 Antioxidant activity of tested herbs analysed by DPPH method expressed in mg of ascorbic acid (AA) per 100 g of sample or per 100 g of dry matter (DM).

Herb	DPPH (mg AA/100 g)	DPPH (mg AA/100 g DM))
Oregano – fresh	1156 ±160	3532 ±450
Oregano – dried	3572 ±481	4189 ±560
Oregano - frozen	1034 ±162	3867 ±607
Greek oregano - fresh	463 ±9	1926 ±39
Greek oregano - dried	2608 ±563	3119 ±674
Greek oregano - frozen	566 ±63	2652 ±294

Table 3 Total phenolics content (TPC) results of tested herbs expressed in mg of Gallic acid (GA) per 100 g of sample or per 100 g of dry matter (DM).

Herb	TPC (mg GA/100 g)	TPC (mg GA/100 g DM))
Oregano – fresh	1248 ±14	3812 ±44
Oregano – dried	2166 ±5	2540 ±6
Oregano - frozen	425 ±6	1588 ±23
Greek oregano - fresh	795 ±6	3309 ±27
Greek oregano - dried	3067 ±43	3668 ±52
Greek oregano - frozen	390 ±1	1825 ±5

Table 2. The results show that both herbs possess significant potential to quench the free radical DPPH which confirmed the work carried out by **Chrpová et al., (2010)**; **Ličina et al., (2013)** or **Skotti et al., (2014)**. Dried herbs had the highest antioxidant activity. The values of frozen and fresh herbs expressed per 100 g of sample were not very different. There can be seen, only a slight difference between fresh, dried and frozen samples of oregano expressed on dry matter content. When comparing oregano and Greek oregano there was always higher antioxidant activity in case of oregano samples.

Table 3 shows the total phenolics content of tested herbs analysed by Folin-Ciocalteu reagent and expressed in mg of Gallic acid (GA) per 100 g of sample or per 100 g of dry matter. It can be observed that fresh, dried and frozen herbs contain reasonable amounts of phenols, which also corresponds to the results of **Ivanova et al., (2005)** and **Skotti et al., (2014)**. The highest amount of phenols per 100 g of the plant was determined on the dried herbs, which correlates with the highest antioxidant activity. After recalculating the results based on dry matter content, fresh oregano samples had the highest phenol content. The lowest amount of total phenols showed frozen herbs even after recalculating to dry matter content. Higher phenol content in dried herbs corresponds with the conclusion of **Ahmad-Qasem et al., (2013)**, who found lower levels of phenols in the frozen sample of olive-tree than in the dried sample. The authors explained this lower content of phenols in a frozen sample as a result of the temporary inactivation of the enzymes during freezing.

Statistical comparison of the results of the fresh, dried and frozen herbs expressed per dry matter content did not show any significant differences in antioxidant activity including the content of total phenols (pDPPH = 0.6509, pTPC = 0.0731). However, significant differences were found comparing the values per 100 g of herbs for both antioxidant activity (p = 0.0339) and in the content of total phenols (p = 0.0266). A more detailed analysis using Scheffé test showed a statistically significant difference in antioxidant activity between fresh and dried herbs (p = 0.0498) and between frozen and dried herbs (p = 0.0493). In the case of TPC, there was only a statistically significant difference between frozen and dried herbs (p = 0.0290). A strong correlation (r = 0.83) was observed between the values of DPPH and TPC expressed per 100 g of herbs.

Higher antioxidant activity by DPPH method in the dried sample may be due to stress of plants accompanied by the formation of phenolic compounds. Also the disruption of tissues during drying could result in the release of phenolics and increasing their content in the extraction process. This is partly confirmed by the results of total phenols, where a strong correlation between the values of TPC and DPPH was found, but only for the results expressed per 100 g of herbs. A significant correlation between the total content of phenols and antioxidant

Table 4 GC-MS analysis of major components of essential oil extracted from oregano.

Component	Content in fresh (%)	Content in dried (%)	Content in frozen (%)
Sabinene	ND	4.30	3.49
β-Phellandrene	1.51	ND	ND
1-Octen-3-ol	0.23	3.09	3.32
β-Myrcene	0.95	ND	1.92
α-Terpinene	ND	2.38	2.36
(E)-β-Ocimene	5.77	5.44	ND
(Z)-β-Ocimene	6.86	ND	5.41
α-Pinen	ND	8.38	8.77
3-Carene	2.54	3.03	0.07
γ-Terpinene	12.20	16.40	15.47
1,6-Octadien-3-ol, 3,7-dimethyl-	0.33	1.71	0.80
Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	5.38	3.36	3.59
Germacene D	10.16	24.44	29.18
Caryophyllene	14.39	5.07	3.86
α-Caryophyllene	2.10	0.77	ND
Cubebol	ND	6.60	5.14
β-Cubebene	16.82	ND	ND
β-Bisabolene	8.34	2.92	2.12

ND = not detected

Table 5 GC-MS analysis of major components of essential oil extracted from Greek oregano.

Component	Content in fresh (%)	Content in dried (%)	Content in frozen (%)
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	1.27	ND	1.76
Bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro deriv.	ND	1.42	ND
Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	ND	ND	1.23
β-Myrcene	1.47	1.73	1.92
Cyclohexene, 1-methyl-4-(1-methylethylidene)-	1.20	0.09	0.08
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	ND	1.70	ND
Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-	ND	ND	1.93
(E)-β-Ocimene	4.61	4.21	4.36
1,3,6-Octatriene, 3,7-dimethyl-, (E)-	0.97	1.41	1.70
γ-Terpinene	6.21	9.33	9.73
Benzene, 2-methoxy-4-methyl-1-(1-methylethyl)-	0.97	ND	1.08
Phenol, 5-methyl-2-(1-methylethyl)-	ND	ND	7.19
Carvacrol	73.09	67.92	59.66
Caryophyllene	1.00	1.46	1.24

ND = not detected

activity of plants has been demonstrated in several studies **(Ivanova et al., 2005; Wojdyło et al., 2007; Chrpová, et al., 2010)**.

Higher antioxidant activity of frozen herbs (values expressed on dry matter) was found compared to fresh herbs. The reason could be damage of herb tissues due to the formation of crystals during freezing and subsequently increased release of secondary metabolites into the solvent **(Keinänen and Julkunen-Tiitto, 1996)**. It should be noted that the results and conclusions of studies investigating the effect of freezing on the antioxidant activity of plants are not always unambiguous and conclusive as mentioned for example by **Chan et al., (2014)**.

A statistically significant difference in antioxidant activity of fresh, dried and frozen herbs determined by DPPH in values calculated per 100 g of plant corresponds with the results of many studies **(Hossain et al., 2010; Kouřimská et al., 2014)**, but they also show a statistically significant difference even after recalculation to dry matter content. **Hossain et al., (2010)** explain the lower antioxidant activity of fresh herbs by the presence of active enzymes that may cause degradation of the antioxidants. Antioxidant activity determined by DPPH was in all cases higher in oregano samples then in Greek oregano samples, concurring with the findings of **Chrpová et al., (2010)**.

The highest content of total phenols was in most cases determined in samples of dried herbs, as reported by **Hossain et al., (2010)** and **Kouřimská et al., (2014)**. It is caused by the release of phenolic compounds and increase of antioxidant activity when the cell tissues of herbs are damaged during the drying process. Comparing the total phenolic content of fresh and dried herbs there was no statistically significant difference which is in line with **Adámková et al., (2015)**.

Comparison of extracted volume of essential oil and its composition

Extraction of the essential oils from the fresh, dried and frozen samples gave significantly higher yields from samples of Greek oregano (6.3, 14.8 and 8.2 $mL.kg^{-1}$ respectively) than for oregano (0.2, 3.2 and 1.7 $mL.kg^{-1}$ resp.). The lowest yield was in case of fresh samples. **Baranauskienė et al., (2013)** also found a higher yield at the Greek oregano compared with oregano and all samples that they harvested in different periods.

The main components of fresh oregano essential oil analysed by GC-MS were β-cubebene, caryophyllene, γ-terpinene and germacene D (Table 4). **Mockutë et al., (2004)** and **Şahin et al., (2004)** also determined germacene D and caryophyllene as the major components of oregano essential oil. The high content of γ-terpinene is consistent with results of **Ličina et al., (2013)**. Other studies found additional components which is influenced by many factors such as growing conditions, locality, time of harvest, extraction method etc. **(Ozkan et al., 2010; Tibaldi et al., 2011; De Falco et al., 2013; Kawase et al., 2013)**. Cubebol, sabinene and α-terpinene were found only in the essential oils of dried and frozen herbs, while β-cubebene and β-phellandrene were only in the samples of fresh oregano.

Carvacrol was the main component of Greek oregano essential oil samples (Table 5), its highest content was in the fresh sample. The second major component was γ-terpinene and (E)-β-ocimene. **Zheng et al., (2009)** and **Stefanakis et al., (2013)** also reported carvacrol and γ-terpinene as major compounds. The results show that the various post-harvest treatments cause the changes in the composition and the content of essential oils components. This corresponds to the studies of **Novák et al., (2011)** who described the changes in the composition of the Greek oregano essential oil treated by various types of drying.

Najafian (2014) observed different representation of ingredients of lemon balm essential oil as a result of the storage of herbs in the freezer, refrigerator and room temperature.

The smallest changes in the composition of essential oils were found during the storage of herbs in the freezer and refrigerator compared to storage at room temperature. The highest level of carvacrol was found in the essential oil of fresh oregano Greek, while the lowest amount was in the frozen sample. Reduced proportion of carvacrol was always associated with increased content of its precursor γ-terpinene. This phenomenon was also highlighted by **Novák et al., (2011)**. High concentration of carvacrol in the essential oil of Greek oregano is responsible for its antioxidant activity.

CONCLUSION

The antioxidant activity and the composition of essential oils of selected plants of the genus *Origanum* were affected by post-harvest treatment of plants. All fresh, dried and frozen herbs showed a considerable potential for quenching the free DPPH radical. Significantly higher antioxidant activity was found in dried herbs comparing to fresh and frozen, but only in case of values calculated per 100 g of the sample. However, the differences were not statistically significant after recalculation when expressed on dry matter content. There was no difference between fresh and frozen samples. The content of total phenols was significantly higher in dried than in frozen herbs in values recalculated per 100 g of sample. A strong correlation between the results of DPPH and TPC was found again only for values expressed per 100 g of the sample. Post-harvest treatment of herbs affects the composition of their essential oils. The dominant essential oil component of Greek oregano is carvacrol with a proportion of 60% or more. On the contrary, there is no such dominating component in oregano essential oil but there are more components with a share of 10 to 20%.

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Contact address:

Lenka Kouřimská, Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Microbiology, Nutrition and Dietetics, Kamýcká 129, 165 21 Praha 6 - Suchdol, Czech Republic, E-mail: kourimska@af.czu.cz.

Kateřina Ešlerová, Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Quality of Agricultural Products, Kamýcká 129, 165 21 Praha 6 - Suchdol, Czech Republic, E-mail: k.eslerova@gmail.com.

Ynus Khatri, Department of Food Science and Agri-food Supply Chain Management, Harper Adams University, Newport, Shropshire, TF10 8NB, United Kingdom, E-mail: ykhatri@harper-adams.ac.uk.

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RELATION BETWEEN SELECTED NUTRIENTS IN THE CHICKEN MEAT DEPENDING ON PHYTOGENIC FEED ADDITIVES

Mária Angelovičová, Martin Mellen, Ondřej Bučko, Jana Tkáčová, Jozef Čapla, Peter Zajác, Marek Angelovič

ABSTRACT

The aim of study was to evaluate the relation between selected nutrients in the breast and thigh muscles after the application of different phytogenic additives in the diet of broiler chickens and between same indicators of meat disregarding additive and parts of carcass, from which muscles originate. We realized an *in vivo* experiment on the Zámostie Company poultry test station with deep litter breeding system. The experiment included 100 pcs of one-day-old hybrid chickens Cobb 500 divided into 2 groups (n = 50): the 1st experimental group with an application of feed additive from chestnut tree and lemon fruit extracts and the 2nd experimental group with an application of feed additive from citrus fruits extract. We used a cereal and soybean basal diet and we divided the fattening period into four phases: starter (1 – 10 days), grower I (11 – 20 days), grower II (21 – 28 days) and finisher (29 – 42 days). We applied a powder form feed mixtures. Nutritive value of feed mixtures was the same in each experimental group during the whole experiment and in accordance with the physiological needs of broiler chickens. We fed the 1st experimental group with a basal diet enriched by feed additive from chestnut tree and lemon fruit extracts (50 g/100 kg). As for the 2nd experimental group, we applied feed additive from citrus fruits extracts through the drinking water (100 mL/100 L). In the 2nd part of our experiment, we compared results obtained from two experimental groups with other four groups of diet. We applied other phytogenic additives to these four groups and we did not take into account the origin of the meat sample. We measured indicators of the chemical composition of protein, fat, water and cholesterol on a sample (50 g) of breast and thigh muscle without skin by the method of FT IR by use of the apparatus Nicolet 6700. Detected relations between nutrients of breast and thigh muscles were defined by correlation coefficient of $-0.6 \leq r \geq +0.6$. When additive with chestnut tree and lemon fruit extracts was used, we detected a negative correlation ($p \leq 0.01$) between protein and cholesterol of breast muscle. In thigh muscle, the negative correlation was observed between protein and energy ($p \leq 0.05$), protein and fat ($p \leq 0.01$) as well as fat and water. The only positive correlation was detected between protein and cholesterol of breast muscle ($p \leq 0.01$), with additive citrus fruits extract. When nutrition and parts of carcass, from which muscles originate, were disregarded, protein of meat increased, energy and fat decreased ($p \leq 0.001$). When fat of meat increased, energy increased ($p > 0.05$) as well, but water decreased ($p \leq 0.05$; $p \leq 0.001$).

Keywords: phytogenic feed additive; breast and thigh muscle; chicken meat; nutrient

INTRODUCTION

Poultry meat is an important source of proteins, but other constituents as fats have an important role in its composition, too. Manipulation in animal feeding **(Kennedy et al., 2005)** or *post mortem* manipulation of carcass body may affect meat quality. 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 **(Kirkpinar et al., 2011)**. Earlier published papers **(Smid and Gorris, 1999)** present that essential oils achieved positive performance in antibacterial *in vitro* studies. We need greater concentration of essential oil to achieve the same effect in foods. High levels of fat and/or protein in foodstuffs protect the bacteria from the action of the essential oil in some way **(Tassou, 1995)**.

This short literature review suggests that an application of phytogenic substances based on essential oils have some unanswered questions in food research.

The aim of our study was to evaluate the relation: a) between selected nutrients, energy and water of breast and thigh muscles regarding to application of two different phytogenic additives in the diet of broiler chickens; b) between the same nutrients, energy and water of chicken meat disregarding parts of carcass, from which muscles originate (breast and thigh muscles together).

MATERIAL AND METHODOLOGY

Experiment, broiler chickens, nutrition

We realized an *in vivo* experiment on the Zámostie Company poultry test station with deep litter breeding system. The experiment included 100 pcs of one-day-old hybrid chickens Cobb 500 divided into 2 groups (n = 50): the 1st experimental group with an application of feed additive from chestnut tree and lemon fruit extracts and the 2nd experimental group with an application of feed additive from citrus fruits extract. Phytogenic substances obtained from chestnut tree and lemon fruit extracts created a base of applied feed additive. The feed additive represents a mixture of taste compounds with supporting antioxidative, antimicrobial and cleansing effects in the digestive tract. Citrus fruits extract included extracts obtained from four species of citrus fruits: grapefruit (*Citrus paradisi*), tangerine (*Citrus reticulata blanco*), bergamot (*Citrus aurantium* ss. Bergamia) and sweet orange (*Citrus sinensis*). We used a cereal and soybean basal diet and we divided the fattening period into four phases: starter (1 – 10 days), grower I (11 – 20 days), grower II (21 – 28 days) and finisher (29 – 42 days). We applied a powder form of feed mixtures. Nutritive value of feed mixtures was the same in each experimental group during the whole experiment and in accordance with the physiological needs of broiler chickens. We fed the 1st experimental group with a basal diet enriched by feed additive from chestnut tree and lemon fruit extracts (50 g/100 kg). As for the 2nd experimental group, we applied feed additive from citrus fruits extracts through the drinking water (100 mL/100 L). In the 2nd part of our experiment, we compared results obtained from two experimental groups with other four groups of diet. We applied other phytogenic additives to these four groups and we did not take into account the origin of the meat sample.

Sample analyses

At the end of the experiment (day 42), we randomly selected 6 pcs from each group with an average live body weight of about 1800 g. We performed a slaughtering of chickens by human rapid cut of the carotid artery (*Ateria carotis communis*). We separated breast and thigh muscle from the carcass and we used them for chemical analysis. We measured indicators of the chemical composition of protein, fat, water and cholesterol on a sample (50.0 g) of breast and thigh muscle without skin by the method of FT IR by use of the apparatus Nicolet 6700. We performed a molecular spectroscopy for infrared spectrum of muscle homogenates analyses. The principle of this method is infrared absorption spectrum of the sample passes and there is a change from the rotary vibrating energy conditions of the molecule depending on the changes of the dipole moment of the molecule. Analytical output is the infrared spectrum, which is a graphical representation of the function of the energy dependence, mostly mentioned as a percentage of transmittance (T) or in units of absorbance (A) at a wavelength of the incident radiation. Permeability is defined as ratio of the radiation intensity which has passed through the sample (I) and of the emission intensity of emitted source (Io). Absorbance is defined as a decimal logarithm of 1/T.

Calculation of the energy value of meat according to the measured values of protein and fat, and the corresponding coefficients: 16.75 ×protein +37.68 ×fat (kJ/100 g).

Statistical analyses

We present our results in the form of mean, standard deviation, minimum and maximum values We used Scheffe´s test at the significance level of $\alpha = 0.05$ to compare a difference between groups. We used a Pearson´s correlation coefficient to reflect a degree of relation between two variables of selected chemical indicators of chicken breast muscle, thigh muscle and meat. Pearson´s r reflects the degree of linear relation between the two data sets. Its value is between -1 and +1. A value of +1 means, that there is a perfect positive linear relation between two data sets. A value of -1 means that there is a perfect negative linear relation and a value of 0 means, that there is no linear relation at all between data sets. We mainly focused our attention on the assessment of relation between two variables defined by correlation coefficient $-0.6 \leq r \geq +0.6$. We supplemented our results of correlation coefficient statistical significance at the significance level of $\alpha = 0.05$, 0.01 and 0.001. We used SAS statistical package (**SAS Institute, 1998**) to perform statistical analyses.

RESULTS AND DISCUSSION

Chemical composition of breast and thigh muscles in relation to feed additive and disregarding parts of carcass from which muscles originate (breast and thigh muscles together)

Chemical composition of breast and thigh muscles in relation to feed additive are shown in Table 1 and disregarding parts of carcass from which muscles originate (breast and thigh muscles together) in Table 2.

In two experimental groups, values of protein content of breast muscles are relatively similar 23.83 and 24.09 g/100 g, respectively. We observed slightly larger difference between the protein content of breast muscle compared to protein content of thigh muscle depending on the type of phytogenic substances in feed mixtures. We did not observe a statistically significant difference ($p > 0.05$) between groups. Similar values of protein content in breast muscles found **Haščík et al., (2012),** but in the hybrid combination of the chickens Ross 308.

We recorded the protein content of 22.73 g/100 g in the group with a feed additive from chestnut tree, and lemon fruit extracts compared with 22.15 g/100 g for the group with the feed additive from citrus fruits extract in the thigh muscles. We observed statistically significant difference ($p \leq 0.05$) between protein content of thigh muscle of the 1st group and the breast muscle of the 2nd group (Table 1). Table 2 presents that, the average protein content of chicken meat was 23.21 g/100 g disregarding parts of carcass, from which muscles originate. The type of phytogenic supplements did not affect the energy value of breast muscle and thigh muscle.

The energy values of breast samples were 435.69 kJ/100 g and 437.26 kJ/100 g in the 1st experimental group and the 2nd experimental group, respectively. Slightly lower energy value of broiler chicken breast muscles Cobb 500 found **Angelovičová and Semivanova (2013)**. We

Table 1 Nutrients, energy and water in the chicken breast and thigh muscles in relation to feed additive.

Variable	n	Breast muscle				Thigh muscle			
		Mean	*SD*	Min.	Max.	Mean	*SD*	Min.	Max.
Feed additive from chestnut tree and lemon fruit extracts									
Protein (g/100 g)	6	23.83	0.49	22.90	24.21	22.73	0.92	21.69	24.40
Energy (kJ/100 g)	6	435.69	6.03	428.72	444.50	458.13	15.82	438.17	474.04
Fat (g/100 g)	6	0.69	0.24	0.46	1.02	1.39	0.60	0.35	2.00
Water (g/100 g)	6	74.85	0.35	74.29	75.32	74.33	0.44	73.77	74.83
Cholesterol (g/100 g)	6	0.23	0.07	0.17	0.34	0.32	0.09	0.22	0.45
Feed additive from citrus fruits extract									
Protein (g/100 g)	6	24.09	0.42	23.57	24.63	22.15	0.49	21.58	22.92
Energy (kJ/100 g)	6	437.26	11.21	424.86	453.78	459.26	12.55	440.66	472.53
Fat (g/100 g)	6	0.69	0.42	0.39	1.50	1.21	0.07	1.09	1.29
Water (g/100 g)	6	74.73	0.24	74.39	75.07	74.09	0.56	73.62	74.92
Cholesterol (g/100 g)	6	0.25	0.11	0.12	0.36	0.36	0.06	0.31	0.44

Legend: n – the number of samples, *SD* – standard deviation, Min. – the minimum value, Max. – the maximum value.

Table 2 Nutrients, energy and water in the chicken meat disregarding parts of carcass from which muscles originate (breast and thigh muscles together).

Variable	n	Mean	*SD*	Min.	Max.
Protein (g/100 g)	72	23.21	0.91	21.58	24.71
Energy (kJ/100 g)	72	448.35	18.42	415.73	492.78
Fat (g/100 g)	72	1.05	0.49	0.21	2.10
Water (g/100 g)	72	74.46	0.59	72.52	75.32
Cholesterol (g/100 g)	72	0.29	0.08	0.10	0.45

Legend: n – the number of samples, *SD* – standard deviation, Min. – the minimum value, Max. – the maximum value.

detected almost the same energy value of thigh muscle in two experimental groups. The energy value of breast muscle and thigh muscle of two experimental groups was not statistically significant (p >0.05), while calculated energy value disregarding parts of carcass, from which muscle originates (Table 2) was 448.35 kJ/100 g.

We obtained interesting results of the fat content of breast muscles in our experiment. We detected the same value of 0.69 g/100 g in the group with feed additive from chestnut tree and lemon fruit extracts, and in the group with feed additive from citrus fruits extract. Fat content values of thigh muscle samples were higher than values of breast muscle samples. In the group with feed additive from chestnut tree and lemon fruit extracts, we detected an average value 1.39 g/100 g of fat in thigh muscle, and 1.21 g/100 g of fat in the group with feed additive from citrus fruits extracts. A difference of fat content was not statistically significant (p >0.05) between groups. The value of fat content in chicken meat was 1.5 g/100 g, when we disregarded the part of carcass, from which the muscle originates and feed additive. Moisture content results in breast samples were almost the same, i.e. 74.85 g/100 g and 74.73 g. /100 g in the 1st experimental group and the 2nd experimental group, respectively.

We did not find statistically significant difference (p >0.05) between experimental groups. Similar values of dry matter of chicken breast muscle of Cobb 500 found **Medveď and Angelovičová (2010).**

Other study with the same hybrid of chickens – Cobb 500 presents lower water content values of 73.73 and 73.29 g/100 g. Authors used commercial feed and they slaughtered chickens of experimental groups at the age of 42 days **(Suchý et al., 2002)**.

Our results of detected water content in the thigh samples were 74.33 g/100 g and 74.09 g/100 g in the 1st experimental group and 2nd experimental group, respectively. The conclusions of other studies **(Al-Sultan, 2003; Latshaw and Moritz, 2009)** confirmed decreased water content of the thigh muscles compared with breast muscle. We obtained same results. Water content of chicken meat was 74.46 g/100 g disregarding parts of carcass, from which the muscles originates and feed additive.

The image of meat and meat products is relatively negative due to their content of fat and saturated fatty acids, cholesterol, sodium and any other substances (e.g. nitrosamines) that somehow can be involved in most prevalent diseases of Western societies like cardiovascular diseases and *diabetes mellitus* **Micha et al., 2010)** and cancer **(Ferguson, 2010)**. Our results of cholesterol content were almost the same in relation to the type of used phytogenic substances. The cholesterol content was slightly increased in the group with feed additive from chestnut tree and lemon fruit extracts compared to group with feed additive from citrus fruits extract. We did not observe statistically significant difference between two experimental groups (p >0.05). The cholesterol content of breast muscles was 0.23 g/100 g in the 1st experimental group and 0.25 g/100 g in the 2nd experimental group. The cholesterol content of thigh muscles was 0.32 g/100 g (the 1st experimental group) and 0.36 g/100 g (the 2nd experimental group). The cholesterol content of chicken meat was 0.29 g/100 g when we disregarded parts of carcass from which the muscles originate.

Within this context, the poultry meat has maintained its identity and a higher value compared to other species for several reasons. Indeed, worldwide poultry meat production and consumption have increased rapidly and, in many parts of the world, it is assumed, per capita consumption of poultry meat will continue to grow

(Cavani, 2009). Relatively low and competitive prices compared to other meats, the absence of cultural or religious obstacles, and dietary and nutritional properties are the main factors explaining poultry meat's attractiveness **(Valceschini, 2006)**.

Relation between selected nutrients, energy and water of chicken breast and thigh muscles depending on feed additive and disregarding parts of carcass from which the muscles originate (breast and thigh muscles together)

A positive correlation coefficient indicates that an increase in the first variable would correspond to an increase in the second variable, thus implying a direct relation between the variables. A negative correlation indicates an inverse relation where as one variable increases, the second variable decreases.

Relation between selected nutrients, energy and water of chicken breast and thigh muscles in the 1st experimental group with feed additive from chestnut tree and lemon fruit extracts

Relation between selected nutrients, energy and water of chicken breast muscle in the 1st experimental group

Table 3 presents a correlation coefficient (r) between nutrients, energy and water of breast muscles in the 1st experimental group.

Table 3 Correlation coefficient (r) between nutrients, energy and water of breast muscles in the 1st experimental group.

Variable	Energy	Fat	Water	Cholesterol
Protein	-0.27	-0.42	0.60	-0.95^{++}
Energy		-0.12	-0.63	0.01
Fat			-0.47	0.43
Water				-0.36

Legend: Numerical data – the correlation coefficient (r) between two variables.
++: value with superscript mark is significantly different ($p \leq 0.01$).

Combined essential oils have additive, synergistic, and antagonistic effects **(Burt, 2004)**. Many commercial products on the market have one or more combined essential oils. Utilization of any feed additive is justified due to the larger beneficial effect compared to the cost of the product. In our experiment, feed additive from chestnut tree and lemon fruit extracts influenced the correlative relation between certain nutrients, energy and water of breast muscle. We detected the relation with correlation coefficient of $-0.6 \leq r \geq +0.6$ between protein and water, between protein and cholesterol and between energy and water. All relations with correlation coefficient value of $-0.6 \leq r \geq +0.6$ were not statistically significant. A positive correlation without statistically significant difference ($p > 0.05$) was between protein and water. We observe statistically significant difference ($p \leq 0.01$) between protein and cholesterol. When protein content of breast muscle increased, cholesterol content decreased. When energy value of breast muscle increased, water content decreased, without statistically significant differences ($p > 0.05$).

We did not find any literature information about effects of essential oils on relations between nutrients of chicken meat, parts of carcass, respectively. Essential oils have antimicrobial, antifungal and antioxidant effects. The effects of several type of essential oils, their combinations, or a combination with other substances might be related to the relations between nutrients of chicken meat, breast and thigh muscles. Citrus species of various origins have been assessed for their phenolic constituents and antioxidant activities **(Guimarães et al., 2009)**. Citrus fruits, citrus fruit extracts and citrus flavonoids exhibit a wide range of promising biological properties including anti-atherogenic, anti-inflammatory and antitumor activity, inhibition of blood clots and strong antioxidant activity **(Middleton and Kandaswami, 1994)**.

Extracts of citrus fruit (e.g. lemon, orange, and grape fruit) are among the most studied natural antimicrobials for food applications. Extracts of citrus fruit effectively decrease the growth of bacteria. Limonoids obtained from *Citrus limon* showed good antibacterial and antifungal activity **(Corbo et al., 2008)**. There are several citrus species, i.e. *Citrus limon* (lemon), *Citrus aurantium* (bitter orange), *Citrus limetta* (sweet lemon), *Citrus jambhiri* (rough lemon) and *Citrus paradise* (grapefruit) **(Al-Ani et al., 2009)**.

Three types of flavonoids occur in citrus fruit, i.e. flavanones (including 3-hydroxyflavanones), flavones (including 3-hydroxyflavones) and anthocyanins **(Horowitz and Gentili, 1977)**.

Eight tested limonoids, i.e. nomilin, limonin, deacetylnomilin, limonol, obacunone, deoxylimonin, isoobacunoic acid and ichangin stimulated the detoxifying enzyme, glutathione S-transferase **(Lam and Hasegawa, 1989)**. Glutathione S-transferase enzymes are one of the major enzyme systems responsible for the detoxification of xenobiotics **(Chasseaud, 1979)**.

Vitamin C and bioflavonoids. Bioflavonoids are a class of water-soluble plant pigments. Vitamin C-rich fruits and vegetables, especially citrus fruits, are often rich sources of bioflavonoids as well. Two small published studies examined the effect of bioflavonoids on the bioavailability of ascorbic acid. In one study, synthetic ascorbic acid given in a natural citrus extract containing bioflavonoids (in the ratio of bioflavonoids to ascorbic acid of 4:1), proteins, and carbohydrates, was more slowly absorbed and 35% more bioavailable than synthetic ascorbic acid alone, based on plasma levels of ascorbate over time and 24-hour urinary excretion of ascorbate. In the other study, there was no difference in the bio-availability of 500 mg of synthetic ascorbic acid and that of a commercially available vitamin C preparation with added bioflavonoids, where the ratio of bioflavonoids to ascorbic acid was 0.05:1 **(Higdon, 2001)**.

Relation between selected nutrients, energy and water of chicken thigh muscles in the 1st experimental group

Table 4 presents a correlation coefficient (r) between nutrients, energy and water of thigh muscles in the 1st experimental group.

Table 4 Correlation coefficient (r) between nutrients, energy and water of thigh muscles in the 1st experimental group.

Variable	Energy	Fat	Water	Cholesterol
Protein	-0.83^{+}	-0.95^{++}	0.70	-0.25
Energy		0.75	-0.64	0.35
Fat			-0.82^{+}	0.10
Water				-0.15

Legend: Numerical data – the correlation coefficient (r) between two variables,
+, ++: value with superscript mark is significantly different ($p \leq 0.05$, $p \leq 0.01$).

Extracts of chestnut tissue display a strong antimicrobial activity against many plant pathogens, which is probably associated with antimicrobial compounds such as flavonol glycoside and several terpenoid substances **(Hao et al., 2012)**. The results presented in study **(Blaiotta et al., 2013)** indicate that chestnut extracts can greatly improve the tolerance of lactobacilli to simulated gastric and bile juice. Chestnut extracts exhibited a surprising effect in improving the tolerance to gastric transit of lactobacilli. The study confirmed that scoparone and scopoletin isolated from chestnut inner shell extract have antioxidant effects, and scopoletin has relatively higher antioxidant capacity than scoparone in an oxidative stress-induced in vitro system.

Chestnut inner shell extract including scoparone and scopoletin as main compounds has the ability to protect against damage due to oxidative stressors including tert-butyl hydroperoxide, carbon tetrachloride (CCl_4), and high-fat diet, by preventing reactive oxygen species generation, decrease of antioxidant enzyme activity, and inhibiting malondialdehydu production. The chestnut inner shell extract might be useful as a natural ingredient for the prevention of oxidative damage in liver cells and tissues **(Noh et al., 2010)**.

In our experiment, the influence of feed additive from chestnut tree and lemon fruit extracts on relation between certain nutrients, energy and water of the thigh muscles was higher compared to breast muscle. We detected a relation with correlation coefficient of $-0.6 \leq r \geq +0.6$ between protein and energy, protein and fat, protein and water, energy and fat, energy and water as well as fat and water. All these correlation relations were not statistically significant. When energy and fat of thigh muscles increased, protein content decreased. These relations were statistically significant ($p \leq 0.05$, $p \leq 0.01$, respectively). When fat content of the thigh muscles increased, water content decreased, with statistically significant difference ($p \leq 0.05$).

Many authors have concluded that essential oils exhibit greater antimicrobial activity than other major components taken together. This could means that either the minor components are critical to the antimicrobial activity or that synergistic effects may occur **(Burt, 2004)**. The major components reflect the biological properties of essential oils, but minor components can modulate their activity, for example the cell penetration, hydrophobicity and fixation on membranes **(Bakkali et al., 2008)**. The composition of essential oil results in interactions between the components that both qualitatively and quantitatively change their evaporation rates **(Saiyasombati and Kasting, 2003)**.

Relation between selected nutrients, energy and water of chicken breast and thigh muscles in the 2nd experimental group with feed additive from citrus fruits extract

Relation between selected nutrients, energy and water of chicken breast muscle in the 2nd experimental group

Table 5 presents a correlation coefficient (r) between nutrients, energy and water of breast muscles in the 2nd experimental group.

We could consider citrus essential oils as suitable alternatives to chemical additives for use in the food industry, attending to the needs for safety and satisfying the demand of consumers for natural components **(Viuda-Martos et al., 2008)**. Since the major component of citrus essential oils is limonene, the chemical, physical and biological properties of this compound greatly affect the properties of the citrus essential oils **(Bakkali et al., 2008)**. For this reason, we can find documented antimicrobial effect of citrus essential oils attributed to the essential oil or to limonene as well, as its main component. Biodegradation essential oils, and in particular limonene; mechanism by which essential oils inhibit anaerobic digestion is not yet understood **(Ruiz and Flotats, 2014)**.

Table 5 Correlation coefficient (r) between nutrients, energy and water of breast muscles in the 2nd experimental group.

Variable	Energy	Fat	Water	Cholesterol
Protein	-0.08	-0.51	-0.40	-0.50
Energy		-0.62	0.08	0.86^{+}
Fat			0.37	-0.40
Water				0.32

Legend: Numerical data – the correlation coefficient (r) between two variables,
+: Value with superscript mark is significantly different ($p \leq 0.05$).

The antimicrobial activity of terpenes and terpenoids (cyclic hydrocarbons) is due mainly to their interaction with the cell membrane **(Bakkali et al., 2008)**. We detected an influence of feed additive from citrus fruits extract only on relations between energy and fat, as well as energy and cholesterol. A correlation coefficient of relation between energy and fat, as well as energy and cholesterol was $-0.6 \leq r \geq +0.6$. An interesting result is the relation between energy and fat. When fat content of breast muscles increased, energy content decreased. The difference was not statistically significant ($p > 0.05$). We detected statistically significant difference ($p \leq 0.01$) of positive correlation between energy and cholesterol.

Relation between selected nutrients, energy and water of chicken thigh muscles in the 2nd experimental group

Table 6 presents a correlation coefficient (r) between nutrients, energy and water of thigh muscles in the 2nd experimental group.

Table 6 Correlation coefficient (r) between nutrients, energy and water of thigh muscles in the 2nd experimental group.

Variable	Energy	Fat	Water	Cholesterol
Protein	-0.41	0.04	-0.27	-0.30
Energy		0.66	-0.25	0.42
Fat			0.05	-0.14
Water				-0.73

Legend: Numerical data – the correlation coefficient (r) between two variables.

The influence of feed additive from citrus fruits extract on relation between some nutrients, energy and water of thigh muscles was lower compared to the effects of feed additive from chestnut tree and lemon fruit extracts. We found a positive correlation with correlation coefficient of $-0.6 \leq r \geq +0.6$ between energy and fat of thigh muscles. The difference was not statistically significant ($p > 0.05$). Citrus essential oils are a complex mixture of volatile compounds that show, among other properties, antifungal activity by reducing or totally inhibiting fungal growth in a dose-response manner **(Sharma and Tripathi, 2006)**. This activity is a resalt of a single major compound or synergistic or antagonistic effect of various compounds **(Deba et al., 2007)**.

Relation between selected nutrients, energy and water of chicken meat disregarding parts of carcass, from which muscles originate

Table 7 presents a correlation coefficient (r) between nutrients, energy and water of chicken meat disregarding parts of carcass, from which muscles originate (breast and thigh muscles together).

Table 7 Correlation coefficient (r) between nutrients, energy and water of chicken meat disregarding parts of carcass from which the muscles originate (breast and thigh muscles together).

Variable	Energy	Fat	Water	Cholesterol
Protein	-0.64^{+++}	-0.75^{+++}	0.42^{+++}	-0.37^{+++}
Energy		0.70^{+++}	-0.67^{+++}	0.39^{+++}
Fat			-0.59^{+++}	0.26^{+}
Water				-0.30^{++}

Legend: Numerical data – the correlation coefficient (r) between two variables,
+, ++, +++: Value with superscript mark is significantly different ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$).

In our experiment, disregarding nutrition and parts of carcass, from which muscles originate, we found a statistically significant relation with correlation coefficient of $-0.6 \leq r \geq +0.6$ between protein and energy, protein and fat, as well as energy and fat, energy and water, fat and water of chicken meat. When protein content of chicken meat increased, energy content and fat content decreased with statistically significant differences ($p \leq 0.001$). These relations are relatively comparable to the relations between protein, energy and water of thigh muscles in the 1st experimental group. When fat content of chicken meat increased, energy content increased as well without statistically significant difference ($p > 0.05$), but water content decreased with statistically significant difference ($p \leq 0.001$). When energy content of chicken meat increased, water content decreased with statistically significant difference ($p \leq 0.001$). We observed a relation with correlation coefficient of $-0.6 \leq r \geq +0.6$ between cholesterol and protein, cholesterol and fat, cholesterol and energy, cholesterol and water, as well as protein and water. The differences were statistically significant ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$, respectively). These relations are relatively comparable to relations between cholesterol, energy and water of thigh muscles in the 1st experimental group.

CONCLUSION

We can confirm based on a statistical evaluation of the results of the experiment that:
a) broiler chicken nutrition is one of the major factors which must be taken into account in the production of chicken meat;
b) various additives of phytogenic substances in the feed mixtures for broiler chicken differently affected relations between protein and cholesterol in breast muscle, and between protein and energy, between protein and fat, and the fat and water in thigh muscle.

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Contact address:
prof. Ing. Mária Angelovičová, CSc., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: maria.angelovicova@uniag.sk.

doc. Ing. PhDr. Martin Mellen, PhD., Hydina Slovakia s.r.o., 065 11 Nová Ľubovňa, 505, Slovakia, E-mail: martin.mellen@gmail.com.

Ing. Ondřej Bučko, PhD., Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Animal Husbandry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: ondrej.bucko@uniag.sk.

Ing. Jana Tkáčová, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.tkacova@uniag.sk.

Ing. Jozef Čapla, PhD. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: capla@potravinarstvo.com.

Ing. Peter Zajac, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zajac@potravinarstvo.com.

doc. Ing. Marek Angelovič, PhD., Slovak University of Agriculture in Nitra, *Faculty of* Enginnering, *Department of* Machinery and Production Systems, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marek.angelovic@uniag.sk.

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EFFECT OF DIET SUPPLEMENTED WITH PROPOLIS EXTRACT AND PROBIOTIC ADDITIVES ON PERFORMANCE, CARCASS CHARACTERISTICS AND MEAT COMPOSITION OF BROILER CHICKENS

Peter Haščík, Lenka Trembecká, Marek Bobko, Miroslava Kačániová, Juraj Čuboň, Simona Kunová, Ondřej Bučko

ABSTRACT

The present research focused on the effects of propolis extract and probiotic preparation based on *Lactobacillus fermentum* (1×10^9 CFU per 1 g of bearing medium) on performance, carcass characteristics and meat composition of broiler chickens. The experiment was performed with 360 one day-old Ross 308 broiler chicks of mixed sex. The chicks were randomly allocated into 3 groups (n = 120 pcs chicks per group), namely, control (C) and experimental (E1, E2). Each group consisted of 3 replicated pens with 40 broiler chickens per pen. The experiment employed a randomized design, and dietary treatments were as follows: 1. basal diet with no supplementation as control (group C), 2. basal diet plus 400 mg propolis extract per 1 kg of feed mixture (group E1), 3. basal diet plus 3.3 g probiotic preparation added to drinking water (group E2). Besides, the groups were kept under the same conditions. Fattening period lasted for 42 days. Feed mixtures were produced without any antibiotic preparations and coccidiostats. As regards performance of broilers, all the investigated parameters were improved after addition of the supplements, especially after probiotic supplementation. However, neither propolis extract nor probiotic in diet of broiler chickens had any significant effect ($p \geq 0.05$) on performance. Meat composition was evaluated as proximate composition (dry matter, crude protein, fat and ash), cholesterol content and energy value in the most valuable parts of chicken meat (breast and thigh muscles). The statistically significant results ($p \leq 0.05$) were attained in fat, ash and cholesterol content, as well as energy value in both breast and thigh muscles after the propolis supplementation. To sum up, the present study demonstrated the promising potential of propolis extract and probiotic to enhance the performance, carcass characteristics and meat composition under conditions of the experiment with, however, statistical significance of results in a few parameters.

Keywords: performance; meat; chicken; propolis; probiotic

INTRODUCTION

Chickens are the most popular amongst different poultry species worldwide. Owing to their relatively low fat and cholesterol contents, chicken meat is considered a healthy animal food. Moreover, chicken continues to be the cheapest among all types of meat consumed in the world and its consumption is expected to increase by 34% by 2018 **(Umaya Suganthi, 2014; Petrová et al., 2015)**. Modern intensive chicken production has achieved phenomenal gains in the efficient and economical production of high quality and safe chicken meat. The use of feed additives has been an important part of achieving this success **(Hashemi et al., 2012)**.

For several decades, antibiotics have been widely used in the chicken diet **(Goodarzi and Nanekarani, 2014)**. However, the use of dietary antibiotics have resulted in controversial problems such as development of antibiotic resistant bacteria and drug residue in the final products which can be harmful to consumers **(Goodarzi et al., 2014)**. As a result, additives such as probiotics and natural substances such as propolis have received increased attention as possible antibiotic growth promoter substitutions in chicken diet **(Haščík et al., 2012; Daneshmand et al., 2015)**.

Propolis is a resinous material elaborated by bees, through the recollection of the exudates from different plant species **(Valenzuela-Barra et al., 2015)** and is used in construction and adaptation of their hives. It possesses many pharmacological activities, such as anti-inflammatory, antibiotic, antiviral and immunostimulant **(Fan et al., 2013)**.

In many studies conducted on propolis, many positive effects like increase in feed intake, body weight, flavonoid content, taste improvement, antioxidant and antimicrobial properties have been reported **(Tatlı Seven et al., 2008)**. The properties of propolis are based on its rich flavonoid, phenolic acid and terpenoid contents **(Seven et al., 2012)**.

An alternative approach to subtherapeutic antibiotics in chicken diet is also the use of probiotic microorganisms **(Alkhalf et al., 2010)**. Probiotics are live, non-pathogenic bacteria that contribute to the health and balance of the intestinal tract **(Giannenas et al., 2012)**. The most important advantage of a probiotic is that it neither has any residues in animal production nor exerts any antibiotic

resistance by consumption **(Alkhalf et al., 2010)**. Several studies showed that dietary supplementation of lactic acid bacteria (e.g. *Lactobacillus*) improve the performance and feed conversion **(Taklimi et al., 2012; Bai et al., 2013)**, stimulate immune response and increase bone strength of broiler chickens. The enhanced growth with probiotics may be partly attributed to the colonisation of the gastrointestinal tract of the chicks, which improved the digestion of essential nutrients **(Khaksefidi and Rahimi, 2005)**.

This study was designed to investigate the effects of dietary addition of propolis extract and probiotic preparation based on *Lactobacillus fermentum* on performance, carcass characteristics and meat composition of Ross 308 broiler chickens.

MATERIAL AND METHODOLOGY

Chickens and dietary treatments

The experiment was carried out in test poultry station of Slovak University of Agriculture in Nitra. A total of 360 one day-old broiler chicks of mixed sex (Ross 308) were randomly divided into 3 groups, namely, control (C) and experimental (E1, E2). Each group consisted of 3 replicated pens with 40 broiler chickens per pen. The experiment employed a randomized design, and dietary treatments were as follows: 1. basal diet as control (group C), 2. basal diet plus 400 mg propolis extract per 1 kg of feed mixture (group E1), 3. basal diet plus 3.3 g probiotic preparation added to drinking water (group E2). Besides, the groups were kept under the same conditions.

The experiment lasted for 42 days. The broiler chickens were reared on breed litter (wood shavings), in a temperature-controlled room; ambient temperature in test poultry station was maintained at 33 °C during the first week and gradually decreased by 2 °C, and finally fixed at 19 °C thereafter. Throughout the entire experimental period, the chickens had *ad libitum* access to feed and water, and were kept under constant light regime.

Table 1 lists the basal diet formulated according to nutrient requirements of broilers. The broiler chickens were fed a starter diet from 0 to 21st day and grower diet from 22nd to 42nd day. The feed mixtures both starter and grower were produced without any antibiotics and coccidiostats.

Propolis had origin in the Slovak Republic. The extract was prepared from minced propolis in the conditions of the 80% ethanol in the 500 cm^3 flasks, according to **Krell (1996)**. Determination of phenolic compounds, namely the phenolic acids (caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid) and flavonoids (routines, quercetin, kaempferol, apigenin, tectochrysin) in propolis extract (Table 2) was performed using an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a degasser, an autosampler and a diode array detector (DAD).

In the experiment, the probiotic preparation based on *Lactobacillus fermentum* (1×10^9 CFU per 1 g of bearing medium) was used.

Slaughter and measurements

At 42 days of age, chickens were weighed and slaughtered at the experimental slaughterhouse of Slovak University of Agriculture in Nitra.

After evisceration, the carcasses were kept at approximately 18 °C for 1 h *post mortem* and thereafter longitudinally divided into two parts. After that, the half-carcasses and giblets were weighed and stored at 4 °C until 24 h *post mortem*. The right half-carcasses were used in order to determinate the parameters as described below, whereas the left half-carcasses were assigned to different analysis. All the weight measurements were performed using the precision balance Kern 440 (Kern & Sohn, Germany) with accuracy of 0.01 g. The carcass yield was calculated by dividing carcass weight with giblets and abdominal fat weight by live body weight.

The chemical analysis of chicken meat (breast muscle without skin, thigh muscle with skin and subcutaneous fat) was performed using an Infratec 1265 Meat Analyzer. The cholesterol content of chicken meat was determined by spectrophotometric method according to **Horňáková et al., (1974)**. The energy value (kJ/100 g) was calculated through the conversion factors for fat and protein **(Strmiska et al., 1988)**.

Statistical analysis

The data processing was performed using a statistical program Statgraphics Plus Version 5.1 (AV Trading Umex, Dresden, Germany). For the determination of significant difference between the tested groups, analysis of variance (ANOVA) was used.

RESULTS AND DISCUSSION

The effects of propolis and probiotic supplementation on performance and carcass characteristics of Ross 308 broiler chickens are shown in Table 3. Live body weight of broilers did not differ statistically between the control and experimental groups ($p \geq 0.05$). Similarly, no differences ($p \geq 0.05$) were found between the groups in carcass weight, giblets weight and carcass yield.

Yet, effect of the supplementation has shown to be favourable since the chickens fed diet containing the propolis extract (2316.9 g) and probiotic preparation (2335 g) had higher live body weight than control chickens (2270.2 g).

The results of the study for performance and carcass characteristics of broiler chickens are in general agreement to those of previous studies where the inclusion of propolis in chicken diet also resulted in slight effect on meat performance.

Tatlı Seven et al., (2008) found higher body weight of chickens fed a diet supplemented with 0.5, 1 and 3 g propolis extract per 1 kg of feed mixture (1975 – 2010 g) than that in control (1940 g).

Shalmany and Shivazad (2006) showed that propolis extract in levels 200 and 250 mg.kg^{-1} has positive effect on growth performance of chickens due to improved weight gain and feed efficiency compared with chickens fed a basal diet.

Table 1 Composition of basal diet and nutrient content.

Ingredients (%)	Starter (HYD-01) (day of age 1 – 21)	Grower (HYD-02) (day of age 22 – 42)
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
Lysine	0.05	0.07
Methionine	0.15	0.22
Palm kernel oil Bergafat	0.70	0.16
Premix Euromix BR 0.5%*	0.50	0.50
Nutrient content ($g.kg^{-1}$)		
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
Linoleic acid	13.51	14.19
ME_N ($MJ.kg^{-1}$)	12.02	12.03

* active substances per kilogram of premix: vitamin A 2 500 000 IU; vitamin E 20 000 mg; vitamin D3 800 000 IU; niacin 12 000 mg; D-pantothenic acid 3 000 mg; riboflavin 1 800 mg; pyridoxine 1 200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 20 000 mg; folic acid 400 mg; biotin 40 mg; kobalamin 8.0 mg; choline 100 000 mg; betaine 50 000 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.

Positive effects of propolis were also observed in the study of **Biavatti et al., (2003)**, where effects of propolis extract, the *Alternanthera brasiliana* extract and lindseed oil as alternative feed additives were evaluated. The researches have suggested the additives in diet of broiler chickens due to improved broiler performance in the same way (similar body weight ($p \geq 0.05$) among the treatments that was higher than that in the control).

In another study **(Ziaran et al., 2005)**, body weight of chickens (47 day-old) fed a diet containing different levels of propolis (oil extract) was not affected when compared to those fed a diet containing no supplement (1916.64 – 1935.67 g vs. 1912.08 g).

Similar to the present findings, **Haščík et al., (2014)** demonstrated that propolis extract (200, 300, 400 $mg.kg^{-1}$) added in feed mixture increased the body weight of broiler chickens (2354.6 – 2382.9 g). However, no major effects on chicken growth performance were observed (2272.89 g in control group).

In contrast, **Açıkgöz et al., (2005)** reported significant decrease in body weight of male broilers after propolis supplementation (powder). The body weight of chickens fed diet containing propolis powder ranged from 2061 to 2229 g compared with that in control group (2302 g). In the study, pine originated propolis, which is characterized by strict genuine odour, volatile compounds and a bitter taste, was used. Because of these specific characteristics, broilers might reject the feed mixture that results in adverse effects on growth performance.

In the study of **Daneshmand et al., (2015)**, the body weight of broiler chickens (42 day-old) fed a diet containing 200 $mg.kg^{-1}$ propolis extract (2395 g) was also lower compared with that in the control (2433 g). On the contrary, probiotic preparation (0.45 $g.kg^{-1}$ of feed mixture) containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* and

Table 2 Concentration of analysed phenolic compounds in propolis extract.

Compound	RT[1] (min)	Concentration ($mg.g^{-1}$)
Caffeic acid	8.48	4.976 ±2.049
p-Coumaric acid	12.83	9.826 ±8.232
Ferulic acid	14.00	7.436 ±6.710
Cinnamic acid	26.47	0.367 ±0.182
Routines	22.33	4.578 ±1.714
Quercetin	29.59	2.963 ±0.762
Kaempferol	32.93	2.503 ±0.502
Apigenin	33.69	3.970 ±2.181
Tectochrysin	37.00	7.523 ±3.959

[1]RT – retention time

Enterococcus faecium used in the same study increased the body weight of experimental chickens (2527 g). However, there was no significant increase ($p \geq 0.05$). Moreover, there was investigated the effects of propolis in combination with the probiotics (0.20 and 0.45 $g.kg^{-1}$ of feed mixture, respectively). Although the combination did not significantly affect performance, the body weight of broiler chickens receiving a combination of these additives was higher than that in control. It may reflect synergetic and complementary effects between the additives in diet of broiler chickens.

As far as the probiotics are concerned, there is considerable variation in published studies that evaluate the effect of probiotic strains on performance of broiler chickens.

There are conflicting reports on the effects of application of probiotics because the response of broiler chickens to probiotics can be affected by different factors such as the duration and method of probiotic feeding, dose and nature of the administered strains and their persistence, variation in the physiological state of the chicken, the actual microbiota balance in the gut of the chicken, as well as the sex and age of chickens **(Aliakbarpour et al., 2012)**.

In the present study, body weight was increased in probiotic-supplemented group compared with that in control and propolis-supplemented group (Table 3), but no significant difference was detected ($p \geq 0.05$).

Many studies have confirmed the positive effect of probiotics on meat performance of broiler chickens. In the study of **Apata (2008)**, addition of probiotic preparation based on *Lactobacillus bulgaricus* to the basal diet (20, 40, 60 and 80 $mg.kg^{-1}$) resulted in improved performance of broiler chickens (35 day-old). Among the dietary treatments, 60 $mg.kg^{-1}$ probiotic preparation elicited the best performance of broiler chickens.

Similar results were observed in the previous study of **Zulkifli et al., (2000)**, who reported that dietary supplementation with *Lactobacillus* cultures improves the performance of chickens.

The significant increase ($p \leq 0.05$) in body weight was demonstrated also by **Ahmed et al., (2014)**, who investigated the effects of *Bacillus amyloliquefaciens* probiotic on growth performance of broiler chickens fed for 35 days. Increasing concentration of probiotic had positive linear effect on the body weight of broilers, with the highest values being observed in broilers offered 20 $g.kg^{-1}$ probiotic.

On the contrary, **Ghasemi et al., (2014)** observed the significant increase ($p \leq 0.05$) in body weight of male broilers only after synbiotic supplementation (probiotic in combination with prebiotic). In the study, the basal diet supplemented with 1 $g.kg^{-1}$ probiotic (combination of *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Enterococcus faecium*) did not result in significant effects on body weight of chickens compared to the control. The findings indicate that after probiotics + prebiotics supplementation may be achieved much better effects on performance of broilers.

Table 3 Effect of propolis extract and probiotic on performance and carcass characteristics of broiler chickens.

Parameter	Group	x	*SD*	SEM	CV (%)
Live body weight (g)	C	2270.20	107.88	34.11	4.75
	E1	2316.90	106.12	33.56	4.58
	E2	2335.00	107.37	33.96	4.60
Carcass weight (g)	C	1629.80	73.64	23.29	4.56
	E1	1669.10	102.48	32.41	6.14
	E2	1674.00	99.54	31.48	5.95
Giblets weight (g)	C	152.08	19.83	6.27	13.04
	E1	155.64	11.53	3.45	7.41
	E2	161.21	12.26	3.88	7.61
Carcass yield (%)	C	78.54	1.41	0.45	1.80
	E1	78.31	1.18	0.37	1.50
	E2	78.58	1.50	0.47	1.91
Abdominal fat (g)	C	22.14^{a}	4.77	1.51	21.54
	E1	21.85^{b}	6.48	2.05	26.66
	E2	24.70^{ab}	7.59	2.40	30.74
Liver (g)	C	40.91	4.63	1.46	11.31
	E1	40.61	5.46	1.73	13.44
	E2	44.50	7.09	2.24	15.93
Gizzard (g)	C	26.00	5.62	1.78	21.62
	E1	25.09	3.30	1.04	13.15
	E2	25.40	4.82	1.52	18.96
Heart (g)	C	10.72	1.10	0.35	10.25
	E1	10.88	1.49	0.47	13.67
	E2	10.77	1.73	0.55	16.10

Legend: C – control group; E1, E2 – experimental groups; x – arithmetic mean; *SD* – standard deviation; SEM – standard error of mean; CV – coefficient of variation; a, b – means with different superscripts within a column differ significantly ($p \leq 0.05$).

The positive effect of probiotic supplementation ($p \leq 0.05$) was reported in the study of **Aliakbarpour et al., (2012)**. The researches demonstrated that supplementation of either *Bacillus subtilis* as the mono-strain probiotic or *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium thermophilum*, and *Enterococcus faecium* as the multi-strain probiotic in the feed mixture has the same potent stimulatory effects on broiler performance. Mono-strain probiotic fed broilers (2672.23 g), as well as multi-strain probiotic fed broilers (2664.92 g), had after 42 days of fattening higher body weight compared with control chickens (2608.99 g).

In the study of **Naseem et al., (2012)**, probiotic supplementation in two different doses (50 and 150 g per 1 ton of feed mixture) resulted in higher ($p \leq 0.05$) and similar body weight of broiler chickens (2141 g and 2120.3 g, respectively) compared with control chickens fed a basal diet (1962.1 g). The probiotic preparation consisted of *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus salivarius*, *Enterococcus faecium*, *Aspergillus oryzae* and *Candida pintolopessii*.

In another study, **Khaksefidi and Rahimi (2005)** also found significant increase ($p \leq 0.05$) in live body weight of chickens. On the one hand, the body weight of chickens in the experimental group (1700 g) at the end of fattening (42 days) was higher than that in the control (1620 g), but on the other hand it was markedly lower than that in the present study. The probiotic preparation used in the study of **Khaksefidi and Rahimi (2005)** consisted of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Aspergillus oryzae*, *Streptococcus faecium* and *Torulopsis* spp. and was fed at 100 $mg.kg^{-1}$ diet. The different results may be thus caused by the dosage and strain of probiotics.

Alkhalf et al., (2010) reported that administration of probiotic (*Pediococcus acidilactici*) in chickens appeared to have noticable effect ($p \leq 0.05$) on final body weight of broiler chickens, which was as low as that in the study of **Khaksefidi and Rahimi (2005)**. Chickens fed on probiotic levels 1 and 0.8 $g.kg^{-1}$ diet (1863.6 and 1844 g, respectively) exhibited higher body weight than control chickens (1661.31 g).

The beneficial effect of probiotic supplementation on chicken diet in terms of increased body weight (2372.50 vs. 1997.5 g) was also observed in the study of **Kabir et al., (2004)**. The probiotic preparation consisted of *Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Streptococcus thermophilus, Enterococcus aecium, Aspergillus oryzae* and *Candida pintolopessi.* It is important to note that broilers were administered the probiotic by drinking water application (consistent with present study).

Promising effect of probiotic ($p \leq 0.05$) as alternative for antibiotics was demonstrated by **Ghahri et al., (2013)**. They used the same probiotic preparation that was used in the study of **Kabir et al., (2004)**. The probiotic (applied into feed mixture) in two different doses increased live body weight of chickens (2475.13 and 2491 g) compared with that of the control group (2243.09 g). The most significant effect ($p \leq 0.05$) was, however, observed in synbiotic-supplemented group compared with that of other groups, which is in agreement with the results of **Ghasemi et al., (2014)**.

Contrary to the above-mentioned studies, no significant effect was observed in the study of **Brzóska et al., (2012)** and **Swiatkiewicz et al., (2014)**, whereas **Ritzi et al., (2014)** found even the negative effect of probiotic supplementation (*Bifidobacterium animalis* subs. *animalis*, *Lactobacillus salivarius* subs. *salivarius* and *Enterococcus faecium*) on performance of broiler chickens.

Regarding carcass yield, neither supplementation of the diet with propolis extract (78.31%), nor the probiotic preparation (78.31%) had any effect on carcass yield of broiler chickens compared to the control (78.54%). Yet, carcass yield of chickens in the present study was higher in comparison to other studies.

Our carcass yield results are consistent with those of **Tatlı Seven et al., (2008)** (76 – 77% vs. 75%), slightly lower were observed in study of **Attia et al., (2014)** (72.1% vs. 68.9%).

Also, **Swiatkiewicz et al., (2014)** reported similar carcass yield, which was, however, not affected when chickens were fed a probiotic bacteria (*Lactobacillus salivarius*) (74.89 vs. 75.53%).

Daneshmand et al., (2015) found much lower carcass yield, 62.77% in the probiotic-supplemented group, 62.86% in the propolis-supplemented group, and 62.93% in probiotic + propolis-supplemented group, that was, however, still higher than that in control (61.9%).

The effects of propolis extract and probiotic supplementation on composition, cholesterol content and energy value of meat of Ross 308 broiler chickens are shown in Table 4. It is evident that the parameters were not absolutely affected by dietary propolis extract and probiotic supplementation.

The results for meat samples of chickens fed the diet with propolis extract and probiotic were similar to those fed the basal diet, which is consistent with results of some experiments where various supplements were used. However, the significant changes ($p \leq 0.05$) were observed in some parameters.

As has been shown by our study, propolis supplementation was the most favourable among the groups, namely as for fat content in both breast (0.93 $g.100\ g^{-1}$) and thigh (9.62 $g.100\ g^{-1}$) muscles, the ash content in both breast (1.19 $g.100\ g^{-1}$) and thigh (1.05 $g.100\ g^{-1}$) muscles, the cholesterol content in breast muscle (86.42 $mg.100\ g^{-1}$), and the energy value in both breast (408.99 $kJ.100\ g^{-1}$) and thigh (664.8 $kJ.100\ g^{-1}$) muscles. Besides, the propolis-supplemented group showed low crude protein content in both breast (22.33 $g.100\ g^{-1}$) and thigh (18.05 $g.100\ g^{-1}$) muscles when compared with the other groups. As regards the probiotic-supplemented group, there was negative effect on the fat content (1.11 $g.100\ g^{-1}$), as well as the cholesterol content (92.17 $mg.100\ g^{-1}$), and the energy value (415.62 $kJ.100\ g^{-1}$) in breast muscle observed. It is noteworthy that the cholesterol content depends mainly on the type of muscle not the diet.

Regarding the meat composition of broiler chickens, some researchers have observed significant positive effects of natural feed supplements, whereas others reported no effect on the meat composition.

Table 4 Effect of propolis extract and probiotic on proximate composition, cholesterol content and energy value of chicken meat.

Parameter	Group	x	*SD*	SEM	CV (%)
		Breast muscle			
Dry matter (g.100 g^{-1})	C	25.11	0.24	0.07	0.95
	E1	24.94	0.39	0.11	1.55
	E2	25.05	0.38	0.11	1.50
Crude protein (g.100 g^{-1})	C	22.52	0.40	0.11	1.76
	E1	22.33	0.58	0.17	2.61
	E2	22.32	0.28	0.08	1.25
Fat (g.100 g^{-1})	C	1.01^{ab}	1.13	1.04	13.02
	E1	0.93^{a}	0.10	0.03	11.28
	E2	1.11^{b}	0.12	0.03	10.66
Ash (g.100 g^{-1})	C	1.18^{ab}	0.03	$8.7.10^{-3}$	2.56
	E1	1.19^{a}	$9.85.10^{-3}$	$2.84.10^{-3}$	0.83
	E2	1.17^{b}	0.01	$4.14.10^{-3}$	1.22
Cholesterol (mg.100 g^{-1})	C	87.06	8.86	3.62	10.18
	E1	86.42	4.37	1.78	5.05
	E2	92.17	4.59	1.87	4.98
Energy value (kJ.100 g^{-1})	C	415.46^{a}	6.10	1.76	1.47
	E1	408.99^{b}	7.17	2.07	1.75
	E2	415.62^{a}	6.85	1.98	1.65
		Thigh muscle			
Dry matter (g.100 g^{-1})	C	29.50	1.37	0.40	4.65
	E1	29.22	0.40	0.11	1.37
	E2	29.10	0.60	0.17	2.05
Crude protein (g.100 g^{-1})	C	18.48^{a}	0.21	0.06	1.17
	E1	18.05^{b}	0.34	0.10	1.88
	E2	18.06^{b}	0.21	0.06	1.16
Fat (g.100 g^{-1})	C	9.81	1.43	0.41	14.54
	E1	9.62	0.40	0.11	4.16
	E2	9.80	0.78	0.22	7.92
Ash (g.100 g^{-1})	C	1.02^{a}	0.02	$6.38.10^{-3}$	2.16
	E1	1.05^{b}	$9.84.10^{-3}$	$2.84.10^{-3}$	0.94
	E2	1.02^{a}	0.02	$6.66.10^{-3}$	2.27
Cholesterol (mg.100 g^{-1})	C	121.25	7.50	3.06	6.19
	E1	118.68	7.68	3.14	6.47
	E2	113.08	10.70	4.37	9.47
Energy value (kJ.100 g^{-1})	C	679.44	54.45	15.72	8.01
	E1	664.80	13.43	3.88	2.02
	E2	671.89	28.34	8.18	4.22

Legend: C – control group; E1, E2 – experimental groups; x – arithmetic mean; *SD* – standard deviation; SEM – standard error of mean; CV – coefficient of variation; a, b – means with different superscripts within a column differ significa ($p \leq 0.05$).

In the study of **Hossain et al., (2014)**, addition of 0.5% fermented water plantain (*Alisma canaliculatum*) increased the crude protein content in both breast and thigh muscles (24.99 and 23.19%, respectively) compared with the control (24.42 and 21.65%, respectively).

The results coincide with the findings of **Skřivan et al., (2012)**, who reported the highest protein content and the lowest fat content in the thigh muscle of broilers fed a diet with vitamin C (720 and 218 g.kg^{-1} of dry mater, respectively) and broilers fed a diet with selenite (724 and 216 g.kg^{-1} of dry mater, respectively). The results are similar to those in the present study (when converting into g/100 g).

Ahmed et al., (2015) found significantly higher crude protein content ($p \leq 0.05$) in the group of broilers fed a diet supplemented with pomegranate in breast (28.55%), as well as thigh muscle (23.44%) than that in non-supplemented group (26.21 and 22.18%, respectively). Moreover, there was a significant decrease ($p \leq 0.05$) in cholesterol content of breast muscle in the pomegranate-supplemented group (62.8 mg.100 g^{-1}) compared with the control (77.44 mg.100 g^{-1}).

On the contrary, **Swiatkiewicz et al., (2014)** noted no effect on the composition of breast muscle after probiotic supplementation, whereby the probiotic-supplemented

group has shown the values very similar to the other groups, with a crude protein content of 23.5%.

Also, the probiotic supplementation in the study of **Haščík et al., (2011)** did not influence the composition of chicken meat significantly despite the slight positive effect in the probiotic-supplemented groups when compared with the control. The researchers have obtained the results similar to those in the present study.

To sum up the previous studies concerning the composition of chicken meat, there is a positive effect on fat content after natural feed additives observed in most of them, while the effect on protein content is not so noticeable.

CONCLUSION

The results of our study demonstrated that none of the experimental supplements (propolis extract and probiotic preparation based on *Lactobacillus fermentum*) caused a significant changes ($p \geq 0.05$) in performance and carcass characteristics of Ross 308 broiler chickens. However, the data have shown positive effect of propolis extract and probiotic due to the higher values of all the investigated parameters (especially in probiotic-supplemented group) than those in the control. The positive fact highlights the importance of evaluating the administration level of supplements in order to maximize the efficacy. As far as proximate composition, cholesterol content and energy value are concerned, there was a significant change ($p \leq 0.05$) in fat, ash and cholesterol content, as well as energy value in both breast and thigh muscles after the propolis supplementation. On the contrary, the probiotic supplementation was rather adverse for meat composition. Therefore, we assume that probiotic supplementation is more applicable for the performance and carcass characteristics, whereas the propolis supplementation is more applicable for meat composition of Ross 308 broiler chickens. Overall, further studies are needed to investigate the effect of the supplements.

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Contact address:

doc. Ing. Peter Haščík, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Products Evaluation and

Processing, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.hascik@uniag.sk.

Ing. Lenka Trembecká, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Products Evaluation and Processing, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xtrembecka@uniag.sk.

Ing. Marek Bobko, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Products Evaluation and Processing, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marek.bobko@uniag.sk.

prof. Ing. Miroslava Kačániová, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@uniag.sk.

prof. Ing. Juraj Čuboň, CSc., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Products Evaluation and Processing, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: juraj.cubon@uniag.sk.

Ing. Simona Kunová, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: simona.kunova@uniag.sk.

Ing. Ondřej Bučko, PhD., Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Animal Husbandry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: ondrej.bucko@uniag.sk.

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THE HEAVY METALS CONTENT IN WILD GROWING MUSHROOMS FROM BURDENED SPIŠ AREA

Marek Slávik, Tomáš Tóth, Árvay Július, Ľuboš Harangozo, Miriama Kopernická

ABSTRACT

In this work, we evaluated the rate of entry of heavy metals into the edible parts of wild mushrooms, from central Spiš area. The area is characterized by extremely high content of heavy metals particularly mercury in abiotic and biotic components of ecosystems. The toxicity of heavy metals is well known and described. Known is also the ability of fungi to accumulate contaminants from substrates in which mushrooms grow. We have collected commonly consumed species of mushrooms (*Russula vesca., Macrolepiota procera, Lycoperdon pyriforme, Lecinum piceinum, Boletus reticulatus*). Sampling was conducted for two years 2012 and 2013. The samples taken mushrooms and substrates on which to grow, we determined heavy metal content (Cd, Pb, Cu), including total mercury content modified by atomic absorption spectrometry (AMA - 254). In the substrate, we determined the humus content and pH value. The heavy metal content in soils were evaluated according to Law no. 220/2004 Z.z The exceedance limit values of Cd, Pb, Cu and Hg was recorded. Most significantly the respective limit was recorded in soil samples in the case of mercury. The determined concentration Hg was 39.01 mg.kg^{-1}. From the results, we evaluated the degree of ability to bioaccumulate heavy metals different kinds of fungi. We also evaluated the health safety of the consumption of these fungi on the comparison with the limit values provided in the food code of SR. We recorded a high rate of accumulation of mercury in the species Boletus reticulatus and Macrolepiota procera. For these types we recorded the most significant than allowed concentrations of mercury in mushrooms. The highest recorded concentration reached 17.64 mg.kg^{-1} Hg in fresh matter. The limit value was exceeded also in the case of copper. We do not recommend to increased consumption of wild mushrooms in the reference area.

Keywords: Mushrooms; heavy metals; soil; food chain; mercury; midle Spiš

INTRODUCTION

The area of middle Spiš is significantly burden by heavy metal in content of soils **Hronec et al., (2008)**. In addition to air pollution, there are other sources of pollution. They came from the extraction of minerals, their modification and processing. From the iron manufactory in Rudňany were emitted into air 120 tons of mercury. These data prepared by measuring groups of Research Institute of Mineral (now Research Institute of Geotechnics SAS) in Košice. These amounts have been challenged by State Inspection of Slovak Republik Based on the balance of atmospheric emission of mercury they calculated, that in the environment received 40 tons Hg per year. It means this is the highest source of mercury pollution in Europe **Závodský, (1991)**. In this area, the specific sources of pollution endogenous geochemical anomalies, especially in the area of Rudňany, Poráč, Gelnice, Slovinky and Krompachy **(Čurlík and Šefčík, 1999)**. In these days, the highest producer of air pollution is town Krompachy, where Kovohuty Krompachy and iron foundry Slovak energy manufactory (SEZ) produce 90% of total emissions (Hronec et al. 2008). Toxic metals can enter the human body by consumption of contaminated food crops, water or inhalation of dust and cause damage to the organism, its toxic efects (**Mahmoode et al. 2013; Timoracká et al., 2011)**. Most dangerous elements in terms of their content in the soil to a possible accumulation of plants in this area appear Hg, Cd, Pb, and Cu. Toxic effect of Hg and its compounds is largely the reaction of Hg ion with SH-groups of biomolecules with subsequent changes in the permeability of cell membranes and damage cellular enzymes. Mercury has the ability to accumulate in the human body and leads to toxic manifestations of brain damage and peripheral nerves **Zahir et al., (2005)**. All compounds of cadmium are toxic. Cd has a high acute toxicity tests and certified short current (short-term) inhalation of high levels may result in the human body resulting in lung damage. It is highly toxic, causing inhibition of sulfhydryl enzymes in particular. Binds in the liver, but also affects the metabolism of carbohydrates and inhibits insulin secretion **Godt et al., (2006)**. Depending on the amount of exposure, lead can adversely affect the nervous system, kidneys, the immune system, reproductive and developmental systems and the cardiovascular system. Its toxic effects vary from subtle changes in neurocognitive function in low-level exposures to a potentially fatal encephalopathy in acute lead poisoning **Gillis et al., (2012)**. Copper is one of the essential elements for humans, but many copper compounds are potentially toxic. Excessive intake of copper is manifested

neurological disorders **(Shaligram and Campbel, 2013)**. As risky part of the food chain can be considered by **Rieder (2011), Kalač (2010)** edible mushrooms due to their properties accumulation characteristics to heavy metals. The ability of fungi to accumulate in their fruiting body of heavy metals relates to the content of heavy metal in the soil, its link to the soil structure (fulvo and humic acids, clay particles etc.) soil pH, as well as the way of nutrition of the species of fungi. It was found significantly higher accumulation of heavy metals in certain species of fungi **(Falandysz and Gucia, 2008; Melgar et al., 2009)**. Heavy metals concentrations in mushrooms are also considerably higher than those in agricultural crop plants, vegetables and fruits. This suggests that mushrooms possess a very effective mechanism that enables them readily to concentrate certain heavy metals from the ecosystem, compared to green plants growing in similar conditions. According to the mechanism by which some heavy metals are accumulated is somewhat obscure although it seems to be associated with a chelation reaction with sulfhydryl groups of protein and especially with methionine. Thus considerable effort has been focused to evaluate the possible risk to human health from the consumption of mushrooms with regard to their heavy metal content **Paraskevi et al., (2007)**. Mushrooms are a popular part of the menu of the population and in particular mushroom pickers are therefore described as a vulnerable population in terms of income food of heavy metals **Ostos et al., (2015)**.

MATERIAL AND METHODOLOGY

Collection and processing of samples

Samples of fungi (n = 20) were collected, then cleaned of soil, and allowed to dry at 45 °C in a tray drier.

Samples of the substrates (n = 20) were taken from the same sites as samples of fungi, we left the air-dry and the final drying (50 °C) the samples were sifted through a sieve (particle diameter = 2 μm). The samples were collected during 2012 and 2013. Mushrooms species were collected: *Russula vesca*, *Macrolepiota procera*, *Lycoperdon pyriforme*, *Lecinum piceinum*, *Boletus reticulatus*.

The water content of the samples was determined by the moisture analyzer DLB 160-3A (Kern, Germany). Homogenized mushroom samples (1.000 g) were mineralized in a closed system of microwave digestion using Mars X-Press 5 (CEM Corp., USA) in a mixture of 5 cm^3 HNO_3 (Suprapur, Merck, Germany) and 5 cm^3 deionized water (0.054 $mS.cm^{-1}$) from Simplicity 185 (Millipore, UK). Digestive conditions for the applied microwave system comprised heating to 160 °C for 15 minutes and keeping it constant for 10 minutes. A blank sample was treated in the same way. The digested substances were subsequently filtered through a quantitative filter paper Filtrak 390 (Munktell, Germany) and filled up with deionized water to a volume of 50 cm^3.

Analytical procedure

Metal determinations were performed in a Varian AA240Z (Varian, Australia) atomic absorption spectrometer with Zeeman background correction. The graphite furnace technique was used for the determination of Cd and Pb, whereas the flame AAS Varian AA240FS (Varian, Australia) was used for the determination of Cu (detection limits for FAAS: 2.0 $mg.kg^{-1}$), and GF-AAS: 10.0 and 10.0 $ng.kg^{-1}$ for Cd and Pb, respectively). The total content of Hg was determined in the homogenized dried samples of mushrooms (0.005 – 0.01 g) using a cold-vapor AAS analyzer AMA 254 (Altec, Czech Republic).

We conducting determination of organic carbon and humus in the soil according to Turin and the modifications by Nikitin.

Soil organic carbon is oxidized with oxygen in chromo-sulfuric mixture. The amount of oxygen consumed in the oxidation is determined by the difference consumed and unconsumed chromo-sulfuric mixture. Humus content was found by calculation according to the following of relations:

$$C_{ox} = \frac{(a-b)\times 0{,}03\times 1{,}17\times f_{mohr}}{n}$$

(a - b) = difference between sample and blank test

f_{mohr} = accurate substance concentration of Mohr salt

n = sample weight in g

humus content = $c_{ox} \times 1{,}724$ [%]

c_{ox} = percentage of oxidizable carbon

1,724 = conversion factor to humus content in the soil sample.

Determination of active reactions in soil (pH/H_2O)

Twenty grams of soil samples were taken. Subsequently 50 ml of H_2O was added. The suspension was allowed 10 minutes to shake by shaker Heidolphpromax 1020 at a frequency of 180 oscilation per minute. After shaking and settled solution we filtered suspension through filter paper *FILTRAK* 390. After filtering the suspension, the pH of the filtrate was measured on the pH meter Metrohm 691, we calibration aparat to two buffers at pH 4 and 7. The resulting values were subtracted from pH meter display with two decimal places.

Bioavailability calculation (Baf): the heavy metal content in mushroom (fresh veight) / heavy metal content in substrat.

RESULTS AND DISCUSSION

The contents of heavy metals (Table 1) in substrates were evaluated according to the Annex. 2 of the Slovak decree no. 220/2004 col. Type of soil samples from the point of delivery 1 was sandy-loam, loam. Soils are predominantly slightly acidic. The humus content is very high. The minimum content of Cd was 0.03 $mg.kg^{-1}$ and maximum content of Cd was was 6.07 $mg.kg^{-1}$. The limit value from Cd in soil exceeded 47% of the samples. Pb minimum in soil we recorded 0,10 $mg.kg^{-1}$ and maximum in value172.50 $mg.kg^{-1}$. Only one sample exceeded the limit value from Pb in soil. Copper minimum in soil was 0.04 $mg.kg^{-1}$ and maximum 145.20 this sample exceeded the limit value from copper in soil 2.42 times. Minimum of mercury content was 0.24 $mg.kg^{-1}$ and maximum was 39.01 $mg.kg^{-1}$. In our study 19 from 20 samples exceeded the limit value for Hg in soil. The soils in middle Spiš area are extremely contaminated with mercury.

Extremely contamined soil by mercury, for which they were collected Wild mushrooms were also recorded in the

Spanish province of Asturias, where the values of Hg concentrations in the soil reach values 1 – 895 mg.kg^{-1}, is also contamination of anthropogenic origin **(Ordóñez et al., 2013)**.

Contents of the monitored heavy metals in mushroom samples varied at different intervals depending on the mushroom species (Table 2). Mushrooms are generally considered extensive accumulators of heavy metals. The level of the transfer of heavy metals into fruiting bodies is affected by a large number of factors, such as mushroom species, chemical parameters of the substrate (substrate composition, heavy metals content, pH, humus content), the age of the mycelium and, probably, the interval between fructification events. **Kalač, 2010**. Content of heavy metals in samples of mushrooms were evaluated according to the Food codex of the Slovak republic (decree of the Ministry of Agriculture and the Ministry of Health no. 608/3/2004-100 of 15 March 2004).

Cadmium

Minimum concentration of cadmium we recorded in sample *Boletus reticulatus* 0.027 mg.kg^{-1} and maximum in (0.48 mg.kg^{-1}) sample *Russula vesca.* No sample exceeded the limit value. The highest mean content we determined in species *Russula vesca*. Biovailability declined from species: *Boletus reticulatus* > *Russula vesca* > *Lycoperdon pyriforme* > *Lecinum piceinum* > *Macrolepiota procera*.

High content of Cadmium (4.27 mg.kg^{-1}) in *Russula* species determined in work from Turkia **(Tüzen, 2003)**.

Lead

The minimum concentration of lead we recorded in sample Boletus reticulatus 0.01 mg.kg^{-1} and maximum in sample Macrolepiota procera 0.51 mg.kg^{-1}.

The highest mean content of lead we determined in species Macrolepiota procera. Analysed samples do not exceed the limit value. Biovailability declined from species: Boletus reticulatus > Lycoperdon pyriforme > Macrolepiota procera > Russula vesca > Lecinum piceinum. High level of lead in species Macrolepiota procera determined also García et al., 2008.

Copper

Minimum content of copper we determined in sample *Lecinum piceinum* 1.61 mg.kg^{-1}. Maximum content (35.86 mg.kg^{-1}) we determined in sample *Lycoperdon pyriforme*, this sample exceede the limit value 3,58 times. The highest mean content we determined in species *Lycoperdon pyriforme.* Three samples exceeded the limit value (*Macrolepiota procera* (2) and *Lycoperdon pyriforme*). Biovailability declined from species: *Boletus reticulatus* > *Macrolepiota procera* > *Russula vesca* > *Lycoperdon pyriforme* > *Lecinum piceinum.* Comparable Cu concentration in mushrooms also mentioned **Kalač 2010.**

Mercury

Minimum content of mercury we determined in sample *Lecinum piceinum* (0.009 mg.kg^{-1}) and maximum mercury content (17.64 mg.kg^{-1}) we determined in sample *Macrolepiota procera* this sample exceeded the limit value 70.56 times.

High mean content we determined in species *Macrolepiota procera* (10.91 mg.kg^{-1}). 90% of samples exceeded the limit value from mercury in wild growing mushrooms. Bioavailability declined from species: *Macrolepiota procera* > *Lycoperdon pyriforme* > *Boletus reticulatus* > *Lecinum piceinum* > *Russula vesca.*

Table 1 Substrate parameters from mushrooms sampling.

Mushroom species	Substrate parameter					
	pH (H_2O)	Humus content %	Cd (mg.kg^{-1}) *LV (0.7)	Pb (mg.kg^{-1}) *LV (70)	Cu (mg.kg^{-1}) *LV (60)	T-Hg (mg.kg^{-1}) *LV (0.5)
Russula vesca						
range	4.44 – 7.29	2.48 –12.28	0.09 – 4.29	0.20 – 61.00	0.71 – 17.80	6.07 – 39.01
mean	5.93	7.70	1.72	25.64	10.33	20.06
SD	1.40	3.74	1.99	30.34	10.55	13.88
Macrolepiota procera						
range	3.73 – 6.18	1.75 – 7.32	0.04 – 4.10	0.10 – 64.50	0.08 – 32.30	0,50 – 18.83
mean	4.72	4.53	2.57	41.32	16.52	10.20
SD	1.15	2.36	1.83	30.05	13.17	7.65
Lycoperdon pyriforme						
range	4.63 – 6.17	2.42 – 6.60	0.03 – 6.07	0.13 – 39.70	0.14 – 145.20	1.26 – 10.66
mean	5.55	4.28	2.05	13.36	48.48	6.39
SD	0.71	1.72	3.48	20.80	83.76	4.75
Lecinum piceinum						
range	4.30 – 6.38	5.33 –14.70	0.09 – 3.44	0.48 – 172.50	0.19 – 37.50	0.24 – 19.30
mean	4.92	7.88	1.39	49.52	11.98	6.98
SD	0.89	3.94	1.59	8.20	17.55	8.66
Boletus reticulatus						
range	4.16 – 5.10	6.29 –10.05	0.14 – 0.14	0.31 – 2.14	0.04 – 0.23	2.33 – 36.35
mean	4.63	8.17	0.14	1.23	0.13	19.34
SD	0.66	2.65	0.00	1.29	0.13	24.05

Note: SD – standard deviation, * LV – limit value (Annex. 2 of the Slovak decree no. 220/2004 Col., Cd, Pb and Cu after aqua regia extraction).

Table 2 The heavy metals content in mushrooms samples (fresh matter).

Mushrooms species	Heavy metals content in fresh matter (mg.kg^{-1})			
	Cd (mg.kg^{-1}) *LV(1.0)	Pb (mg.kg^{-1}) *LV(1.0)	Cu (mg.kg^{-1}) *LV(10.0)	T – Hg (mg.kg^{-1}) *LV(0.25)
Russula vesca				
range	0.05 – 0.48	0.02 – 0.11	3.66 – 6.12	1.65 – 3.54
mean	0.18	0.06	4.65	2.33
SD	0.20	0.03	1.04	0.83
Macrolepiota procera				
range	0.08 – 0.16	0.16 – 0.51	3.09 – 20.93	1.24 – 17.64
mean	0.10	0.37	11.46	10.91
SD	0.03	0.16	9.03	7.14
Lycoperdon pyriforme				
range	0.08 – 0.21	0.12 – 0.26	7.71 – 35.86	0.56 – 6.96
mean	0.13	0.20	17.10	3.61
SD	0.06	0.07	16.24	3.20
Lecinum piceinum				
range	0.03 – 0.11	0.03 – 0.11	1.61 – 7.14	0.009 – 2.86
mean	0.06	0.06	3.28	1.32
SD	0.03	0.03	2.58	1.49
Boletus reticulatus				
range	0.027 – 0.10	0.01 – 0.07	1.78 – 2.10	1.62 – 14.35
mean	0.06	0.04	1.94	7.99
SD	0.05	0.04	0.22	9.00

Note: SD – standard deviation, * LV – limit value (Decree of the Ministry of Agriculture and the Ministry of Health of the Slovak republic no. 608/3/2004-100 of 15 March 2004).

Table 3 Pearson corelation coeficients between heavy metals conntent in mushrooms and substrates

corelation	Cd (mushrooms)	Pb (mushrooms)	Cu (mushrooms)	Hg (mushrooms)	Cd (substrates)	Pb (substrates)	Cu (substrates)	Hg (substrates)
Cd (mushroom)								
Pb (mushrooms)	0.03							
Cu (mushroom)	0.16	0.27						
Hg (mushroom)	0.015	0.67*	0.09					
Cd (substrates)	0.27	0.44	0.47	0.18				
Pb (substrates)	0.17	0.21	0.08	-0.04	0.65*			
Cu (substrates)	0.29	0.22	0.82*	0.04	0.76*	0.35		
Hg (substrates)	-0.10	-0.08	-0.14	0.41	-0.22	-0.34	-0.16	1

Note: * p <0.05

Contamination of wild mushroom species (*Boletus* and *Agaricus*) with mercury dealt **Melgar et al. (2009)**, with the highest concentration of mercury found in species of the genus *Boletus* and *Agaricus* in the range of 2.0 to 6.9 mg.kg $^{-1}$. The lowest concentrations reported in species of the genus Fistulina 0.22 mg.kg^{-1}. Bioavailability was also recorded the highest in the species of the genus *Boletus* and *Macrolepiota*. The observed area was northwest Spain. Their findings are comparable to our resultes.

We demonstrated high correlation (Table 3) between content of lead and mercury in mushrooms. We found high correlation between copper content in mushrooms and substrates on which they grow. We found high correlation between the cadmium content and lead, copper content in substrates.

CONCLUSION

Mushrooms as a popular culinary raw material is in our country a source of heavy metals, particularly mercury, in the food chain. This is due to their ability to accumulate well heavy metals from substrates on which they grow. This is confirmed by their biovailability. Mushrooms are therefore a real risk to human health following exposure to heavy metals in the target area. We don´t have known data about year-round consumption of mushrooms for better risk assessment. It would be appropriate to continue to complement those data and thus continue in this direction of research.

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Contact address:

Marek Slávik, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marekslavik87@gmail.com.

Tóth Tomáš, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: tomas.toth@uniag.sk

Árvay Július, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: julius.arvay@gmail.com

Harangozo Ľuboš, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: harangozolubos@gmail.com

Kopernická Miriama, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: m.kopernicka@gmail.com

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DIAGNOSTICS OF SUBTROPICAL PLANTS FUNCTIONAL STATE BY CLUSTER ANALYSIS

Oksana Belous, Valentina Malyarovskaya, Kristina Klemeshova

ABSTRACT

The article presents an application example of statistical methods for data analysis on diagnosis of the adaptive capacity of subtropical plants varieties. We depicted selection indicators and basic physiological parameters that were defined as diagnostic. We used evaluation on a set of parameters of water regime, there are: determination of water deficit of the leaves, determining the fractional composition of water and detection parameters of the concentration of cell sap (CCS) (for tea culture flushes). These settings are characterized by high liability and high responsiveness to the effects of many abiotic factors that determined the particular care in the selection of plant material for analysis and consideration of the impact on sustainability. On the basis of the experimental data calculated the coefficients of pair correlation between climatic factors and used physiological indicators. The result was a selection of physiological and biochemical indicators proposed to assess the adaptability and included in the basis of methodical recommendations on diagnostics of the functional state of the studied cultures. Analysis of complex studies involving a large number of indicators is quite difficult, especially does not allow to quickly identify the similarity of new varieties for their adaptive responses to adverse factors, and, therefore, to set general requirements to conditions of cultivation. Use of cluster analysis suggests that in the analysis of only quantitative data; define a set of variables used to assess varieties (and the more sampling, the more accurate the clustering will happen), be sure to ascertain the measure of similarity (or difference) between objects. It is shown that the identification of diagnostic features, which are subjected to statistical processing, impact the accuracy of the varieties classification. Selection in result of the mono-clusters analysis (variety tea Kolhida; hazelnut Lombardsky red; variety kiwi Monty and Hydrangea forma rosea) shown as a helpful tool to detect drastically different varieties.

Keywords: subtropical crops; cluster analysis; diagnostics; adaptability; water regime; pigments; enzymes activity

INTRODUCTION

Recently in Sochi, the importation of tropical and subtropical plants has increased, in the fruit and ornamental areas. These crops are deservedly popular among the local residents, especially given the existence of the municipal program "Development within territories of municipal formation the city-resort Sochi» **(Resolution of the Sochi city administration, 2014)**. At the same time, in light of recent political events and the imposition of sanctions, Russia is actively carrying out activities on import substitution, which implies the saturation of the farmer's market local adapted varieties, process of restoring tea plantations is in full swing **(Resolution of the Russian Federation Government, 2012; Decree of the Russian Federation President, 2014)**. The subtropics of Krasnodar region are in undoubted interest and as well as a year-round family resort direction, therefore, the issues of urban green space in settlements are required as a must. In this regard, of paramount importance in the development of horticulture in Russia is the development of a scientifically-based selection of crops and use of better varieties and garden forms that meet modern requirements and the most adapted to the conditions of damp subtropics.

However, often in the cultivation of new varieties, we have to face failure, because of the classical approach to plant care carried out by analogy with well known established varieties. At the same time, it is known that the choice of crop conditions is individual and depends on the varietal characteristics. All this leads to the need for a thorough, comprehensive research not only by each culture but by each class. Research is often lengthy; require large analysis, which in connection with the use of phenological observations and the large number of morphological, anatomical, physiological indicators, sometimes is difficult. Thus, before researchers there is a question about the search for more rapid methods of assessment introduced new material.

In this regard, in the laboratory of biotechnology, plants physiology and biochemistry in recent years conducted a comprehensive study of adaptive reactions of various subtropical and tropical crops (tea, kiwi, hazelnut, gidrangea, weigela, etc.) aimed at the search of diagnostic criteria for their evaluation.

For a number of crops already established diagnostic indicators, and established scales for assessment of drought resistance varieties and crops such as tea, kiwi, gidrangeya, and weigela **(Belous, 2009; Malyarovskaya and Belous, 2012; Klemeshova and Belous, 2013; Malyarovskaya and Belous, 2015)**. However, attracting new assortment leads to more specific research in respect

of obtaining a large number of indicators. As is known, methods of multivariate analysis are effective quantitative tools for the study of fundamental processes described by a large number of characteristics. Cluster analysis most clearly reflects the characteristics of a multivariate analysis in classification and its use allows you to quickly organize the extensive material available in the laboratory. In this case, combining objects into clusters so that way, similar classes maximally got into one class, and objects from different classes would maximally differ from each other.

MATERIAL AND METHODOLOGY

As objects of researches were made by different varieties of the following plants: tea (*Camellia sinensis* L); filbert (*Corylus pontica* C. Koch); kiwi (*Actinidia deliciosa*); gidrangeya large-leaved (*Hydrangea macrophylla* (Thunb.) Ser.); weigela (*Weigela* X *Wagnera*). The accounting of plants resistance to adverse climate conditions was determined visually (in balls) by Technique of the State grades researches **(Technique, 1968)** and methods of laboratory and field assessment of water regime parameters: water deficiency (for a drought resistance assessment) **(Pochinok, 1976)**; the concentration of cell sup (CCS) (for heat tolerance testing) **(Filippov, 1975)**; express diagnostics of change parameters of leaf blade (for a drought resistance assessment) **(Goncharova, 2005)**; activity of enzyme catalase - gasometrical method **(Gunar, 1972)**; thickness of leaf was determined by field turgor meter, coefficient of heat resistant - method express diagnosing **(Kushnirenko, et al., 1986)**.

When processing the data and evaluating the results of the research used the statistical software package STATGRAPHICS Centurion XV standard mathematical software package MS Excel XP. To construct the dendrograms and partition the varieties into homogeneous the adaptability of the group used clustering k-average, or «nearest neighbor».

RESULTS AND DISCUSSION

The establishment of the indicator organ that can serve as a reliable diagnostic authority in the evaluation of the adaptability of cultures was the paramount importance. In the result of conducted research we have established that when long diagnosis culture of the kiwi should consider the existence of tiers in plants (to take away the leaves from the middle tier) and the location of leaves in relation to inflorescences and fruits; for the diagnosis of plant resistance gidrangea and weigela gives a more accurate physiologically formed the third, starting from the terminal of a leaf bud; while the research on tea should be selected physiologically Mature leaves that are after the so-called «fishy».

The following moment was the selection of diagnostic indicators to assess the sustainability of the studied cultures. In the order of the country's scientific institutions developed different methods of diagnosis of plant resistance, recommending for practical use a variety of techniques for evaluation of resistance to extreme factors **(Tsukanov, 2007; Goncharova, 2011)**. Their analysis showed that the entire diversity of ways to diagnose plant resistance lies with a small number of general principles of evaluation based on views on the adaptation mechanisms of plants to stresses **(Tsukanova, 2007; Goncharova, 2011)**.

As a rule, when determining the resistance of the varieties we used two or three well-known varieties (grown in the area), clearly differing from each other in terms of resistance to a specific type of stress: highly resistant, moderately resistant and unstable. However, variety, highly resistant to extreme factors, but not with great productivity potential (realized only in optimal conditions) and gives the highest absolute yield. Most often, the introduction of such varieties in production is recognized as inappropriate; however, it retains its value for breeding as a genetic source of high resistance to stress, which does not preclude his selection as recommended.

We also took into account the fact that the sustainability of any plant organism changes in ontogenesis: it is low at a young age, then gradually increases. From this overall biological patterns should be that a comparative assessment of crops for resistance to stress factors is possible only on the basis of the same age.

Since the main disadvantage of our subtropical zone is the uneven distribution of rainfall, with recurrent drought periods and high temperatures, often accompanied by hair dryers, the main focus of the research was done on plant resistance to drought. In particular, we used evaluation on a set of parameters of water regime. These settings are characterized by high liability and high responsiveness to the effects of many abiotic factors that determined the particular care in the selection of plant material for analysis and consideration of the impact on sustainability. Methods of using identified indicators that are closely related to water status of crops, are: determination of water deficit of the leaves, determining the fractional composition of water and detection parameters of the concentration of cell sap (CCS) (for tea culture flushes).

To confirm the validity of the studies conducted statistical processing of experimental data summarizing and averaging of results and involvement analysis of variance, according to the methodical instructions on conducting field experiments specific to perennial crops.

As a result, on the basis of the experimental data calculated the coefficients of pair correlation between climatic factors and used physiological indicators. The result was a selection of physiological and biochemical indicators proposed to assess the adaptability and included in the basis of methodical recommendations on diagnostics of the functional state of the studied cultures **(Ryndin at all 2014)**. The tea culture is established that a significant relationship exists between enzyme activity – temperature and activity of the enzyme – solar insulation; the culture of hazelnuts dependence between temperature and physiological indicators of above average or even high, the strong correlation observed between temperature and amount of carotenoids (r = 0. 98), the temperature rise causes a decrease in the synthesis of chlorophylls (r = -0. 73) and indicators related to water regime: the water content (r = -0. 81), water-holding capacity (r = -0. 83) and the amount of free water (r = -0. 78); revealed close correlations between the indicators of water status weigela (water deficit, the concentration of cell sap (CCS) and the activity of the enzyme catalase **(Belous, 2008; Belous, Ryndin and Pritula, 2009; Klemeshova and Belous**

2011; Kozhevnikova, 2014; Malyarovskaya and Belous 2015).

However, as already mentioned, the analysis of complex studies involving a large number of indicators is quite difficult, especially does not allow to quickly identify the similarity of new varieties for their adaptive responses to adverse factors, and, therefore, to set general requirements to conditions of cultivation. In this case, for the separation of the studied cultivars on the most similar in agility classes (clusters), it is desirable to connect the cluster analysis, which is the most effective way to solve this problem.

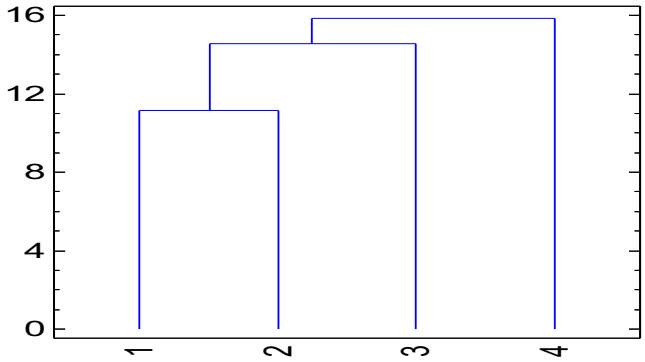
As known, cluster analysis is a multivariate statistical procedure that performs the data collection that contains information about the sample objects, and then marshalling them into homogeneous groups **(Gorsky and Orlov, 2002; Bessokirny, 2003; Savvina, 2013)**. Thus, the objective of cluster analysis is that the newly introduced varieties appear to refer to one of the already defined classes.

Use of cluster analysis suggests that in the analysis of only quantitative data; define a set of variables used to assess varieties (and the more sampling, the more accurate the clustering will happen), be sure to ascertain the measure of similarity (or difference) between objects. In addition, it is necessary that the sample should be homogeneous (not to contain «emission») and the distribution of indicators should be close to normal **(Acopov at all 2013; Sidorenko, 2001)**.

Cluster analysis of the tea plants allowed determining similar to the adaptive potential of varieties. In close clusters are of local plant populations, varieties, and Kimyn and Karatum (Figure 1). Moreover, the more similar the sustainability of local plant populations (sufficiently adapted to growing conditions) and grade Karatum (anthocyanin pigment flushes which is an indirect confirmation of its stability). At the same time the cultivar Kolkhida is located in a separate cluster, as a highly variable grade. High temperature instantly results in drying of young flushes, which are unsuitable for the collection and production of the drink.

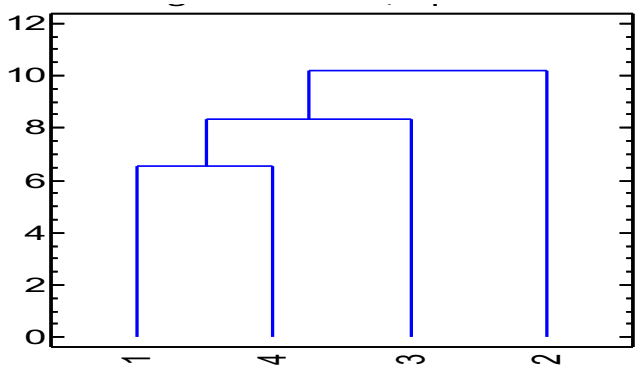
According to the results of the cluster analysis were divided into classes that demonstrate their adaptive capacity, these varieties of hazelnut, as the Cherkessky -2, Lombardsky red, President and Futkurami (Figure 2).

As can be seen from the dendrogram 2 varieties Cherkessky -2, President and Futkurami form a group similar in terms of resistance to stress factors, while variety Lombardsky red according to the degree of adaptability differs from the others. At the same time, considering the combined group of varieties within this cluster are the President that differ in their adaptive potential of varieties Cherkessky -2 and Futkurami.



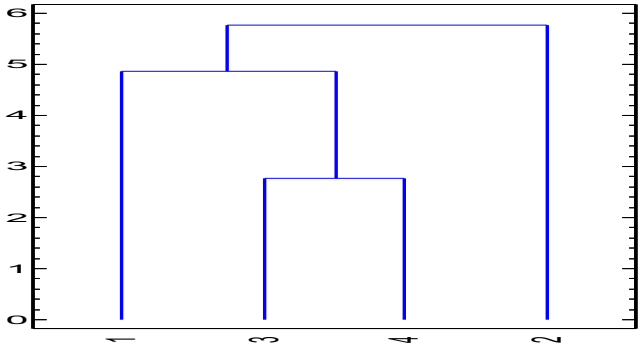
1 – Karatum, 2 – local plants, 3 – Kimyn and 4 – Kolhida

Figure 1 Dendrogramm of tea plants.



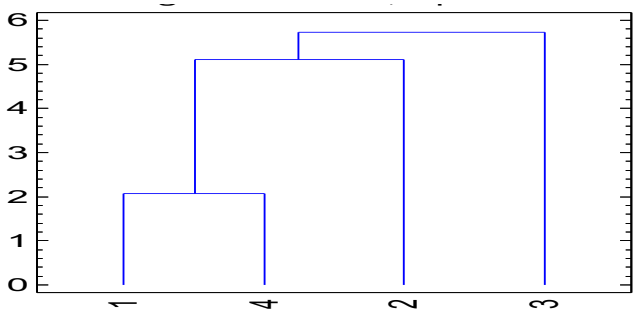
1 – Cherkessky-2, 2 – Lombardsky red, 3 – President and 4 – Futkurami

Figure 2 Dendrogramm of varieties filbert.



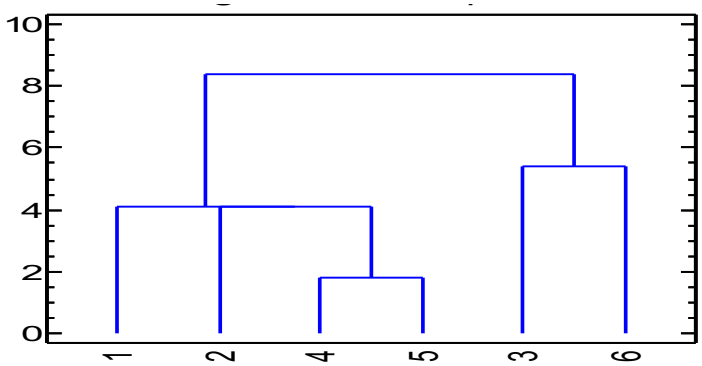
1 – Hayward, 2 – Monty, 3 – Allisson and 4 – Bruno

Figure 3 Dendrogramm of varieties kiwi.



1 – Sester Teresa, 2 – Bichon, 3 – f. rosea and 4 – Draps Wonder

Figure 4 Dendrogramm of varieties gidrangeya.



1 - Eva Ratke, 2 - Gustav Male, 3 – Avgusta, 4 – Arleqin, 5 - Mon Blanc, 6 - Variegata

Figure 5 Dendrogramm of varieties weigela.

As in previous stat analysis using the method of «Nearest neighbor» we managed to get the explanation for the greater stability of the Monti varieties compared to other varieties of kiwi (Figure 3). As can be seen from the dendrogram 3, this variety occupies a position in a separate cluster, which causes the difference of metabolic reactions, including the action of stress factors.

Similarly, the distribution of the studied varieties hydrangea large groups, characterized by similar adaptive potential (Figure 4). As can be seen from the dendrogram 17 variety Draps Wonder, be determined by us on the basic physiological indicators as the most responsive, reasonably is a separate cluster, variety Sester Teresa close to it in terms of resistance to hydrothermal factors, occupying a total cluster.

While form rosea is unstable, rapidly losing its decorative qualities when exposed to high temperatures and lack of water availability (Figure 4). It is not surprising, since form rosea genotypic and phenotypic different from other varieties.

According to the results of the cluster analysis all the studied varieties weigela were divided into the following groups that demonstrate their adaptive capacity (Figure 5).

As can be seen from the dendrogram 5 varieties of Gustav Male, and Arleqin form a cluster, characterized by high resistance to stress factors, while the varieties Avgusta and Variegata according to the degree of adaptability differ greatly, being unstable and leaving a separate group. At the same time, variety Eva Ratke as

melatonin, stands in its phenotypic characteristics closer to the group Gustav Male and Arleqin.

CONCLUSION

Thus, it is possible to establish the fact that the cluster analysis reliably and sufficiently illustrates the analyzed material, which gives the opportunity to use it in classification purposes. Moreover, this method is confirmed by the findings on sustainability of cultures that we have done on the results of physiological and biochemical tests. In the end, we propose a two-stage analysis to select the most informative features and classification of the studied cultivars, where the first phase involves a correlation analysis, the second step - cluster analysis. In this case, the value of the correlation coefficient affects the accuracy of further classification. The resulting dendrogram can be used for inter cluster distance quickly assess the differences in functional state of the species and its place in the classification of resistance to stress factors. In addition, the selection in the result of the analysis of mono clusters (as in the case of dendrograms: 1 - variety tea Kolkhida, 2 – hazelnut Lombardsky red; 3 – variety kiwi Monty and 4 – hydrangea forma rosea is a good tool to detect drastically different varieties.

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Contact address:

Belous Oksana, Dr. Sci. Biol., associate professor, major researcher of biotechnology, plants physiology and biochemistry laboratory of All-Union Scientific research institute of floriculture and subtropical cultures, Fabritius

St., 2/28, Sochi, Russia, 354207, E-mail: oksana191962@mail.ru.

Malyarovskaya Valentina, PhD, head of biotechnology, plants physiology and biochemistry laboratory of All-Union Scientific research institute of floriculture and subtropical cultures, Fabritius St., 2/28, Sochi, Russia, 354207, E-mail: malyarovskaya@yandex.ru.

Klemeshova Krystina, PhD, head of laboratory phytotechnologies of All-Union Scientific research institute of floriculture and subtropical cultures, Fabritius St., 2/28, Sochi, Russia, 354207, E-mail: Klemeshova_KV@mail.ru.

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^{137}Cs MONITORING IN THE MEAT OF WILD BOAR POPULATION IN SLOVAKIA

Katarína Beňová, Petr Dvořák, Martin Tomko, Marcel Falis

ABSTRACT

Currently, due to the elapsed time and the nature of the Chernobyl accident, the only artificial radionuclide present in the soil is ^{137}Cs, with a physical half-life conversion of 30.17 years. The ^{137}Cs is quickly integrated into a biological cycle, similar to potassium. Generally, radionuclides are characterized by their mobility in soil. Contamination of materials and food by radionuclides represent a serious problem and has a negative impact on human health. The threat of international terrorism and the inability to forestall the impact of natural disasters on nuclear energetic (Fukushima accident), are also reasons for continuous monitoring of food safety. According screening measurement performed in European countries, high radioactivity levels were reported in the wild boars muscles from Sumava (Czech Republic). Seasonal fluctuation of ^{137}Cs activity in the wild boar meat samples was observed in the forests on the southern Rhineland. Monitoring of ^{137}Cs activity in the wild boar meat samples in the hunting grounds in Slovakia was initiated based on the reports on exceeding limits of the content of radiocaesium in the meat of wild boar from the surrounding countries. The aim of this study was to determine the ^{137}Cs post Chernobyl contamination of wild boars population in different hunting districts of Slovakia during 2013 – 2014. A total of 60 thigh muscle samples from wild boars of different age categories (4 months – 2 years) were evaluated. ^{137}Cs activity was measured by gamma spectrometry (Canberra). Despite the fact Slovakia is closer to Chernobyl as Czech Republic and Germany, the ^{137}Cs activity measured was very low and far below the permitted limit. The highest radiocaesium activity level measured in muscle was 37.2 Bq.kg^{-1} ±4.7%. Wild boar originated from Zlate Moravce district. The measurement results show, that ^{137}Cs contamination levels of game in Slovakia are low. Radiocaesium activity in examined samples was very low and therefore consumption of wild boar meat does not represent a health risk problem.

Keywords: wild boar; contamination; radiocaesium; Slovakia

INTRODUCTION

In the terrestrial environment of Europe the contamination by anthropogenic radionuclides comes from two different sources. The first was the global atmospheric fallout, which appeared after the start of intensive atmospheric nuclear weapons tests in the 50-ies of the last century, and has been observed for a long time after their completion in 1963.

From the radio-ecological perspective are relevant only the long-living components of nuclear weapons tests (^{90}Sr, ^{137}Cs, ^{238}Pu, ^{239}Pu). The most significant long-lived contaminant was the cesium ^{137}Cs isotope **(Högberg, 2013)**. The level of contamination in a certain area was dependent on the latitude and a long-term weather situation, particularly precipitation **(Csupka et al., 1978)**. Currently, the ^{137}Cs from this source, is already largely immobilized in the clay fraction of the soil, with limited access for plant roots **(Nimis, 1996)**.

The second source of the terrestrial contamination was the fallout after the nuclear reactor accident at Chernobyl in 1986. The Chernobyl nuclear accident on April 26, 1986, caused the release of radioactive caesium in the amount up to 3.8 x 10^{16} Bq. The ratio of ^{137}Cs to ^{134}Cs long-term radionuclides released was approximately 2:1 **(UNSCEAR, 1988)**. The radioactive cloud passed three times over the continent. On some territories with intensive rainfalls an increased soil contamination by large amounts of radionuclides was observed. Among most affected parts of Europe, except for Ukraine, Belarus and Russia, belongs despite the distance Norway, where surface activity values reached up to 500 kBq.m^{-2} **(Pedersen et al., 1998)**.

Currently, due to the elapsed time and the nature of the Chernobyl accident, the only artificial radionuclide present in the soil is ^{137}Cs, with a physical half-life conversion of 30.17 years **(Krolak et al., 2010)**.

High mobility of radiocaesium gradually decreased after its deposition to the soil. Differences in mobility were observed especially in the non-cultivated meadow and forest soils, and were associated with the soil depth. In mineral soils by increasing depth, the amount of ^{137}Cs accessible by plants may rise, however it depends on many factors and circumstances **(Schimmack and Bunzl, 1996)**. In the Central Europe, the most contaminated animal product by Chernobyl accident was the meat of game **(Sprem et al. 2013)**.

Based on information from the neighboring countries on exceeding limits of radiocaesium content in wild boar meat, monitoring of the hunting grounds in Slovakia was initiated.

MATERIAL AND METHODOLOGY

Thigh muscle samples of 60 wild boars (*Sus scrofa*) of different age categories (4 month – 2 years) collected from different hunting districts in Slovakia were evaluated in the period of 2013 – 2014. Thigh muscle samples of 500g collected from wild boars weighing from 15 up to 35 kg and originated from 26 hunting grounds were used in the study.

^{137}Cs activity was measured by gamma spectrometry (Canberra) consisting of a Ge detector (GC 3520: 20% effectiveness, 2.0 keV resolution) and a multichannel analyzer (Desktop Inspector) in 450 mL geometry Marinelli beakers and 200 mL PE bottle. Results of mass activity Bq.kg^{-1} were determined by a 2000 Genie (Canberra) and Gamvin (Envinet) software systems. It is further stated the % of relative standard uncertainty for each sample. All geometres including the gamma-spectrometric system were certified in the Czech Metrological Institute.

RESULTS AND DISCUSSION

The highest radiocaesium activity level measured in muscle was 37.2 Bq.kg^{-1} ±4.7%. Wild boar (Table 1) sample originated from Zlate Moravce district. In 8 muscle samples the highest value was less than 3.6 Bq.kg^{-1} ±2.2%. In all other muscle samples, the radiocaesium activity levels were below the minimum detectable activity.

Due to the fact that Scandinavia was heavily contaminated by Chernobyl radiocaesium fallout, within the food chain considerable attention was paid to elk **(Palo et al., 2003)** and reindeer meat **(Skuterud et al., 2004)**. Similar attention was paid also to marine organisms (seafood), such as crab from the north of Ireland **(Copplestone et al., 2004)**. In the Central Europe, the most contaminated animal product by Chernobyl accident, was the meat of game **(Sprem et al., 2013; Škrkal et al., 2015)**. Relatively higher activity of ^{137}Cs in the wild animals is based on the mosaic pattern of contamination area after the Chernobyl accident, in the way the wild animals searches and acquires food (especially wild boars), and in a significantly greater mobility and persistence of radiocaesium in the forest ecosystems, compared to intensively used agricultural land **(Vaaramaa et al., 2009)**. The ^{137}Cs is quickly integrated into a biological cycle, similar to potassium. Generally, radionuclides are characterized by their mobility in soil **(Gadd, 1996)**. Analysis has shown, that the diffusion coefficient of radionuclides in the soil is affected by the soil moisture, presence of chemical homologs, which determine the capacity of the exchange process in the soil, soil acidity, soil humus content and temperature.

Fungi, as one of the most important constituents of forest ecosystem are capable to accumulate a significant amount of radionuclides including ^{137}Cs **(Škrkal et al., 2013; Guillen and Baeza, 2014)**. It is due to their heterotrophic metabolism, significantly different from green plants, and dependence on the supply of final organic compounds **(Yoshida and Muramatsu, 1994)**.

Some species of fungi, including edible ceps (*Boletaceae family*) growing in deciduous forests of Central Europe, are not only bio indicators of environmental contamination by radiocesium (and heavy metals as well), but do to their consumption represent a potential hygiene and health risks problem **(Linkov et al., 2000; Dvořák et al., 2006)**. Radiocesium distribution in different mushroom body parts was uneven, nevertheless higher activity was determined in the mushroom caps **(Mukhopadhyay et al., 2007)**. Ability to accumulate radionuclides from the environment differs among different species of ceps (*Boletaceae family*). This corresponds with the finding, that increased accumulation of ^{137}Cs in bay bolete (*Xerocomus badius, syn. Boletus badius*) is mainly due to the presence of a pigment norbadion A, present in the mushroom cap **(Aumann et al., 1989)**. It is assumed that, as in higher plants, the radiocesium activity in fungi is associated with the growth phase, and the total radiocesium activity decreases with the gradual growth. Mushrooms samples collected in coniferous forests, compared to deciduous forests, are characterized by a high content of radionuclides **(Čipáková, 2004)**.

Table 1 Radiocaesium activity levels in muscles.

Hunting districts, sample data	^{137}Cs (Bq.kg^{-1})	^{40}K (Bq.kg^{-1})
Uhrinc (Kosice) Juvenile female, 1 year, 35 kg	0.60 ±27.6%	81.2 ±4.8%
Jablonov n/T (Roznava) Juvenile female, 1 year, 35 kg	2.03 ±10.0%	47.4 ±4.8%
Rakos, Camovce II (Lucenec) Squeaker female, 6 month, 17 kg	1.1 ±16.6%	51.2 ±2.6%
Vidoslav, Camovce II (Lucenec) Squeaker female, 4.5 month, 15 kg	0.9 ±23.8%	73.0 ±2.6%
Sklens (Turcianske Teplice) Squeaker male, 5 month, 15 kg	0.8 ±33.0%	77.0 ±4.8%
Smolnik (Gelnica) Juvenile female, 1 year, 30 kg	1.5 ±15.5%	75.7 ±4.8%
Lovce (Zlate Moravce) Wild boar female, 2 years, 35 kg	3.6 ±2.2%	63.6 ±2.2%
Topolcianky (Zlate Moravce) Wild boar female, 15 month, 25 kg	37.2 ±4.7%	57.9 ±4.8%
Velky Krtis (Velky Krtis) Squeaker female, 8 month, 20 kg	0.47 ±34.5%	96.8 ±4.8%

Results of the radiocesium mass activity from different areas of the Czech and Slovak Republic in 2000 – 2004, were published by **Dvořák et al., (2006)**. The highest ^{137}Cs activity of 6,263 Bq.kg^{-1} dry weight (measured by gamma spectrometry method) was found in the *Xerocomus badius* from the Old Ransko area (Czech-Moravian Highlands). The highest measured ^{137}Cs level in Slovakia was 966 Bq.kg^{-1} dry weight (*Suillus luteus*), in the area of Senica. However, when converted to fresh weight this value do not exceed permitted limit. For comparison, the ^{137}Cs activity in the sample of mixed dried ceps (*Boletaceae family*) coming from the 30 km Chernobyl border zone, was 6,000 Bq.kg^{-1} dry weight. Results for the dried mushrooms show, that currently there is no ^{137}Cs activity dependency related to the distance from the place of radioactive accident or the location altitude. These results also indicate significantly lower values of ^{137}Cs activity in the Slovak Republic compared to the Czech Republic, despite the fact Slovakia is closer to Ukraine. Explanations should be sought in the airborne radioactive cloud movement through the various parts of Europe after the Chernobyl disaster. In the fresh mushrooms collected in the French Alps in 1999 – 2002, the value of the ^{137}Cs activity ranged from 273 – 1,165 Bq.kg^{-1} **(Pourcelot et al., 2003)**.

After a gradual decline of the radiocaesium (^{137}Cs) activity in the game muscles in the 1990s, unexpected activity increase occurred after the floods in North-East Moravia in 1997. In the meat of wild boars, the activity exceeded the hygienic limit set for EU countries (600 Bq.kg^{-1}), especially in the age category up to 1 year. Since 2000 the ^{137}Cs activity was reduced back to the level before the flood **(Obzina, 2002)**. Seasonal fluctuation of ^{137}Cs activity in the wild boar meat samples was observed in the forests on the southern Rhineland. The stomach contents were examined as well. A positive correlation (0.66) for the activity of the stomach contents and the activity of muscle was found, but the stomach contents were usually less contaminated compared to muscles; the median of the stomach content was 22 Bq.kg^{-1}, the maximum 1,749 Bq.kg^{-1}, while the median of muscle was 129 Bq.kg^{-1}, and the maximum 5,573 Bq.kg^{-1}. No difference in the specific activities of female and male muscles was proved **(Hohmann and Huckschlag, 2005)**. From 1998 to 2008, 656 samples from the wild boars were analysed in the district of Ravensburg (southern Germany). The activity was variable from less than 5 up to 8,266 Bq.kg^{-1}, depending on the season, weather conditions and the associated changes in dietary habits and food availability **(Semizhon et al., 2009)**. High radioactivity levels (up to 10,699 Bq.kg^{-1}) were reported in the wild boars muscles from Sumava (Czech Republic) **(Latini, 2011)**. In 2012, the highest measured value was 14,252 Bq.kg^{-1} **(Kouba et al., 2013)**. In Croatia, some locations were monitored during the years 2000 and 2002 **(Vilic et al., 2005)**; radiocesium in boar muscles ranged from 0.4 to 611.5 Bq.kg^{-1}. The highest muscle contamination was observed in autumn. The authors interpreted this fact as a result of higher mushroom intake by boars at that time. Our muscle samples were collected during different annual periods, however no significant seasonal differences in the radiocaesium activity we observed. This was probably due to low radiocaesium activity in fungi in the observed area, and thus subsequently in our samples as well.

The **Recommendation of the European Commission (2003)** highlights the existence of limit exceeding activity of radiocaesium in the meat of game, and call the Member States to act, in order to protect consumers. Member states should implement steps to ensure that the limits set by **Directive no. 737/90 / EEC (2000)** for placing the game meat, wild berries, mushrooms, and predatory lake fish to the market, are respected. At the same time it recommends to warn the inhabitants of affected regions on the health risks resulting from contaminated food consumption. Member States are asked to prepare for the European Commission and other EU Member States a feedback on the implementation of this Directive.

Wild boar muscles contamination is mainly due to the consumption of the underground fruiting bodies of the mushroom genus of *Elaphomyces sp.* (*E.granulatus* - deer truffle, hart's truffles) **(Hohmann and Huckschlag, 2005; Dvořák et al., 2010)**. The highest ^{137}Cs specific activity of 4,743 Bq.kg^{-1} was detected in the mushroom fruiting bodies in the area of Šabrava, while the other components of the food chain of feral pigs do not exceed few tens of Bq.kg^{-1} **(Dvořák et al., 2010)**. The results of stomach content analysis showed the considerable importance of additional feeding and beechnuts in the food of boars while rootlets and sprouts were less important, and the importance of animal components was minimal. Mushrooms were not identified in any stomach, probably due to their relatively high digestibility in boars. Furthermore, the following food components of boars manifested the ^{137}Cs specific activities higher then MDA in the samples tested: 16 Bq.kg^{-1} in meadow earthworms, 200 Bq.kg^{-1} in rootlets at the Šabrava location, and finally 4,743 Bq.kg^{-1} and 2,858 Bq.kg^{-1} in *Elaphomyces granulatus* fruiting bodies. The fruiting bodies were not found in the boar stomach as other mushrooms, however, they were found in the marginal areas around the rooted locations. We may assume that this mushroom with a vegetation season from September to April is searched and consumed by boars, similarly as e.g. the truffles in France. For example, considering the high activity of 4,743 Bq.kg^{-1} measured in one sample and taking into account the high mushroom digestibility in boars, the specific activity of chyme can be estimated to about 237 Bq.kg^{-1} only due to the intake of the mushroom when 5% intake in 1 kg of the stomach content was taken into account. For monogastric animals, the radiocaesium resorption can reach up to 100% while excretion is about 25%, which would mean a daily growth of specific activities of 21 Bq.kg^{-1} in the net muscle weight of 25 kg (for a boar life weight of 100 kg and food consumption of 3 kg daily). However, such chyme usually does not consist only of the fruiting bodies of the above mentioned mushroom and other components with the specific activities below MDA. The following example based on our results (muscle sample with the specific activities of 4,743 Bq.kg^{-1}, soil elements 173 Bq.kg^{-1}, rootlets 200 Bq.kg^{-1}, earthworms 16 Bq.kg^{-1}, all at the Šabrava location) shows the importance of other ^{137}Cs sources. The proved presence of soil, earthworms, rootlets and other components increases the ^{137}Cs activity in the stomach content. In case of food consumption of 3 kg.day^{-1} at the

Šabrava location with the individual contributions, i.e. 5% fruiting body of *Elaphomyces granulatus* (total 711 Bq), 5% soil elements (26 Bq), 20% rootlets (120 Bq), 2% earthworms (1 Bq) and other components with the specific activities below MDA, the total activity of the stomach content would be 858 $Bq.day^{-1}$. For the stomach content of 1 kg, this means the specific activity of 286 $Bq.kg^{-1}$. The daily increase of the specific activity in muscle would be 26 $Bq.kg^{-1}$. However, the increase due to other components in the food chain is only 5 $Bq.kg^{-1}$, i.e. an increase by 25%. *Elaphomyces granulatus* is mainly found in soils with high content of humus, especially in sandy pine and spruce forests. It grows under the ground and appears on the ground surface usually due to wild game (wild boar) activity. The main period of the fungi growth is from September to April. This is the time when animals have limited access to food sources. In Slovakia, the occurrence of this fungus is very rare, and is mainly present in protected landscape areas, where hunting is not allowed. Compared to the situation in livestock, limited possibility of protective measures implementation represents the biggest problem of ^{137}Cs contamination in game. The only possible solution remains to check each hunted animal individually. One possibility of preventing the game contamination is the supplementary feeding.

The use of alternative food sources as prevention, may result in reduced consumption of natural food, including *Elaphomyces granulatus*, to cover organism energy needs. Slovakia has so far monitored very limited number of hunted animals. The value of ^{137}Cs samples activity collected are still very low **(Beňová et al., 2014)**, what is consistent with our results.

CONCLUSION

In recent years, the wild game meat is more frequently present on menus of variety of restaurants and is becoming very popular in consumers. Despite the fact Slovakia is closer to Chernobyl as Czech Republic and Germany, the ^{137}Cs activity measured was very low and far below the permitted limit. The measurement results show, that ^{137}Cs contamination levels of game in Slovakia are so low, that they do not represent an increased health risk for humans.

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Contact address:

Katarína Beňová University of Veterinary Medicine and Pharmacy in Košice, Department of Biology and Genetics, Komenského 73, 041 81 Košice, The Slovak Republic, E-mail: katarina.benova@uvlf.sk.

Petr Dvořák University of Veterinary and Pharmaceutical Sciences in Brno, Faculty of Veterinary Hygiene and Ecology, Department of Biochemistry and Biophysics, Palackeho 1-3, 612 42 Brno, The Czech Republic, E-mail: dvorakp@vfu.cz.

Martin Tomko University of Veterinary Medicine and Pharmacy in Košice, Department of Biology and Genetics, Komenského 73, 041 81 Košice, The Slovak Republic, E-mail: martin.tomko@uvlf.sk.

Marcel Falis University of Veterinary Medicine and Pharmacy in Košice, Department of Pharmacology and Toxicology, Komenského 73, 041 81 Košice, The Slovak Republic, E-mail: marcel.falis@uvlf.sk.

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THE EFFECT OF FEEDING MILK THISTLE SEED CAKES ON QUALITY INDICATORS OF BROILER CHICKENS MEAT

Ondřej Šťastník, Miroslav Jůzl, Filip Karásek, Hana Štenclová, Šárka Nedomová, Leoš Pavlata, Eva Mrkvicová, Petr Doležal, Alžbeta Jarošová

ABSTRACT

This study was conducted to evaluate the effect of feeding milk thistle *(Silybum marianum L.)* seed cakes at dose 5% and 15% in feed mixture on quality indicators of broiler chickens meat. The used milk thistle seed cakes contained 3.73% of flavonolignans and 129.83 mg.kg^{-1} of cyanidin-3-glucoside. A 150 cockerels of Ross 308 were divided into three equal groups. The chickens were fattened on conventional deep litter system. The experimental groups received feed mixtures containing 5% of milk thistle seed cakes (MT5), 15% of milk thistle seed cakes (MT15) and third group was control – without milk thistle seed cakes (C). The trial lasted 37 days. At the end of trial was observed significant higher average weight of chickens (2,320.31 g) in control group. Compare to that the experimental group MT5 achieved significant lower mean bodyweight 2,166.69 g. From the perspective of fattening was decreased growth of chickens where a higher percentage of milk thistle seed cakes (MT15). The group MT15 was up to 420 g lower slaughter weight compared to the control group. This was probably due to the higher content of fiber in the feed. At the end of experiment 15 birds were selected randomly from each group, weighed and slaughtered. Feathers were removed and chickens were eviscerated. Carcass yield was calculated for each group like as percentage of live weight. The MT5 and MT15 group had significantly higher breast meat tenderness that the control group. Initial pH1 was highest in group with its middle addition of milk thistle seed cakes (MT5). Significant differences were not observed between control and group MT15. Breast meat was rated as the best in parameter flavour in control and MT15 group. The thigh meat was evaluated significantly best for colour parameter in MT15 group. Fibreness was rated as the finest in MT15 group. The addition of milk thistle seed cakes do not worsened sensory characteristic of breast or thigh meat of broilers and reflects optimal sensory quality traits.

Keywords: *Silybum marianum L.*; meat quality; growth; texture; colour; flavour

INTRODUCTION

Milk thistle *(Silybum marianum* L.; Asteraceae) have been used for almost 2 000 years as a natural treatment for the liver diseases **(Ding et al., 2001)**. The seeds of milk thistle contain flavonoids (anthocyanins) and flavonolignans (silymarin) in an amount of 1.5 – 3% **(Opletal and Šimerda, 2015)**.

Flavonoids are widespread in nature, including the anthocyanins commonly found in dark-coloured fruits and vegetables. Anthocyanins are produced by plants as secondary metabolites to protect against environmental stress factors and fungal infections **(Chalker-Scott, 1999)**. And they also promote health status **(Wallace, 2011; Pojer et al., 2013)**. Phenolic compounds, mainly anthocyanins have antioxidant and anti-inflammatory activities **(Jung et al., 2014)**. This anthocyanin, cyanidin-3-glucoside (CG) respectively, have been reported to be bioavailable **(Miyazawa et al., 1999)**. CG decreased obesity and circulating triglycerides in an in vivo study **(Wei et al., 2011)**. In vitro, CG decreased inflammation in isolated vascular endothelial cells and monocytes and produced an insulin-like effect in human adipocytes **(Luo et al., 2012; Scazzocchio et al., 2011)**.

The main active substances occurring in milk thistle are flavonolignans, which are hepatoprotective substances. The mixture of silydianin (10%), silychristin (20%), silybin A and silybin B (50 – 60%), isosilybin A and isosilybin B is known as silymarin **(Opletal and Skřivanová, 2010; Ding et al., 2001; Zahid and Durrani 2007; Comelli et al., 2007)**. Silymarin complex exhibits chemopreventive activity against chemical, viral, bacterial and fungal toxins, inhibits lipid peroxidation, and stabilizes the cell membranes of the liver parenchyma **(Opletal and Skřivanová, 2010)**. Various trials showed that silymarin addition in diet or silymarin administration increased productive and reproductive performances and improved livestock health status of animals **(Tedesco, 2001)**.

Many works investigate the effect of herbs addition to feed mixtures for broiler chickens and their influence to the meat quality **(Haščík et al., 2015; Bobko et al., 2009)**.

The rapid growth of modern broilers hybrid and toxic substances in the feed mixtures can lead to metabolic and oxidative stress. It can worsen feed conversion ratio, growth parameters and it can affect the quality of chicken meat **(Erdogan et al., 2005; Carreras et al., 2004)**. The consumers have very high requirements on their food. It

must be natural, healthy, quality, safe and, on top of that, it should have pleasant appearance, texture, odour and taste **(Drobná et al., 2006)**.

This study was conducted to evaluate the effect of feeding milk thistle seed cakes at dose 5% and 15% in feed mixture on quality indicators of broiler chickens meat.

MATERIAL AND METHODOLOGY

Growth performance, body and chemical composition

The used milk thistle seed cakes contained 3.73% of flavonolignans and 129.83 mg.kg^{-1} of cyanidin-3-glucoside. Table 1 shows chemical composition of used milk thistle seed cakes.

Table 1 Chemical composition of milk thistle seed cakes.

Dry matter (%)	100
Gross energy (MJ.kg^{-1})	17.44
Crude protein (%)	18.65
Crude fat (%)	8.66
Crude fibre (%)	25.13
Crude ash (%)	5.84

The experiment was performed with cockerels of Ross 308 hybrid (n = 150) which were fattened on conventional deep litter system. Wood shavings were used as bedding material. The trial was conducted from day 12 to day 37 of chick's age. Room temperature and humidity were controlled. Lighting system was 16 hours light and 8 hours dark. Cockerels were divided into three equal groups. The two experimental groups received feed mixtures containing 5% and 15% of milk thistle seed cakes (groups MT5 and MT15, respectively). The third group was without milk thistle seed cakes (Control group). The rations were calculated according to the Recommended nutrient content in poultry diets and nutritive value of feeds for poultry **(Zelenka et al., 2007)**. The composition of feed mixtures is shown in Table 2.

The chickens were fed *ad-libitum*. Health status was evaluated daily and live weight measured every week during the trial. Body weight gain was measured individually.

At the end of experiment fifteen birds were selected randomly from each group, weighed and slaughtered. Feathers were removed and chickens were eviscerated. Carcass yield was calculated. The breast muscle and leg muscle were deboned and weighed in these selected chickens. These values were calculated by the percentage of live weight.

Texture, colour and pH of meat

The tenderness of the fillets was determined through the application of the Meullenet–Owens razor shear (MORS) test, using a texture analyzer (Model TA-XT2*Plus*, Texture Technologies, Scarsdale, N.Y., U.S.A.) as described by **Meullenet et al., (2004)** and **Cavitt et al., (2005)** during which Razor Blade Shear Force (N) were recorded. Tests using the MORS blade are conducted on whole intact right fillets with 5 replicates. The sharp blade was replaced every 80 measurements for optimum shearing performance. Test Settings: test speed 10 mm.s^{-1}, distance 20 mm.

Table 2 Composition of feed mixture (g.kg^{-1}).

Component	C	MT5	MT15
Wheat	378.2	271.8	269
Corn	247	282.4	251
Milk thistle seed cakes	0	50	150
Soybean meal	105	120	128
Soybean extruded	190	190	78
Rapeseed oil	20	30	40
Wheat gluten	18.8	15.2	40
Premix*	30	30	30
Monocalciumphosphate	7	6.5	7
Limestone milled	4	4	5
L-lysine	0	0	2
Chemical composition (per kg of diet)			
Dry matter (%)	100	100	100
Gross energy (MJ)	18.59	18.83	19.07
Crude protein (%)	21.41	21.73	22.43
Crude fat (%)	7.60	9.60	8.96
Crude fibre (%)	2.81	4.07	7.03
Crude ash (%)	5.96	5.84	6.65

* Premix contains (per kg): lysine 60 g; methionine 75 g; threonine 34 g; calcium 200 g; phosphorus 65 g; sodium 42 g; copper 500 mg; iron 2,500 mg; zinc 3,400 mg; manganese 4,000 mg; cobalt 7 mg; iodine 30 mg; selenium 6 mg; tocopherol 450,000 mg; calciferol 166,700 IU; tocoferol 1,500 mg; vit K 350 mg; B_1 140 mg; B_2 230 mg; B_6 200 mg; B_{12} 1,000 mg; biotin 7 mg; niaciamid 1,200 mg; folic acid 57 mg, calcium pantothenate 450 mg; choline chloride 6,000 mg; salinomycin sodium 2,333 mg.

Colour measurement was performed by CIE L*a*b* colour space. L* (lightness), a*(redness) and b* (yellowness) values from the breast muscle sample surface on the dorsal side were measured using a Spectrophotometer CM-3500d (Konica Minolta Sensing Inc., Osaka, Japan) in SCE mode (specular component excluded), angle 8°, 8 mm slit. Each sample was measured at three places 1-hour *post-mortem*. Average value was taken as the final result. ΔE^*_{ab} **(CIE, 2007)** was calculated according next formulas **(Valous et al., 2009)**:

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

$$\Delta L^* = L^*_{control} - L^*_{group}$$

$$\Delta a^* = a^*_{control} - a^*_{group}$$

$$\Delta b^* = b^*_{control} - b^*_{group}$$

The samples was measured using pH meter Portavo 907 Multi (Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany) with a needle-type electrode (SE104N; Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany) immediately (inicial pH, abbreviation pH1) after chicken´s slaughter and 1 hour *post-mortem* (abbreviation pH2).

Sensory analysis

Sensory analysis of breast and thigh muscle samples was evaluated by 10 panellists in special sensory laboratory (Department of Food Technology, MENDELU) according ISO 8589. Each sample (breast and thigh) was packed into plastic case and frozen (freezer, -18 °C). After two weeks was thawed (cold storage room, 4 °C) and boiled in convection oven (200 °C, 60% humidity, 1 hour). Professional evaluation group was represented by a panel of trained panellists under ISO 8586-1. We used a graphic non-structured scale (100 mm) to compare experimental group of descriptors (odour, colour, fibreness, chewiness, juiciness, flavour, fatty taste) with control group.

Statistical analysis

Data has been processed by Microsoft Excel (USA) and Statistica version 12.0 (CZ). We used one-way analysis (ANOVA). To ensure evidential differences Scheffe´s test was applied and p <0.05 was regarded as statistically significant difference.

RESULTS AND DISCUSSION

Growth performance, body and chemical composition

At the end of trial was observed significant (p <0.05) higher average weight of chickens (2,320.31 g) in control group. Compare to that the experimental group MT5 achieved significant lower mean bodyweight 2,166.69 g. The significant lowest mean bodyweight was achieved in the group MT15 with value 1,988.78 g at the end of trial (Table 3).

Table 3 Live weight at the day of slaughter (g).

Group	Mean ±standard error
C	2,320.31 ±29.24[c]
MT5	2,166.69 ±36.43[b]
MT15	1,988.78 ±30.09[a]

[a,b,c] – different letters are statistically significant differences (p <0.05).

According to the technological procedure for ROSS 308, the average body weight of cockerels would be 2,493 g at 37 days of age **(Aviagen Group, 2014)**. This is much closer to the value of the control group (2,320 g) in our trial. **Suchý et al., (2008)** in their experiment observed then the addition of 0.2% and 1% *Sylibum Marianum* seed cakes caused a decrease in the weight gain. **Gawel et al., (2003)** found an increase in the slaughter weight in broilers when supplied silymarin.

From the perspective of fattening was decreased growth of chickens where a higher percentage of milk thistle seed cakes (MT15). The group MT15 was up to 420 g lower slaughter weight compared to the control group, which was probably due to the higher content of fiber in the feed.

Table 4 present the carcass yield parameters of chickens. The carcass yield was not show significant (p >0.05) differences. Carcass yield stated in the technological procedure for ROSS 308 **(Aviagen Group, 2014)** is 72.08% for 2,200 g live weight. The highest carcass yield showed the control group with a value 72.09%. It is comparable with technological procedure for ROSS 308 **(Aviagen Group, 2014)**.

The higher breast meat yield was found in the group 5% of milk thistle seed cakes (22.11 ±0.42% SE). The differences among groups were not statistically significant (p >0.05). The manual for hybrid Ross 308 **(Aviagen Group, 2014)** is stated similar percentage of breast muscle

Table 4 Body composition (%).

Group	n	Carcass	Breast meat	Leg meat
		Mean ±standard error		
C	15	72.09 ±1.05	21.62 ±0.63	14.84 ±0.33
MT5	15	71.44 ±0.95	22.11 ±0.42	14.77 ±0.28
MT15	15	70.51 ±0.75	20.70 ±0.49	15.21 ±0.37

Differences between groups are not signifficant (p >0.05).

Table 5 Chemical analysis of breast and thigh (%).

			C	MT5	MT15
		n	Mean ±standard error		
Dry Matter	Breast meat	6	23.97 ±0.62	24.20 ±0.25	23.56 ±0.54
	Leg meat		24.62 ±0.37	24.12 ±0.29	23.95 ±0.18
Crude Protein	Breast meat	6	20.94 ±0.77	21.39 ±0.28	21.68 ±0.57
	Leg meat		18.68 ±0.19	18.46 ±0.22	18.89 ±0.31
Crude Fat	Breast meat	6	1.24 ±0.19	1.10 ±0.11	0.96 ±0.15
	Leg meat		4.17 ±0.25	4.31 ±0.28	3.88 ±0.34

Differences between groups are not signifficant (p >0.05).

Table 6 Effect of addition milk thistle seed cakes into feed on texture, pH and colour of breast meat (means ±SE).

Parameter	Control	MT5	MT15
Razor Blade Shear [N]	11.23 ±0.47[a]	8.94 ±0.27[b]	9.52 ±0.36[b]
L*	62.00 ±2.20[a]	61.25 ±1.82[a]	62.58 ±2.07[a]
a*	4.42 ±0.35[a]	4.86 ±0.71[a]	5.16 ±0.87[a]
b*	11.33 ±0.44[a]	13.19 ±0.62[b]	13.51 ±0.27[b]
ΔE^*_{ab}	0[a]	2.06[a]	2.38[a]
pH1	6.40 ±0.08[a]	6.64 ±0.09[b]	6.47 ±0.12[a]
pH2	6.12 ±0.10[a]	6.38 ±0.11[b]	6.33 ±0.20[ab]

pH1 values were measured just after slaughter in breast, likewise pH2 values were measured after 1 hour post-mortem.
ΔE^*_{ab} is compared with control group.
[a,b] Means in a row within effect with no common superscript differ significantly (p <0.05).

of body weight to our results.

The highest non-significant difference (p >0.05) in thigh meat yield was observed in the MT15 group (15.21 ±0.37%) compared to the experimental groups. The manual for the hybrid Ross 308 **(Aviagen Group, 2014)** indicates a yield of leg meat 16.03% for 2,200 g of live weight.

Schiavone et al., (2007) performed an experiment with the addition of silymarin into feed mixture for broilers chickens. They found that the control group (without silymarin) achieved significantly highest carcass yield 75.04%. The breast muscle was reached highest weight (29.62%) in group with addition of 40 ppm of silymarin. And the leg muscle reached the highest yield (29.34%) in the group with addition of 80 ppm of silymarin. The lipid content of breast (1.19%) and thigh (3.81%) muscle was affected (p <0.05) by silymarin supplementation, and the lowest amount of lipid content was observed in group with 40 ppm of silymarin.

The chemical composition of breast and thigh muscles is shown in the Table 5. Differences between groups are not significant (p >0.05).

Chemical composition of breast and thight muscles of chickens was not found statistically significant differences. The breast meat of MT15 group contain the most of protein and minimum of fat. The nutrient composition of leg muscle is comparable across all three groups.

Table 7 Sensory analysis of breast meat (mm).

Group	C	MT5	MT15
	Mean ±standard error		
Sensory trait n	60	60	60
Odour	63.97 ±2.75[a]	70.80 ±1.75[a]	69.27 ±1.60[a]
Colour	73.18 ±1.45[a]	75.50 ±1.71[a]	77.40 ±1.39[a]
Fibreness	55.18 ±2.61[a]	56.43 ±2.38[a]	56.97 ±2.06[a]
Chewiness	62.75 ±2.51[a]	59.78 ±3.05[a]	58.60 ±2.20[a]
Juiciness	51.22 ±2.77[a]	44.03 ±3.08[a]	47.33 ±2.07[a]
Flavour	74.00 ±1.50[b]	65.02 ±3.02[a]	73.97 ±1.73[b]
Fatty taste	78.77 ±2.09[a]	81.08 ±1.39[a]	83.40 ±1.06[a]

[a,b] – different letters on one line - statistically significant differences (p <0.05).

Table 8 Sensory analysis of thigh meat (mm).

Group	C	MT5	MT15
	Mean ±standard error		
Sensory trait n	60	60	60
Odour	70.98 ±1.94[a]	69.07 ±1.92[a]	72.02 ±1.42[a]
Colour	50.08 ±1.19[a]	53.05 ±1.51[ab]	57.72 ±1.52[b]
Fibreness	56.67 ±1.39[a]	61.58 ±1.46[b]	65.55 ±1.24[b]
Chewiness	64.83 ±1.59[a]	65.47 ±1.60[a]	66.13 ±1.35[a]
Juiciness	66.90 ±1.97[a]	64.30 ±1.58[a]	67.70 ±1.54[a]
Flavour	74.25 ±1.87[a]	69.42 ±2.16[a]	72.78 ±1.72[a]
Fatty taste	76.35 ±2.56[a]	77.15 ±2.62[a]	79.13 ±2.21[a]

[a,b] – different letters on one line - statistically significant differences (p <0.05).

Texture, colour and pH of meat

The Razor Blade Shear Force results (n = 30) are shown in Table 7. The MT5 and MT15 groups had significantly (p <0.05) higher breast meat tenderness that the control group. Presented in Table 6.

The colour change is not significant in all coordinates (lightness L*, a* and b*), see Table 6. There were no significant differences between all three groups. However, compared with the control group, the yellowness (b*) was higher in both experimental groups. The total colour change (ΔE^*_{ab} from 1.5 to 3.0) is clearly perceptible but not yet discordant and it is acceptable for consumers **(Saláková, 2012)**.

The pH values from control group and groups with addition of *Silybum marianum* into feed is illustrated in Table 6. Initial pH1 was highest (p <0.05) in group with its middle addition of milk thistle seed cakes (MT5). Significant differences were not observed between control and group MT15. The highest pH decrease was noticed in control group breasts. pH2 values measured after 1-hour post mortem is obviously higher in MT5 and MT15 than control group. Between control and MT5 group was significant difference (p <0.05). Some authors **(Zhang et al., 2011, Salami et al., 2015)** confirm the slower post mortal process of muscle acidification on the grounds of various feeding supplementation, but still with acceptable sensory traits.

Sensory analysis

Breast meat was rated as the best in parameter flavour in control and MT15 group (p <0.05). The odour parameter was the best evaluated in MT5 group. Chewiness and juiciness were the best rated in the control group. The fibreness parameter was best rated in the group with the highest addition of milk thistle seed cakes (MT15). This data was not significant (p >0.05) (Table 7).

Some people prefer to consume leg meat of chickens, because it´s more fatty and therefore contains more of flavour substances **(Komprda et al., 2002)**. Table 8 shown sensory analyses of thigh meat. The leg meat was evaluated significantly (p <0.05) best for colour parameter in MT15 group. Fibreness was rated as the finest in MT15 group (p <0.05). The most typical flavour of chicken meat was evaluated in the control group and the chewiness parameter was the best in MT15. There are no significant differences.

Overall assessment of sensory analysis of breast and leg meat shows that the flavour was the best evaluated in the control group. The color and fibrous parameters of meat were the best in the MT15 group. The fatty taste was the lowest in the control group.

Cook and Homer (1996) classified chewiness, juiciness and flavour intensity as the important sensory traits in sensory analyses. This claim is also confirms **Poste et al., (1996)** who advise that flavour is one of the most important sensory traits.

CONCLUSION

The MT5 and MT15 group had significantly (p <0.05) higher breast meat tenderness that the control group. Overall, the total colour change of meat is not significant differences between all three groups. Initial pH1 was highest (p <0.05) in group with its middle addition of milk thistle seed cakes (MT5). Significant differences were not observed between control and group MT15. Breast meat was rated as the best in parameter flavour in control and MT15 group (p <0.05). The leg meat was evaluated significantly (p <0.05) best for colour parameter in MT15 group. Fibreness was rated as the finest in MT15 group (p <0.05).

In this study, the presence of milk thistle seed cakes at dose 5% and 15% in feed mixture were evaluated. The addition of this do not worsened sensory characteristic of breast or leg meat of broilers and reflects optimal sensory quality traits.

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Contact address:

Ondřej Šťastník, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: ondrej.stastnik@mendelu.cz.

Miroslav Jůzl, Mendel University in Brno, Faculty of Agronomy, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: miroslav.juzl@mendelu.cz.

Filip Karásek, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: filip.karasek@mendelu.cz.

Hana Štenclová, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: hana.stenclova@mendelu.cz.

Šárka Nedomová, Mendel University in Brno, Faculty of Agronomy, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: sarka.nedomova@mendelu.cz.

Leoš Pavlata, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: leos.pavlata@mendelu.cz.

Eva Mrkvicová, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: eva.mrkvicova@mendelu.cz.

Petr Doležal, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: petr.dolezal@mendelu.cz.

Alžbeta Jarošová, Mendel University in Brno, Faculty of Agronomy, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: alzbeta.jarosova@mendelu.cz.

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DETERMINATION OF ASCORBIC ACID IN PHARMACEUTICAL PREPARATION AND FRUIT JUICE USING MODIFIED CARBON PASTE ELECTRODE

Simona Žabčíková, Dai Long Vu, Libor Červenka, Vojtěch Tambor, Martina Vašatová

ABSTRACT

Acrobic acid is key substance in the human metabolism and the rapid and accurate determination in food is of a great interest. Ascorbic acid is an electroactive compound, however poorly responded on the bare carbon paste electrodes. In this paper, brilliant cresyl blue and multi-walled carbon nanotubes were used for the modification of carbon paste electrode. Brilliant cresyl blue acts as a mediator improving the transition of electrons, whereas multiwalled carbon nanotubes increased the surface of the electrode. Both brilliant cresyl blue and multiwalled carbon nanotubes were added directly to the composite material. The electrochemical behavior of modified electode was determined in electrolyte at various pH, and the effect of the scan rate was also performed. It was shown that the electrochemical process on the surface of the modified carbon paste electrode was diffusion-controlled. The resulted modified carbon paste electrode showed a good electrocatalytic activity towards the oxidation of ascorbic acid at a reduced overpotential of +100 mV descreasing the risk of interferences. A linear response of the ascorbic acid oxidation current measured by the amperometry in the range of 0.1 – 350 μmol.L^{-1} was obtained applying the sensor for the standard solution. The limit of detection and limit of quantification was found to be 0.05 and 0.15 μmol.L^{-1}, respectively. The novel method was applied for the determination of ascorbic acid in pharmaceutical vitamin preparation and fruit juice, and the results were in good agreement with the standard HPLC method. The presented modification of carbon paste electrode is suitable for the fast, sensitive and very accurate determination of ascorbic acid in fruit juices and pharmaceutical preparation.

Keywords: ascorbic acid; carbon nanotubes; amperometry; brilliant cresyl blue

INTRODUCTION

Ascorbic acid (AA) is a significant vitamin in the diet of humans which prevents scurvy and takes part in several biological reactions **(Oguntibeju 2008)**. In recent studies, it has been found that ascorbic acid may be used as a supporting agent for the treatment of cancer **(Du et al., 2012)** and seems to be interesting in research of Alzheimer's disease **(Bowman 2012)**. However, AA cannot be synthesized by humans and must be supplied from various natural and prepared foods, drugs and physiological fluids, fruit juices, soft drinks and vegetables. For that reason, the determination of AA is very important for biological and agro-industry. At present, vitamin C is determined using widely different techniques including colorimetric and titrimetric measurement, UV spectrophotometry, as well as fluorimetric, chromatographic and other spectroscopic methods **(Ötleş and Karaibrahimoglu, 2012)**. The most common method for analysis of vitamin C is HPLC which is more accurate, selective and sensitive than other methods mention above. However, HPLC methods require specific equipments which are very expensive, difficult in monitoring and generally time-consuming. A need has arisen for a fast, sensitive and inexpensive method for the detection of AA. Recently, a portable strip used for the rapid determination of ascorbic acid has been described **(Kudrnáčová a Kouřimská, 2015)**. The electrochemical determination is one of the approaches as was described in literature **(Skrovankova et al., 2015; Pisoschi et al., 2014)**. Research and development in amperometric sensors for the determination of AA based on the carbon paste composite material have gained increasing importance in the last few years for their advantageous properties as analytical tools, namely the easy of application, lower cost, providing direct, sensitive and fast detection of AA, in comparison with well-established, lab-based methods **(Weng et al., 2013; Li et al., 2011; Huang et al., 2014; Heli and Sattarahmedy 2015; Chang et al., 2014)**. The carbon paste electrodes have attractive advantages, such as simple preparation, low-cost implementation, renewability, low background current, and wide potential window **(Švancara et al., 2012)**. Carbon nanotubes (CNTs) modified CPE have been applied in many studies due to the unique properties of CNTs such as large active surface area, high electronic conductivity, anti-fouling capability and their ability to reduce over potential **(Jacobs et al., 2010; Huang et al., 2014; Bijad et al., 2014)**.

Brilliant cresyl blue (BCB) is a cationic quinine-imide dye with a planar and rigid structure which has been proven to possess promising properties as a redox catalyst. BCB can absorb strongly on the electrode surfaces and these chemically modified electrodes have been used for the determination of various organic compounds **(Lin et al., 2012; Ding et al., 2016; Shaikh et al., 2013)**. The

reduction of BCB by ascorbic acid has been described **(Ulusoy et al., 2011)** therefore we chose this organic dye as a suitable redox mediator for amperometric determination of ascorbic acid.

MATERIAL AND METHODOLOGY

Reagents and equipment

All the reagents were purchased from Sigma-Aldrich (Czech Republic). Deionized water was used in this study (G ≤0.055 μS). Dissolved oxygen was removed from all the solutions by purging with argon for 5 min (purity 99.99%, Linde Technoplyn, Prague, Czech Republic).

A solution of ascorbic acid (10^{-2} mol.L^{-1}) was daily prepared in deionized water and was kept in a dark bottle during the experiments. Briton Robinson (0.04 M, B-R) buffer solution containing 0.1 M KCl was used as a supporting electrolyte.

A three electrode system consisting of CPEs (working), Ag/AgCl/3.0 M KCl (reference) and platinum wire (counter electrode) connected to PalmSens (Ivium Technologies, Netherland) was used for electrochemical measurement. The surfaces of CPE were regenerated by renewing and polishing them on wet filter paper before each measurement.

Preparation of CPE

Both bare and modified electrodes were prepared by mixing of 0.5 g of graphite powder 5.5 – 7.0 μm (CR-5, Maziva Týn n. L., s.r.o., Czech Republic) with 130 μL of mineral oil (M5904, Sigma-Aldrich, Germany). The modified CPE electrode contained 1.0% of multiwalled carbon nanotubes (40 – 60 nm, Shenzhen NanoTech Port Co., China) (MWCNTs), and 3.0% of brilliant cresyl blue powder (BCB). The resulting paste was packed into the Teflon piston holder (3.0 mm inner diameter) (**Švancara and Metelka 2000)**. The resistance of the composite material was always ≤15.0 Ω.

Electrochemical procedure

The effect of modifiers in carbon paste was investigated using cyclic voltammetry in 0.04 M Britton-Robinson buffer solution (B-R) at the pH 5.0 containing 0.1 M KCl at a scan rate of 50 mV.s^{-1} in the range of potentials from -400 mV to +1000 mV. The influence of pH between 3.0 and 9.0 was also investigated at the scan rate of 50 mV.s^{-1} in the same potential window. In order to study the effect of the scan rate on the peak potentials and peak currents, the cyclic voltammograms of the BCB-MWCNTs/CPE were recorded at different scan rates in the potentials ranged from -300 to 800 mV in B-R buffer solution at the pH 5.0 containing 0.1 M KCl.

The amperometric detection of ascorbic acid based on its electrocatalytic oxidation was studied by using BCB-MWCNTs/CPE. Aliquots of a stock solution of AA were added to the supporting electrolyte solution (B-R buffer solution at the pH 5.0 plus 0.1M KCl) after the background current reached a steady state value at an applied potential +100 mV.

Sample preparation

The proposed method was applied for the determination of AA in pharmaceutical sample (tablet, Celaskon 250 mg, Zentiva, Czech Republic) and fruit juice sample (Toma Juice Multivitamin, Pepsico CZ s.r.o., Czech Republic). The tablet (15 mg) was dissolved in deionized water, transferred to a 250 mL volumetric flask, and then was diluted by deionized water until the mark. Juice sample was used directly without any treatment. The amount of ascorbic acid was also determined using HPLC/UV (LC-10AD, Shimadzu Co., Japan) equipped with column LiChrospher RP-18e (250 × 4 mm, 5 μm). A mixture of 250 μL of dilute sample and 50 μL of internal standard (isoascorbic acid in metaphosphoric acid) was vigorously shaken following by centrifugation (13.000 rpm, 5 min). The supernatant (5 μL) was injected into the mobile phase (sodium phosphate/phosphoric acid, pH 2.0, flow rate 1 mL.min^{-1}) with detection wavelength at 263 nm.

Student´s t-test was used for determination of statistical differences between results at the probability level $p = 0.05$ (Origin Pro v. 9, OriginLab Corp., USA).

RESULTS AND DISCUSSION

Electrochemical behavior of BCB-MWCNTs/CPE

As can be seen from Figure 1, an anodic and cathodic peak was observed at +125 mV and +75 mV, respectively. The formal potential of the redox process was +100 mV, and a peak-to-peak separation was +50 mV. The reverse-to-forward current peak ration was approximately unity, which reflects the reversible electrochemical behavior of BCB.

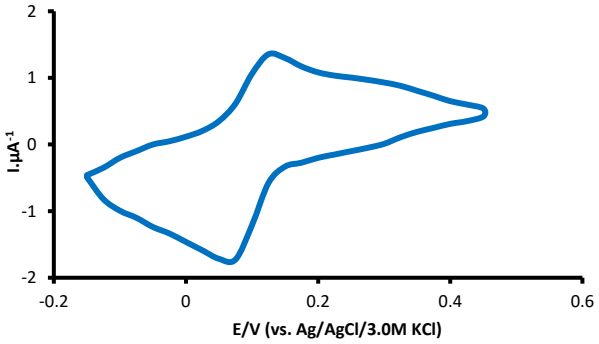

Figure 1 Cyclic voltammograms of BCB-MWCNTs/CPE in the absence of ascorbic acid in B-R buffer solution (pH 5.0) containing 0.1M KCl at the scan rate 50 mV.s^{-1}. Potential range from -150 to +450 mV, potential step 25 mV.

The electrochemical behavior of BCB-MWCNTs/CPE was investigated by recording of cyclic voltammograms in a B-R buffer solution at the pH 3.0 containing 0.1M KCl at various scan rates. The anodic (I_{pa}) and cathodic (I_{pc}) peak currents were proportional to the scan rates (ν) in the range from 10 to 100 mV.s^{-1}. The equations and the regression coefficients were found to be: $I_{pa} = 0.11v^{1/2} + 0.02$ ($R^2 = 0.998$) and $I_{pc} = -0.11v^{1/2} + 0.04$ ($R^2 = 0.994$), respectively.

These results indicate that the electrochemical process on the BCB-MWCNTs/CPE is diffusion-controlled. The shifting of the potentials was not observed between 10 and 100 mV.s^{-1}.

In order to study the effect of pH on the electrochemical behavior of BCB-MWCNTs/CPE, B-R buffer with the pH ranged from 3.0 to 9.0 as a supporting electrolyte was used

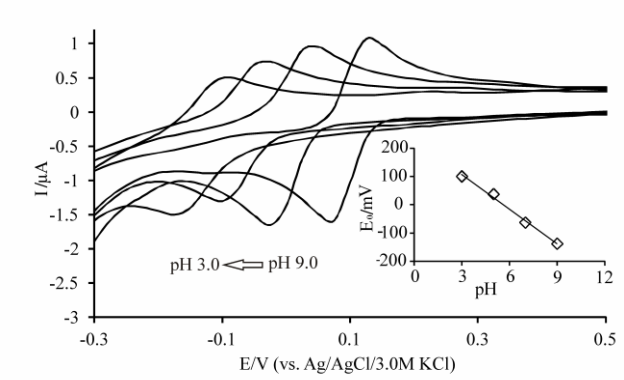

Figure 2 Cyclic voltammograms of BCB-MWCNTs/CPE in B-R buffer solutions (pH 3.0, 5.0, 7.0 and 9.0) containing 0.1M KCl, scan rate 50 mV.s^{-1}, potential range from -500 to +900 mV. Inset: Effect of formal potential (E_0) of BCB-MWCNTs/CPE on the pH.

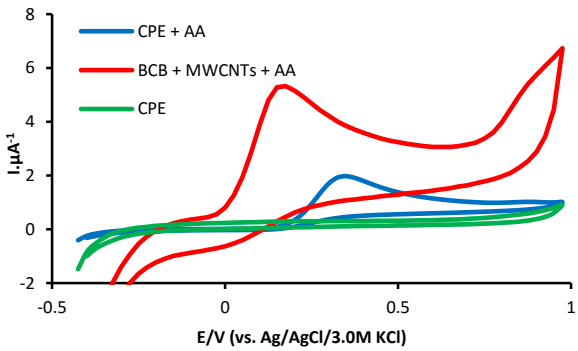

Figure 3 Cyclic voltammograms of BCB-MWCNTs/CPE and CPE in the presence of 250 μmol.L^{-1} of ascorbic acid in B-R buffer (pH 5.0) containing 0.1M KCl, scan rate 50 mV.s^{-1}, potential step 25 mV. Bare CPE (green) in buffer solution served as control.

for determination of cyclic voltammograms of BCB-MWCNTs/CPE at the scan rate of 50 mV.s^{-1}. It was seen that the pH significantly influenced the behavior of BCB-MWCNTs/CPE, since the shape and the position of the peak for redox pair was better in acidic conditions (3.0 and 5.0) in comparison with those obtained in the pH 7.0 and 9.0 (Figure 2).

The inset in Fig. 2 shows the formal potential (E^0) of the BCB-MWCNTs/CPE plotted against pH in the range from 3.0 to 9.0. It shows a slope -40.63 mV.pH^{-1}, which is close to that given by the Nernstian equation for equal number of electrons and protons transfer process. Moreover, the both oxidation and reduction currents proportionally decreased with increasing pH. The electrochemical behavior has been documented in electrodeposited BSB film on the surface of multi-walled carbon nanotubes modified glassy carbon electrode **(Lin et al., 2012)**.

Ascorbic acid oxidation at BCB-MWCNTs/CPE

Cyclic voltammograms of BCB-MWCNTs/CPE were recorded both in the absence and presence of ascorbic acid in order to study the electrocatalytic activity of modify electrode towards AA oxidation. As shown in Figure 3, anodic and cathodic peaks were obtained at +125 and 75 mV, respectively. Oxidation of AA in the bare CPE and BCB-MWCNTs/CPE gave peak potentials at about 350 and 150 mV, respectively. The overpotential for AA oxidation was found to shift by about 200 mV.

The modification of CPE by each modifier separately was also investigated. The oxidation peak potential of AA using CPE with 1.0% of MWCNTs shifted to more negative potentials by about +50 mV with increasing (p *<0.05*) of oxidation current of AA to 3.06 ±0.02 μA in comparison with that obtained in bare CPE (2.13 ±0.02 μA). On the other hand, the separate addition of 3.0% of BCB to CPE resulted in similar oxidation current of AA (2.91 ±0.05 μA) but more progressive shift of the peak potential to +175 mV was observed. It confirms that MWCNTs just increased the electroactive surface area of the CPE electrode (with slight increase of overpotential) whereas BCB acts as a redox mediator. This result clearly indicates that BCB-MWCNTs/CPE in B-R buffer solution (pH 5.0) containing 0.1M KCl exhibited a significant supporting electrocatalytic activity towards AA oxidation. In the study of **Zhang et al., (2013)**, the film of poly(bromcresol purple) at glassy carbon electrode also showed good electrocatalytic effect towards AA oxidation with reduced the oxidation overpotential for about 240 mV with increasing current. In view of oxidation current and the targeted analyte (AA), pH 5.0 was chosen as the best buffer since it offered a relatively high oxidation current and a low oxidation potential.

Various values of applied potential from -50 to +400 mV were used for the amperometric detection of AA. The oxidation current of AA exhibited steep increase from -50 to 100 mV. An applied potential is considered suitable when it offers relatively high oxidation current and low oxidation peak potential. The applied potential +100 mV was chosen for the amperometric determination of AA at BCB-MWCNTs/CPE since it gave the highest current (4.86 ±0.01 μA) compared with that obtained from the lower applied potentials. Besides, using low applied potential can avoid the interferences of some compounds from the matrices. Figure 4 shows the current-time curves for the amperometric responses at various concentration of AA.

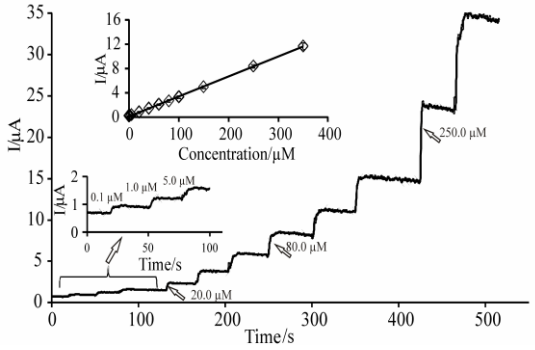

Figure 4 Amperometric current-time curves of AA with various concentrations (μM) using BCB-MWCNTs/CPE. Inlet: the plot of maximum oxidation current vs. concentration of AA. B-R buffer solution at pH 5.0, constant potential +100 mV, stirring speed 400 rpm. The vertical bars represent standard deviation (n = *6*).

Table 1 Comparison of carbon paste electrode (CPE) based electrochemical sensors for amperometric determination of ascorbic acid.

Electrode modification	Applied potential (mV)	LOD ($\mu mol.L^{-1}$)	Dynamic range ($\mu mol.L^{-1}$)	Reference
AF-MWCNT/EPI[1]	+420	4.1	NA	**Huang et al., 2014**
Gold decorated SiO_2@PANI[2] core-shell microsphere	+400	3.8	150.0-8000.0	**Weng et al., 2013**
Graphene doped CPE	+310	0.07	0.1-106.0	**Li et al., 2011**
Graphene oxide CoHCF[3] nanocomposite	+430	0.29	2.5-62.5	**Heli and Sattarahmady 2015**
trans-PEPACC[4]	+450	2.27	0-550	**Chang et al., 2014**
BCB/MWCNT[5]	+100	0.05	0.1-350.0	This work

[1] amino-functionalized multi-walled carbon nanotube electroactive polyamide, [2] polyaniline, [3] cobalt hexycyanoferrate. [4] photoactive and electroactive azo-based polyimide/amino-functionalized multiwalled carbon nanotubes, [5] briliant cresyl blue/muti-walled carbon nanotube.

Table 2 Ascorbic acid content ($mg.L^{-1}$) in real sample using BCB-MWCNTs/CPE and standard HPLC method (n = 20).

Sample	Amperometric method	HPLC method
Juice	120.1 ±5.2*	120.5 ±4.7
Tablet	25.8 ±0.5	25.2 ±0.4

* standard deviation

A linear relationship between the AA concentration and the peak oxidation current was obtained over the concentration range $1 \times 10^{-7} – 3.5 \times 10^{-4}$ $mol.L^{-1}$ (Figure 4, inlet) with equation:

$$I_{pa}\ (\mu A) = 0.033 \times c\ (\mu mol.L^{-1}) + 0.166\ (R^2 = 0.993)$$

The repeatability of the method was investigated by amperometric measurements of 5 $\mu mol.L^{-1}$ and 300 $\mu mol.L^{-1}$ of AA (n = 20) and the relative standard deviation (RSD) was found to be 4.5% and 5.2%, respectively. The limit of detection (LOD) using the equation $LOD = 3s_b/m$, where s_b is the standard deviation of the blank response and m is the slope of the calibration plot, was found to be 5.0×10^{-8} $mol.L^{-1}$. The limit of quantification (LOQ) using the equation $LOQ = 10s_b/m$ was found to be 1.5×10^{-7} $mol.L^{-1}$ (signal/noise = 10). Various modified carbon paste electrodes used for ascorbic acid determination were compared (Table 1). Among amperometric sensors based on CPE, our modification allowed the detection of AA at lower applied potential avoiding the interference species. BCB-MWCNTs/CPE electrochemical sensor is proven to be extremely sensitive, simple renewable and easy in preparation and storage.

After 45 days of storage at room temperature, the amperometric responses of AA at the concentration of 5.0 $\mu mol.L^{-1}$ was not significantly ($p > 0.05$) different in comparison with those obtained using the freshly prepared electrode. The low detection limit is an advantageous character of BCB-MWCNTs/CPE compared with the most CPE modified electrodes mentioned in Table 1. BCB-MWCNTs/CPE is also applicable for the analysis of AA in fruit juices and pharmaceutical preparations. As described in Table 2, the contents of AA in real samples did not differ from those determined by HPLC method.

CONCLUSION

This study demonstrated that BCB-MWCNTs/CPE showed significant electrocatalytic activity towards the oxidation of AA. It was observed that the oxidation peak potential of AA shifted from +375 mV at CPE to +100 mV at BCB-MWCNTs/CPE together with increasing of the oxidation current. The electrocatalytic activity of BCB-MWCNTs/CPE was investigated to detect AA by amperometry with a good linearity and sensitivity. We may conclude that BCB-MWCNTs/CPE represents a steady electrode material for electrocatalytic oxidation of AA.

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Contact address:

Simona Žabčíková, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentska 573, 532 10 Pardubice, Czech Republic, E-mail: simona.zabcikova@student.upce.cz.

Libor Červenka, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentska 573, 532 10 Pardubice, Czech Republic, E-mail: libor.cervenka@upce.cz.

Dai Long Vu, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentska 573, 532 10 Pardubice, Czech Republic, E-mail: longvu1182@gmail.com.

Vojtěch Tambor, Biomedical Research Center, University Hospital Hradec Králové, Sokolská 581, 500 05 Hradec Králové, Czech Republic, E-mail: vojtech.tambor@fnhk.cz.

Martina Vašatová, Institute of Clinical Biochemistry and Diagnostics, University Hospital Hradec Králové, Sokolská 581, 500 05 Hradec Králové, Czech Republic, E-mail: martina.vasatova@fnhk.cz.

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ADSORPTIVE STRIPPING VOLTAMMETRY IN LIPOPHILIC VITAMINS DETERMINATION

Milan Sýs, Simona Žabčíková, Libor Červenka, Karel Vytřas

ABSTRACT

The aim of this contribution was to check if adsorptive stripping differential pulse voltammetry (AdSDPV) is suitable tool for sensitive simultenous electrochemical detection of lipophilic vitamins. Retinol (vitamin A_1), cholecalciferol (vitamin D_3), α-tocopherol (vitamin E) and phylloquinone (vitamin K_1) were selected as representatives. All electrochemical measurements were performed in two separate steps due to the lipophilic character of the analytes. In the first step, an accumulation of lipophilic vitamin on the surface of glassy carbon electrode (GCE) was done by immersing working electrode into the aqueous-acetonitrile solutions (50%, *v/v*) of each vitamin (50.0 μmol.L^{-1}) at 400 rpm for 5 min. In the second one, differential pulse voltammetry of accumulated vitamins was performed in 0.01 mol.L^{-1} acetate (pH 4.5) buffer at potential step (E_{step}) 5 mV, potential of amplitude (E_{ampl}) 25 mV, interval time (t) 0.1 s and scan rate (v) 50 mV.s^{-1}. It was observed that electrochemical behaviour of lipophilic vitamins adsorbed on surface of solid GCE in the aqueous electrolyte was very similar to those performed in organic/aqueous electrolyte in literature. Due to reversible electrochemical behaviour of vitamin K_1 (phylloquinone/phyllohydroquinone redox couple), it was possible to detect all lipophilic vitamins only in one analysis. Observed values of peak potentials (E_p) were sufficiently different for their recognition which was confirmed by the analysis of real sample. The results obtained in this study showed that simultaneous determination of some lipophilic vitamins is possible requiring further optimization study. For this reason, it is necessary to understand this work as an initial step in simultaneous determination of lipophilic vitamins without application of any chromatographic technique.

Keywords: lipophilic vitamin; glassy carbon electrode; adsorptive voltammetry; margarine analysis

INTRODUCTION

It is known that lipophilic vitamins are nonpolar organic compounds essential for proper functioning of the human metabolism which have to be received through diet **(Cockburn, 2003)**. Thus, their detection and quantification in different kinds of samples in a great importance in nutrition, medicine, cosmetics and food technology **(Gonnet et al., 2010)**. Unfortunately, analysis of lipophilic vitamins is quite complicated and time consuming due to their lipophilic character. The main disadvetage is the use of organic solvents.

The determination of lipophilic vitamins is not practically possible without using chromatographic techniques, especially by high performance liquid chromatography (HPLC) followed by extraction of lipophilic vitamins into organic solvent. It is necessary to remind that HPLC analysis of fats may take up to several hours.

All lipophilic vitamins contain conjugated system of double bonds in their structures, therefore a normal-phased HPLC with combination of UV detection is common way of their determination **(Kamal-Eldin et al., 2000)**. Moreover, they were also determined in human serum by reversed-phase HPLC with electrochemical detection **(Wang et al., 2001)**.

Generally, lipophilic vitamins are classified in four main groups **(Webster, 2012)**. In our experiment, the most biologically active forms (all-trans-retinol; vitamin A_1, cholecalciferol; vitamin D3, α-tocopherol, vitamin E and phylloquinone, vitamin K_1) were selected as standards to explore if an adsorptive stripping differential pulse voltammetry (AdSDPV) is suitable electrochemical method for their sensitive simultanenous detection in model sample and selected margarine.

Adsorptive stripping voltammetry (AdSV) is similar to anodic stripping voltammetry (ASV) and cathodic stripping voltammetry (CSV) with the preconcentration step being not controlled by electrolysis **(Wang, 1990)**. In our case, the preconcentration step is controlled by adsorption of analytes on solid glassy carbon electrode (GCE). Their electrochemical detection was performed by differential pulse voltammetry (DPV) which is the most commonly used electrochemical technique for simultaneous determinations **(Baranowska et al., 2008)**.

For comparison, the declared contents of lipophilic vitamins in selected traditional Czech margarines are shown in Table 1. Contents of all present lipophilic vitamins were only copied from nutrition facts of corresponding labels. Additionally, it was observed that tested margarines always contained a mixture of several plant (palm, sunflower and rapeseed) oils whose volume ratios were not surprisingly listed.

Table 1 Declared contents of lipophilic vitamins in several traditional Czech margarines.

Margarines (types)	*Vitamin A (μg/100g)*	*Vitamin D (μg/ 100g)*	*Vitamin E (mg/ 100g)*
Flora light	*800*	*7.5*	*10*
Flora gold	*800*	*7.5*	*—*
Flora original	*800*	*7.5*	*14*
Flora pro-active	*800*	*7.5*	*11*
Perla plus vitamíny	*800*	*7.5*	*—*
Perla tip	*800*	*7.5*	*—*
Rama classic	*800*	*7.5*	*9.2*
Stella	*800*	*3.5*	*—*

It is evident that concentration levels of present lipophilic vitamins are mutually very different (~10 mg E, ~1 mg A and ~0.01 mg D). Therefore, it can be assumed that simultaneous electrochemical determination of lipophilic vitamins in real samples, especially in margarines, remains a challenge for further scientific research.

MATERIAL AND METHODOLOGY

Standards of lipophilic vitamins

Vitamin A_1 as retinol (crystalline), vitamin E as (+)-*α*-tocopherol (from vegetable oil; 1000 IU.g^{-1}), vitamin K_1 as phylloquinone (viscous liquid) and acetonitrile (ACN) of HPLC purity (99.8%) were purchased from Sigma Aldrich (Vieanna, Austria). Vitamin D_3 as cholecalciferol (40×10^6 IU.g^{-1}; crystalline) was obtained from Merk (Darmstadt, Germany).

Instrumentation

All electrochemical measurements were carried out at conventional three-electrode system consisting solid GCE with surface diameter 2 mm from, Ag/AgCl and 3.0 mol.L^{-1} KCl as salt bridge (reference) and platinum wire (auxiliary) electrode which were together connected to potentiostat Autolab PGSTAT101 from Metrohm (Prague, Czech Republic) which is also compatible with software Nova (Prague, Czech Republic).

Pretreatment of glassy carbon electrode

Surface of solid GCE was renovated by polishing pad with presence of wet Al_2O_3 powder for 30 s. After subsequent rinsing of the surface by distilled water, the GCE was ready for new electrochemical experiment.

Sample preparation

The sample preparation is consisted only by dissolving of 2 g margarine type "Perla plus vitamíny" from UNILEVER ČR, spol. s r.o. (Prague, Czech Republic) in pure ACN and filled to the mark of 50 mL volumetric flask.

Procedure

Adsorptive stripping voltammetry of lipophilic vitamins was performed in two separate steps. In the first step, the analytes adsorption on GCE surface was done by immersing working electrode in aqueous-acetonitrile solutions (50% content of ACN) containing 50 μmol.L^{-1} of each vitamin 10 min at 400 rpm. In second one, repettive CV of accumulated lipophilic vitamins in 0.01 mol.L^{-1} acetate (pH 4.5) buffer was done to examine their electrochemical behaviours at potential step (E_{step}) 5 mV, scan rate (v) 50 mV.s^{-1} and fivecycles repetition.

Analogically, DPV of accumulated vitamins (100 μmol.L^{-1} of each vitamin in 25% ACN at 400 rpm for 10 min) was performed in 0.01 mol.L^{-1} acetate (pH 4.5) buffer with deposition potential (E_{dep}) –0.6 V for 120s, potential step (E_{step}) 5 mV, potential of amplitude (E_{ampl}) 25 mV, interval time (t) 0.1 s and scan rate (v) 50 mV.s^{-1}.

RESULTS AND DISCUSSION

Cyclic voltammetry of accumulated vitamins

Electrochemistry of retinol (vitamin A) film deposited on GCE surface

Vitamin A1 deposited on surface GCE provided only one sensitive oxidation peak at +0.708 V whose current response dramatically decreased with the number of cycles. For demonstration, typical repetitive CV of vitamin A1 accumulated at GCE in acetate buffer is shown in Figure 1.

Similar electrochemical behaviour was observed at GCE in a methanol/acetate (pH 5.0) buffer at scan rate 50 mV.s^{-1} **(Wring et al., 1988)** which corresponds to irreversible electrochemical oxidation of retinol to the retinaldehyde with participation of two protons and electrons **(Ziyatdinova et al., 2010)**.

However, it is important to note that a background

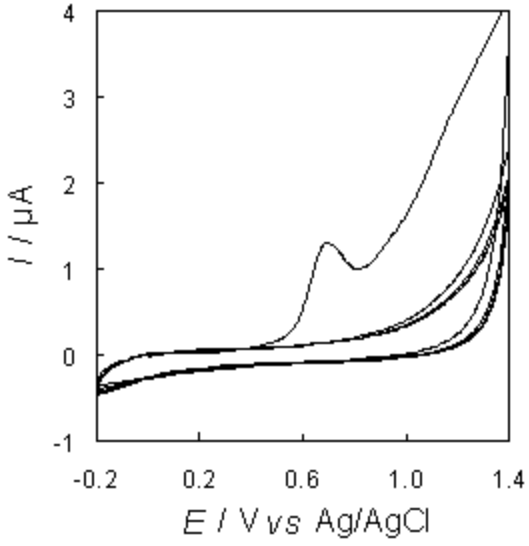

Figure 1 Repetitive cyclic voltammetry of vitamin A_1 (50.0 μmol.L^{-1}) in 0.01 mol.L^{-1} acetate (pH 4.5) buffer at v = 50 mV.s^{-1}.

current increased after oxidation of vitamin A1, probably due to adsorption of the oxidized products. Unfortunately, this phenomenon can negatively affect an electrochemical detection of other lipophilic vitamins which could be oxidized at higher values of potential than present vitamin A1.

Electrochemistry of cholecalciferol (vitamin D_3) film deposited on GCE surface

Electrochemically similar behaviour as in previous situation was observed also for vitamin D3 which also provided only one oxidation peak at +1.032 V which was not visible under following repetitions. According to obtained cyclic voltammogram shown in Figure 2, the oxidation process of cholecalciferol appeared to be irreversible. In fact, the same electrochemical behaviour has been obtained at GCE in in a methanol/acetate (pH 6.0) buffer at scan rate 50 $mV.s^{-1}$ **(Hart et al., 1992)**.

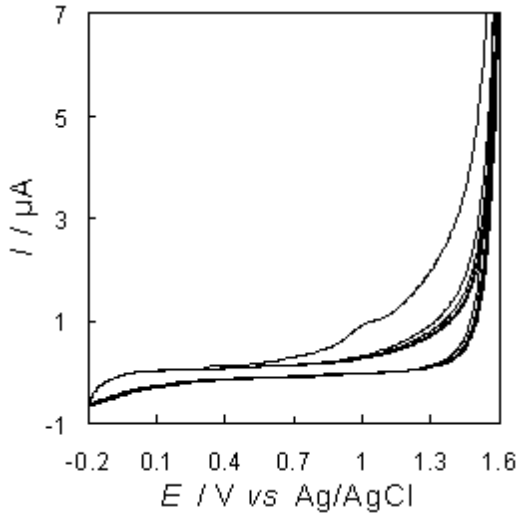

Figure 2 Repetitive cyclic voltammetry of vitamin D_3 (50.0 $\mu mol.L^{-1}$) in 0.01 $mol.L^{-1}$ acetate (pH 4.5) buffer at v = 50 $mV.s^{-1}$.

Electrochemistry of α-tocopherol (vitamin E) film deposited on GCE surface

Thin layer electrochemistry of α-tocopherol (α-TOH), known as the most active form of vitamin E in aqueous electrolytes was investigated resulting in formation of lipid multilayer **(Yao et al., 2009)** or modification of carbon paste **(Kim and Kusuda, 1994)**. Electrochemical behaviour of α-TOH deposited on surface of solid GCE in aqueous electrolytes was also published by our research group **(Sýs et al., 2016)**.

Electrochemistry of phylloquinone (vitamin K_1) film deposited on GCE surface

In comparison to previous measurements, cyclic voltammetry of vitamin K_1 always began with cathodic scan due to content of quinone unit in its structure **(Wang et al., 1994)**. Thus, the electrochemical behaviour of vitamin K_1 was very similar to redox couple quinone/hydroquinone. According to Figure 3, the vitamin K_1 provided typical two reversible electrochemical peaks at –0.325 and –0.006 V. Moreover, another sensitive cathodic peak was observed at –0.832 V.

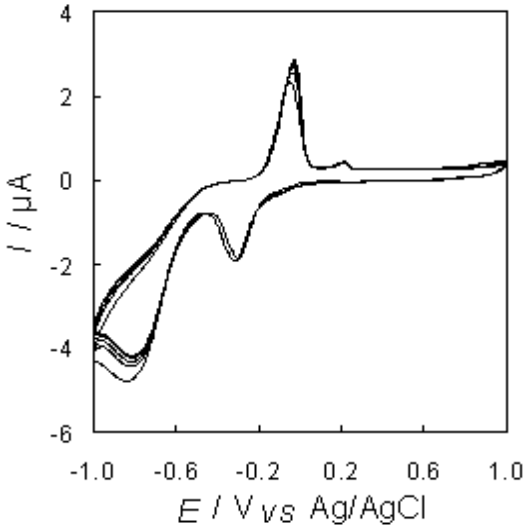

Figure 3 Repetitive cyclic voltammetry of vitamin K_1 (50.0 $\mu mol.L^{-1}$) in 0.01 $mol.L^{-1}$ acetate (pH 4.5) buffer at v = 50 $mV.s^{-1}$.

Simultenous differential pulse voltammetry of accumulated vitamins

Form the previous section, it states that only vitamin K_1 can not be electrochemically oxidized because it usually occurs in its oxidation form as naphthoquinone with long alkyl chain. Based on this finding, it was necessary to reduce the phylloquinone to phyllohydroquinone with participation of two protons and electrons. Additional lipophilic vitamins accumulated together with phylloquinone on surface of working electrode were present in their corresponding reduction forms. Therefore, applying of deposition potential –0.6 V for 120 s did not cause any electrochemical changes of these vitamins (A_1, D_3 and E).

Only after electrochemical reduction of vitamin K_1, anodic DPV can be used for simultaneous electrochemical detection of all presented lipophilic vitamins in potential window from –0.6 to +1.4 V. The evidence that all selected lipophilic vitamins can be determined together in one analysis is demonstrated in Figure 4. Moreover, it shows that distance of individual voltammetric peak was satisfactory for their sufficient resolution without using any chromatographic technique due to sufficiently different values of the appropriate peak potentials.

It is very important to realize that the electrochemical method presented in this contribution has not been optimized yet. It can be assumed that whole optimization will be very time consuming because it is always based on the finding the optimal working conditions to obtain high sensitivity such as selection of suitable electrode material, organic solvent and many others.

For example, an amount of deposited analytes on solid electrode material is limited by surface area. Therefore, it is obvious that linearity range of developed analytical method will be very narrow and the sensitivity will be completely dependent on the time of accumulation. The

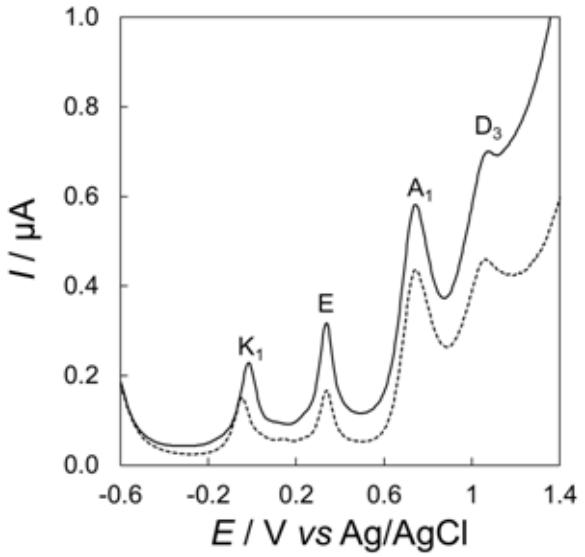

Figure 4 Simultaneous adsorptive stripping voltammetry of lipophilic vitamins deposited on GCE surface from their 100 µmol.L^{-1} solution containing 25% ACN at 400 rpm for 10 min; then detected by DPV in 0.01 mol.L^{-1} acetate (pH 4.5) buffer at E_{dep} = –0.6 V at 120 s, E_{step} = 5 mV, E_{ampl} = 0.025 V, v = 25 mV.s^{-1} (dashed line) and v = 50 mV.s^{-1} (solid line).

solution can be found in using of suitable kind of carbon paste electrode (CPE) which can be classified from physical point of view as a dispersion of solid carbon powder particles in a viscous lipophilic binder **(Švancara et al., 1996)**. In this case, the amount of accumulated analyte is controlled by corresponding extraction equilibrium.

According to publication **(Žabčíková and Červenka, 2015)**, carbon paste can be prepared from plant oils which are commonly used in technology of margarines. Thus, it can be another way how lipophilic vitamins also could be electrochemically detected.

Using carbon nanomaterials offers another possibility. Especially, carbon nanotubes (CNTs) immobilized on some carbon-based electrode material usually cause dramatical increasing of electrode surface due to their specific physical properties **(Volder et al., 2013)**.

Analysis of margarine

Analysis of margarine (Perla plus vitamíny) was only based on qualitative determination of present lipophilic vitamins. Therefore, any sophisticated statistical treatment was not necessary to use. Values of peak potentials are usually presented as arithmetic mean ($\bar{x}$) of minimally five repetitions (n) and corresponding standard deviations (σ) were less than 2% due to polishing of electrode surface after each measurement.

In this case, 2.5 mL sample solution was added into the 7.5 mL pure water in order to obtain 25% ACN in total volume. After that, GCE was immersed into resulting solution and deposition occurred at 400 rpm for 20 min. Obtained voltammograms of accumulated analytes performed in acetate buffer is shown in Figure 5.

It is interesting that only vitamin K_1 and vitamin E were qualitatively determined in the sample of margarine, although these vitamins were not listed on the product label. An explanation lies in the basic ingredients of all margarines. From physical point of view, they can be defined as emulsions of water in edible plant oil which are natural resources of these lipophilic vitamins **(Piironen et al., 1997)**. According to manufacturer, the analyzed margarine contains sunflower and rapeseed oils.

Mentioned rapeseed oil usually contains relatively high amounts of oleic and linoleic acids **(Frančáková et al., 2015)** which are very important like lipophilic vitamins. It is maybe reason why these compounds beneficial for health are very often abused in commercials.

It is clear from Figure 5 that second peak at +0.332 V (anodic oxidation of vitamin E) is not symmetric like oxidation peak of vitamin K_1 at –0.080 V. It is necessary to remember that vitamin E is not chemical individual but group of eight isomers known as tocopherols **(Gliszczyńska-Świgło1 et al., 2007)** which have similar electrochemical properties. Therefore it is quite possible that not only α-TOH was present in the sample of the margarine.

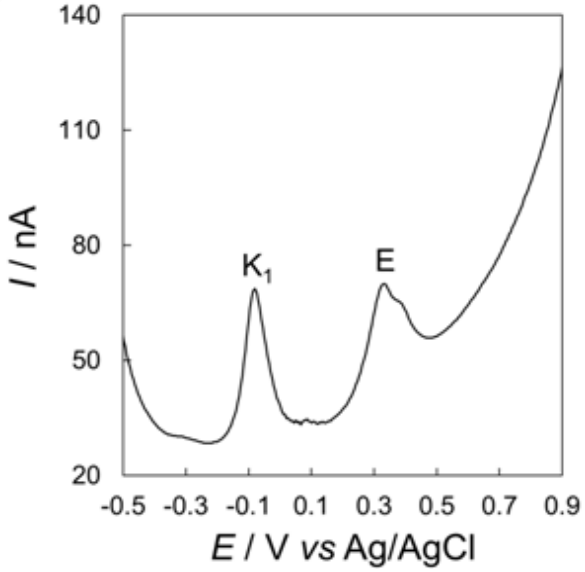

Figure 5 Qualitative determinations of lipophilic vitamins in margarine (Perla plus vitamíny) by adsorptive stripping voltammetry of at solid GCE.

CONCLUSION

According to our experimental results, it may be concluded that simultaneous qualitative determination of lipophilic vitamins is possible using adsorptive stripping differential pulse voltammetry. Unfortunately, it is clear that deposition of analytes on solid glassy carbon electrode and their following electrochemical detection does not provide satisfactory sensitivity, especially, in determination of vitamin D_3. However, it can be assumed that the sensitivity to all lipophilic vitamins can be improved using carbon nanomaterials or heterogeneous carbon materials which are known as carbon pastes. It is necessary to understand this work as an initial step in simultaneous determination of lipophilic vitamins.

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Contact address:

Milan Sýs, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: milan.sys@upce.cz.

Simona Žabčíková, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: simona.zabcikova@student.upce.cz.

Libor Červenka, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: libor.cervenka@upce.cz.

Karel Vytřas, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: karel.vytras@upce.cz.

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INFLUENCE OF TYPE AND SHELF-LIFE ON TWO BRANDS COMPLEMENTARY FOOD IN COLOR, VITAMINS, AND SENSORY EVALUATION

Vladimír Sýkora, Hana Šulcerová, Michal Mihok, Roman Pytel

ABSTRACT

The aim of our study was to measure the color by system CIELAB, sensory analysis, and determination of vitamins in children vegetable complementary feeding (carrot, vegetable mix) with the option to extend shelf life from eighteen to twenty-one months. Complementary children food was obtained from private factory in the Czech Republic. In this research there were used only carrot and vegetable mix samples. To determine the color changes by system CIELAB and determination of vitamins, samples of mash were analyzed before filling into jars and sterilization, and then immediately after sterilization. Further analyzes were performed for twenty-one months, with run of every three months (p <0.05). The comparison of color CIELAB parameter L* (lightness) for two process steps: raw mash and sterilized mash; there were significant differences when processing (p <0.0001, r^2 = 0.9983). Mainly, the parameter L* (Lightness) showed statistically significant differences in carrot and garden mix (p <0.05). β-carotenes such as provitamin A, is in food of plant origin stable substance in the absence of air. Storing time had significant influence on contain of β-carotenes, the mean content during twenty-one months was 0.862 mg.100g^{-1} (p <0.05, r^2 = 0.2300). There were no significant differences in dark storing (p >0.05, r^2 = 0.1097). The sensory evaluation showed statistical differences in all descriptors (color saturation, uniformity of color, consistency and homogeneity) (p <0.05) in course of months of storage time and storage conditions (daylight-dark). The results can be recommended to manufacturers, extending the period of minimum shelf life of the required three months to twenty-one months due to instability as characteristics of color and textural properties which were obtained.

Keywords: carrot; vegetable; colorimetry; storing; baby food; sensory analysis; vitamins

INTRODUCTION

The period of transition from exclusive breastfeeding to family foods, referred to as complementary feeding, covers a child from 6 – 23 months of age. Complementary feeding embraces all solid and liquid foods other than breast milk or infant formula and follow-on formula **(ESPGHAN, 2008)**. Malnutrition in young children can be prevented by feeding them with enough nutritious and safe complementary foods. The Association of United Kingdom Dietitians (BDA) and its Department of Health (DH) guidelines recommend the introduction of complementary feeding at around six months **(BDA, 2013)**. Furthermore BDA guidelines correspond with WHO (World Health Organization) statement. Most infants are developmentally ready for other foods at about 6 months. During the period of complementary feeding, children are at high risk of undernutrition **(WHO, 2009)**.

Complementary foods are known as weaning foods, which are semi-solid or solid foods **(Bond et al., 2005)**. Complementary food should be thick enough so that it stays on a spoon and does not drop off. Generally, such food is thicker or more solid with more energy- and nutrient- rather dense than thin. Malnutrition is common problem in infants, between age of 6 – 8, 9 – 11, and 12 – 23 months should intake than 200, 300, and 550 kcal per day, respectively **(WHO, 2009; Bronský et al., 2014)**. Home complementary food can hold lower amount of energy than processed food, with unstable content of minerals and vitamins. Infants should be fed by some fortified food. Fortified food-based products meant to be added to other foods or eaten alone to improve macronutrient, micronutrient, and vitamin intake **(Thurnham, 2013)**. On the other hand, if commercially prepared foods are used to increase micronutrient intake, their packaging instructions should clearly show purpose of feeding (age, increasing of micro-, macronutrient, etc.).

In the Czech Republic and Germany, complementary feeding usually starts with a single vegetable mash, (mostly carrot), or single fruit puree. Especially, fresh vegetables and fruits offer a high potential of taste and flavor variety and therefore the opportunity to get used to the taste of vegetable and fruits early in life depending on the season. On the other hand, commercial complementary food products provide a broader range of taste, flavors, and texture **(Foterek, Hilbig and Alexy, 2015; Bronský et al. 2014; MZČR, 2015)**.

In the past, baby foods were carefully prepared at homes. However, modern lifestyles have led to the commercialization of ready-made complementary food. Currently commercial baby jars have become an important part of baby food due changes in lifestyle. People do not have enough time for homemade alternatives of baby food

and they also tend to increase consumption of ready-cooked foods **(WHO, 2009; Mir-Marqués, 2015)**.

In general, complementary foods are made of fruit, vegetable, and meat from different animals, such as pork **(Nebot et al., 2014)**, chicken, beef, rabbit, calf, turkey, or tuna meat. Complementary food is defined as all semi-solid, pureed or mashed foods. Commercial complementary food is defined as all industrially processed, pre-packed foods in the jars or packets. Homemade complementary food is defined as all home prepared semi-solid, pureed or mashed food **(Foterek, Hilbig and Alexy, 2015)**. Complementary food can be classified in couple of groups, for example according to nutrient content, color, shape, texture, and consistency **(Rodriguez-Oliveros, Bisogni, Frongillo, 2014)**.

In recent years, commercial complementary foods have become an important part of baby dairy food. An increasing number of mothers feed their infants by processed complementary food in jars or plastic pots. The assortment of products offered has grown significantly. The processed complementary food is standardized rather than homemade complementary food, mainly incontent of vitamins, minerals, proteins, lipids, and carbohydrates **(Foterek, Hilbig and Alexy, 2015)**. Despite the benefits of infant complementary food as a major source of food for infants, the presence of contaminants, such as heavy metals may pose health risks to children **(Pandelova et al., 2012)**. Infants are exposed to daily intake of established by PTWI (Provisional Tolerable Weekly Intake) receive in complementary food. In addition, the amount of contaminants as lead, mercury and others in the European Union basket of complementary food descended in last decades **(Agostoni, Brunser, 2007; EFSA, 2012a; EFSA, 2012b)**.

Commission Directive 2006/125/EC (as amended) "on processed cereal-based foods and baby foods for infants and young children" gives an account of essential composition for baby foods, for infants and young children like protein, fat, sodium, vitamins, and minerals. Further information about specific maximum residue of pesticides or metabolites of pesticides in processed complementary food is in the annex VI. In scientific field, there are studies about the content of minerals, vitamins, contaminants **(Melø et al., 2008; Carbonell-Barrachina, 2012; Pandelova et al. 2012, Juan et al., 2014; Mir-Marqués 2014)** further researches are focused on e.g. mycotoxins, tetracyclines, acryl-amid etc.

In children diets a major role play basic characteristics of raw material (texture, pigments, etc.) that react with electromagnetic radiation in the visible spectrumand give the response to evaluators **(Figura, Teixeira, 2007)**. In food products, it was found around 10,000 inhalants **(Berger, 1995)**. Functional element which predominates is the main indicator of intra- and intermolecular interactions in the food system and its amount, gives resulting values for appearance, color, aroma, taste, and texture **(McGorrin, 2006; Stępniewski, Grundas 2013)**. On the other hand, if it uses objective measurements, the result may not be the overall color effect, therefore, still uses classical sensory analysis **(Pomerancz, Meloan, 1994)**.

The aim of our study was to measure the color by system CIELAB, sensory analysis and determination of vitamins in children vegetable complementary feeding with the option to extend shelf life of 18 to 21 months.

MATERIAL AND METHODOLOGY

Material

Complementary children feeding were obtained from private factory in Czech Republic. In this research there were used two kinds as carrot and vegetable mix samples.

Composition of samples was:

Carrot: carrot (70%), water, rice flour, citric concentrate. Nutrition per 100 g: energy 180 kJ, 0.3g of fat, carbohydrates 0.1 g, and protein 4.5 g.

Vegetable mix: potato purée (water, spray potato flocs, emulsifier: mono- and diglycerides of fatty acids), water, garden pea, carrot, spinach, leek, vegetable oil, rice flour, citric concentrate. Total vegetable content is 64%. Nutrition per 100 g: energy 287 kJ, 2.2 g of fat, carbohydrates 10.4 g, protein 1.7 g.

Methodology

To determine the color changes by system CIELAB and determination of vitamins, samples were analyzed before filling into jars and sterilization, and then immediately after sterilization. Further analyzes were performed for 21 months every 3 months. Sensory analysis was performed immediately after production – sterilization, and then again 21 months, in a run of every 3 months. The last determining of all parameters were already 3 months after the expiry date of minimum shelf life.

Color

Color of the complementary food samples were determined as reflectance values based on the L*a*b* system (lightness, redness, yellowness) using a spectrophotometer CM-3500d (Konica Minolta, Osaka, Japan) containing an integrated spectral component, a D65 illuminator and a 10° observer. Samples were measured in Petri dish with flat surface at room temperature with SCE. The L*a*b* values were determined in duplicate; the average value from these three determinations was used in the statistical evaluation.

Determination of β-carotene

5 g of homogenous samples were placed to a vial with volume of 40 mL and added 10 mL of metanolic KOH solution (KOH p.a., Merck, Czech Republic; Methanol for HPLC, Merci, Czech Republic), shaked 60 min at 350 RPM in Vortex. Next, adding of 10 mL deionization water and 3 mL of hexan p.a. (Merck, Czech Republic), vial was placed into the shaker for 10 min and 350 RPM. To 5 mL of vial was moved the top surface of hexan take out by micropipette and evaporated in blow of nitrogenous. To vial there was added next 3 mL of hexan and placed to shaker for 10 min. Upper surface was placed out and put to previous 5 mL of hexan, the content of vial was dried by nitrogenous, this steps were used triplicate. Into vial there was added 0.5 mL of methanol and 0.5 mL of dichlormethan (Merck, Czech Republic), solution was mixed in Vortex. Ready samples were place for HPLC analysis. Standard of β-carotene for HPLC was solution of 20 mg β-carotene (Fluka, Czech Republic) in 10 mL of methanol and it was shaken in Vortex and calibration

curve was papered. For HPLC analysis Shimadzu set with column Exlipse XDB-C8, 50 x 4.6 mm, 1.8 µm, 55 °C was used, mobile phase methanol-water, gradient of methanol 0 min 90%, 2 min 90%, 3 min 95%, 6 min 100%, 13 min 100%, 14 min 90%, 15 min 90%, flow of mobile phase 0.8 $mL.min^{-1}$, analysis was resulted in 15 minutes. The volume of samples 20 µL, UV/VIS detector at 450 nm was measured. Results were obtained by programme Chemstation.

Sensory analysis

Sensory evaluation of two samples of complementary food was performed immediately after the production, and for storage with minimum durability of 18 months every 3 months and 3 months after the storage by 10 trained members in sensory laboratory equipped according to ISO 8589. Determination of color saturation, uniformity of color, homogeneity and consistency were evaluated using a continuous unstructurated scale (100 mm) without references. There were evaluated 4 descriptors: color saturation, uniformity of color, consistency and homogeneity.

Statistical evaluation

Panel data were collected by Excel and tested with one-way analysis of variance (ANOVA, Statistica 12) by means of Duncan's test (p <0.05) for multiple comparisons.

RESULTS AND DISCUSSION

When comparing color CIELAB parameter L* (lightness) for two process steps: raw mash and preserved mash; there were significant differences between them p <0.0001, r^2 = 0.9983. L* parameter of carrot mash showed direct decrease of lightness. On the other hand, parameter a* (redness) and b* (yellowness) were saturated in color p <0.0001, r^2 = 0.9995, and p <0.0001, r^2 = 0.9850, respectively. The samples garden mix appeared before heat treatment and after as color stable for human eye. However, the significant statistical differences were calculated for all CIELAB parameters L*a*b* p <0.05 they showed significant differences of processing raw mash compared preserved mash which was filled in to jars. Lightness tendency were decreasing in the parameter L* in both samples; regardless, redness and yellowness parameters a* and b* were more saturated after-processing in both cases.

Majority of vegetable-based complementary food is carrot and mixes of carrot with other vegetable and other products, these similary tendencies are in the Middle Europe such is Germany **(Mesch et al. 2014)**. Studied carrot samples were measured in following storing conditions: room temperature with daylight storing. The samples were observed in raw mash, after preservation and every 3 months to "use before" and furthermore 3 months for last measurement run. The main differences between raw and preserved mash were carried out previously, the advance measurement shows significant differences between storing periods. In most cases, in parameter L* there were calculated statistically significant differences p <0.05. The lightness has changed with advance storing, however, no significant trend was observed. Variability in lightness was observed in raw mash, over L* 52, but after

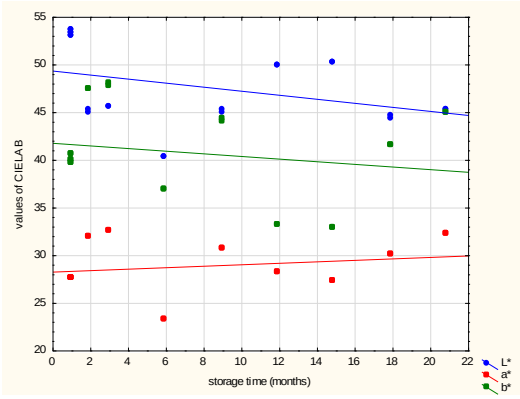

Figure 1 Monitoring changes color co-ordinates L*a*b* in relation to the length of storage in daylight for carrot samples.

heat treatment decreased to L* 44, for instance, high differences were measured during shelf-life (Figure 1).

Significant differences p <0.05 were observed in a* parameter. This parameter was relatively stable because the variation of a* was not as high as in L* parameter. The raw mash was less saturated before preservation, on the other hand saturation of a* parameter was variable in advanced storing. In redness parameter b* there were calculated significant difference in most cases. Regardless, for preservation and 3 months, 12 and 15 months, two pairs of homogenous groups p <0.05 were carried out. For instance, could be obtained non-significant data in food where the shelf-life is only one month **(Kročko et al., 2015)**.

Carrot samples were stored in the dark and in room temperature and the measure runs were the same as carrot storing in the daylight. Significant differences (p <0.05) were calculated for these samples in most cases. However, in this kind of storing there were observed more homogenous groups in parameter L*. Similar tendencies in variability of parameter L* ware observed with advance storing (Figure 2). In comparison of two storing mode were investigated no high differences. The color stability was no stable with advance storing, but for dark storing were calculated more homogenous groups in all parameters L*a*b*.

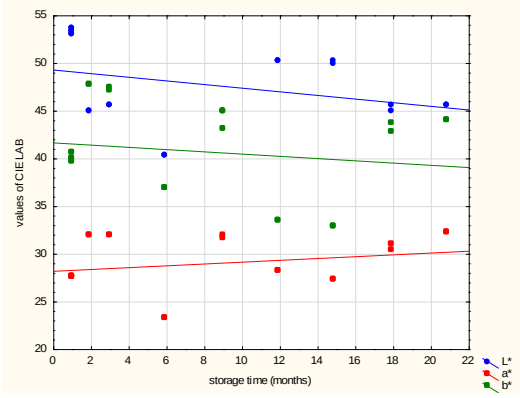

Figure 2 Monitoring changes color co-ordinates L*a*b* in relation to the length of storage in dark for the samples carrot.

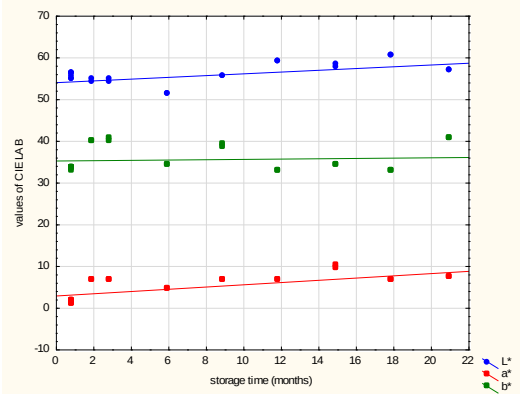

Figure 3 Monitoring changes color co-ordinates L*a*b* in relation to the length of storage in daylight for the samples vegetable mix.

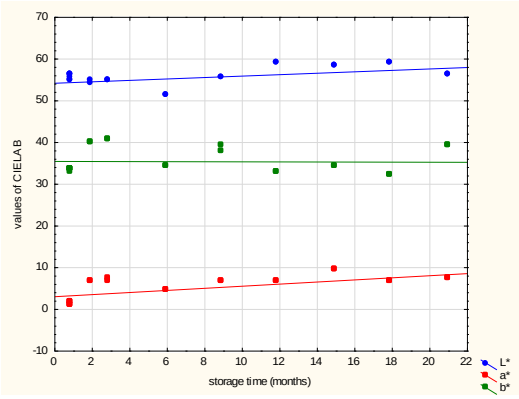

Figure 4 Monitoring changes color co-ordinates L*a*b* in relation to the length of storage in dark for the samples vegetable mix.

Furthermore, samples which called garden mix were investigated in same parameters as previous carrot mash. Lightness for daylight and dark storing had similar behavior, where lightness tendency in both were decreasing till 6 months and for the rest of runs were increasing (Figure 3 and Figure 4). On the other hand, for statistical analysis were calculated in most cases paramount significance differences p <0.05, except for preservation and 3th month and for raw and nine months for both types of storing. For instance, in dark were calculated next homogenous group for twelve and eighteen months.

For parameter a* (daylight storage) were calculated the most homogenous groups p <0.05 for preservation, 3, 9, 12, and 18 months. On the other hand, this trend was not investigated for dark storage, where were two main groups preserved, 9, and 21 months and 9, 21, and 3 months, respectively.

B-carotene

β-carotene such as provitamin A is in food of plant origin stable substance in the absence of air. During food preservation, it can isomerize to neocarotenes (still counting to vitamin A) which are less intensely colored **(Velíšek, 2014)**. It was not corresponded with our obtained data for carrot, because the color on redness axis a* was higher after preservation than before; and contain of β-carotene decrease from 0.186 to 0.015 mg.100g^{-1}, and the redness increase from L* 27 to 31, respectively. Storing time had significant influence to contain of β-carotenes, the mean content during 21 months was 0.862 mg.100g^{-1}, in the case of light storing there were calculated statistical differences p <0.05, r^2 = 0,2300. Samples of vegetable mix mean contains 0.135 mg.100g^{-1} of provitamin β-carotene and the statistically significant differences were calculated in light storing p <0.05, r^2 = 0.2300; on the other hand, no significant differences were obtained in dark storing p >0.05, r^2 = 0.1097. The obtain results shows nonsignificant connection between contain of β-carotene to redness parameter a* p <0.05.

Sensory evaluation

After assessing the results of sensory analysis, it was found that the length and type of storage affect the color changes of children complementary food and is perceptible to the human senses.

Carrot: When the sensory analysis of samples carrot compared to the daylight-dark were in the first 9thmonths of storage found statistically significant differences between the samples. In the ninth month of storage (p <0.05), as well as in 15th (p <0.01), there was a change in the descriptor consistency.At 18th months, again there were no statistically significant differences between the samples stored in daylight and in darkness. In the last month of monitoring, three months after the expiry date of minimum durability, changes have occurred only in the descriptor uniformity of color (p <0.05). From the results we can conclude that storage of the product carrot is only minimally affected when exposed to daylight.

The relative proportions of the samples were stored in daylight for 21st months (Figure 5). At color saturation occurred two homogeneous groups were created. The biggest differences that separated the groups were between 12th and 15th months of storage. The inferior results were recorded in the 21st months of storage, so three months after the expiry date of minimum durability. This change was highly significant (p <0.0001, r^2 = 0.3978). At descriptor uniformity of color, occurred to separation of homogeneous groups between 12th and 15th months of storage. Again, a statistically significant difference between all groups was evaluated with the last assessment in the 21st months (p <0.0001, r^2 = 0.3892). For descriptors consistency and homogeneity, the results were very inconsistent and have failed to form strictly according to

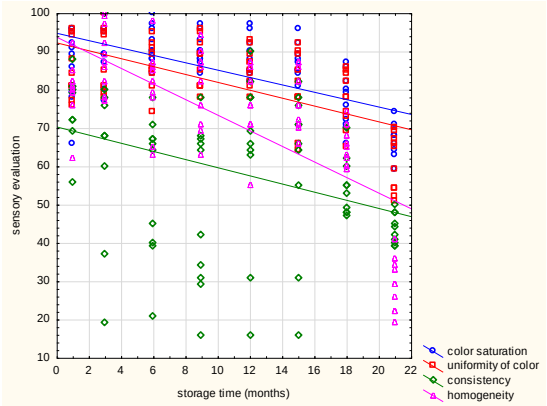

Figure 5 Dependence changes observed in sensory quality descriptors on the duration of storage of complementary food carrot for children in daylight.

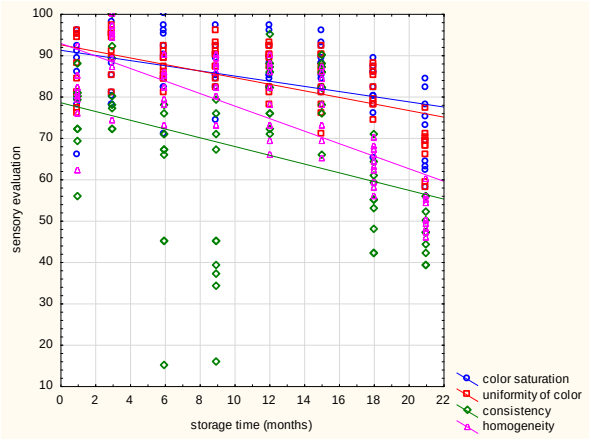

Figure 6 Dependence changes observed in sensory quality descriptors on the duration of storage of complementary food carrot for children in dark.

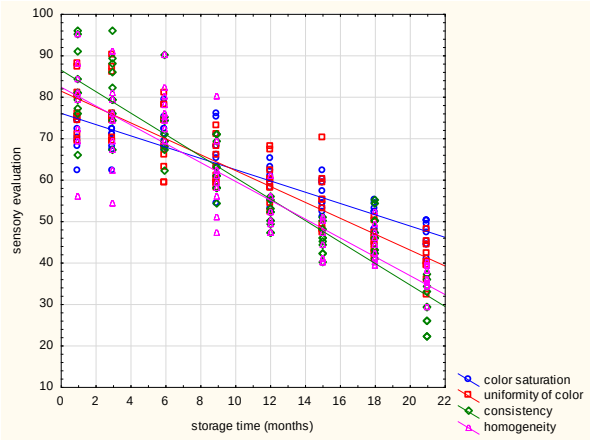

Figure 7 Dependence changes observed in sensory quality descriptors on the duration of storage of complementary food vegetable mix for children in daylight.

the length of storage. Even when descriptor consistency (p <0.001, r^2 = 0.1316) and a descriptor of homogeneity (p <0.0001, r^2 = 0.5027) showed a statistically significant difference, it can not be said that it was quite adequate to the storage time.

For samples stored in the dark (Figure 6), it was found that the color saturation created two homogeneous groups, from1st to 15th, respectively 18th month, with a fluctuation in the sixth month. Evaluation of the 21st month was quite different from the others (p <0.0001, r^2 = 0.1989). The same tendency was observed also in the descriptor uniformity of color (p <0.0001, r^2 = 0.3657), consistency (p <0.001, r^2 = 0.1393), and homogeneity (p <0.0001, r^2 = 0.5387). In the last mentioned descriptor a significant improvement occurred in the 3rd month, not only when stored in the dark, but also in the light.

Vegetable mix: By comparing storage at daylight-dark there was found no influence on color change after 3 months. Changes occurred at the rising trend in following months. After 6 months of storage there were changes (p <0.05) in the descriptor uniformity of color and after 9 months there were noticeable changes in color saturation (p <0.05) and uniformity of color (p <0.01). After 12 months of storage, there were found statistically significant differences between samples (p <0.01) for all measured descriptors –color saturation, uniformity of color, consistency, and. Fifteen months of storage significantly influenced the evaluation. There were found statistically significant differences between light and dark with descriptors color saturation (p <0.01), the uniformity of color (p <0.001) and homogeneity (p <0.001). When assessing influence of storage at samples after 18 and 21 months, there were statistically significant differences at all investigated descriptors (p <0.05). For descriptors of color uniformity in the 18th month and color saturation descriptors, consistency and homogeneity was detected very high, with statistical significance difference (p <0.001) between the samples stored in the light and in the dark.

In the samples storage by 21 months in daylight were found relatively significant differences (p <0.0001). There was a significant decline in sensory quality, particularly towards the end of the storage period (Figure 7). At descriptor color saturation (r^2 = 0.7872), there were created two homogeneous groups, one to 6 months of storage, and the second one from 9th to 21th months of storage. The most significant changes in sensory quality of descriptor for color saturation were between 6th to 9thmonths of storage. Further significant degradation of color saturation occurred between 18th and 21thmonths of storage. At descriptor uniformity of color there were established two homogenous groups, one from 1st to 15th months storage; and the second one from18th to 21stmonths. The most important changes were at 6th months and then between 15th and 18th months (r^2 = 0.8213). For consistency there was found the largest difference between 9th and 12th months, leading to their deterioration (r^2 = 0.8603). Significant changes also occurred in the descriptor of homogenity (r^2 = 0.7732). Two separate homogeneous groups were obtained and separated by 6thto 9th months. The biggest changes were occurred between 9th and 12th months of storage. At two last mentioned descriptors the samples had only low sensory evaluation after 21 months of storage.

Samples storage in the dark there were found significant differences (p <0.0001) between months again (Figure 8). Differences during storage time were not so significant such as during storage in the daylight. At descriptor color

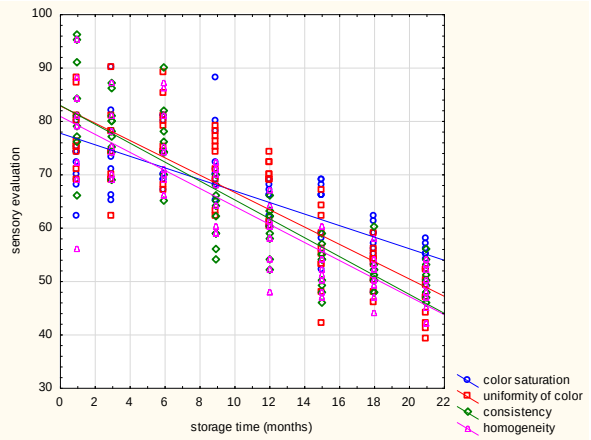

Figure 8 Dependence changes observed in sensory quality descriptors on the duration of storage of complementary food vegetable mix for children in dark.

saturation there were found two homogeneous groups, and the most significant changes occurred between 12th and 15th months of storage (r^2 = 0.5974). In another reference descriptor uniformity of color, the most significant changes were between 9th and 12th months and 12th and 15th months of storage. The 21st month exceeded by its value to other phases of evaluation (r^2 = 0.7179). Descriptor consistency recorded the most significant deterioration in sensory quality between the 12th and 15th month (r^2 = 0.7915). However, very important sensory quality deterioration was also in the 9th month. The same evaluation is also evident in the descriptor homogeneity (r^2 = 0.7552).

Generally, the color of complementary food has not so much investigated; some papers were issued **(Palazón et al., 2009)**. The objective measurement of color is not so common but sensory evaluation is wide used. In last decade, the scope of researches is on food safety and product quality such as: content of vitamins, minerals, etc. **(Bosh et al., 2013; Mir-Marqués et al., 2015; Melø et al., 2008; Mesh et al., 2014)**. Sensory quality and changes in the descriptor are affected by materials, processing, and foodstuffs **(Trejo Arayaa et al., 2009; Berger et al., 2008)**.

CONCLUSION

There were found significant differences in all monitored descriptors by sensory evaluation in storage time for samples in daylight and dark in 21 months. The samples were evaluated every 3 months. The paramount statistically differences were carried out in daylight storage and in last third of sensory evaluation in all descriptors from 15th to 21st months. The obtained data shows the same results as a CIELAB. The samples from dark were more stable than daylight storage. On the other hand, the storage time had significant influence to complementary food in both storing conditions. β-carotene was affected by storage time and there was found significant differences. Sensory analysis plays an important role in the selection of food in general, but especially for infant food. From the results of objective measurement, were found that the color and storage conditions (light/dark) statistically varied over time. However, consumer preferred changes obtained by sensory analysis to select and purchase food before changes detected by measuring CIELAB.

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Contact address:

Vladimír Sýkora, Mendel Universtiy in Brno, Czech Republic, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, E-mail: vladimir.sykora@mendelu.cz.

Hana Šulcerová, Mendel Universtiy in Brno, Czech Republic, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, E-mail: hana.sulcerova@mendelu.cz.

Michal Mihok, Mikrofarma, s.r.o., Udolni 567/33, 602 00 Brno, Czech Republic, E-mail: michal.mihok@mikrofarma.cz.

Roman Pytel, Mendel Universtiy in Brno, Czech Republic, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, E-mail: roman.pytel@mendelu.cz.

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THE EFFECT OF REDUCED ZINC LEVELS ON PERFORMANCE PARAMETERS OF BROILER CHICKENS

Hana Štenclová, Filip Karásek, Ondrej Šťastník, Ladislav Zeman, Eva Mrkvicová, Leoš Pavlata

ABSTRACT

The experiment was conducted to determine the effect of reduced supplemental zinc levels on broiler growth and carcass yield. A total of 160 male broiler chicks (Ross 308) divided into four groups were allotted to 16 cages with 10 birds per cage in each of group and kept in a temperature-controlled room. During the trial, chicks were ad libitum access to feed and water. The experiment started at 11 days of broiler age and chicks were fattened up to 35 days of age. It consisted of 4 dietary treatments with 4 replications per treatment. A corn-wheat-soybean meal basal diet containing 25.84 mg $Zn.kg^{-1}$ was formulated and zinc levels of 120, 40 or 20 $mg.kg^{-1}$ was supplied as zinc oxide to give four dietary treatments. At the end of the feeding trial, 24 birds from each group were randomly selected, slaughtered and carcass evaluation was performed. The results show that different levels of zinc had no significant effect on body weight of broilers or feed consumption ratio. These parameters increased by decreasing zinc levels from 120 to 20 mg $Zn.kg^{-1}$ similarly as the carcass yield, percentages of breast meat and leg meat, but differences between these groups were not significant. In case of relative liver weight and zinc concentration in liver there were significant difference (p <0.05) between group given supplemented zinc of 40 $mg.kg^{-1}$ and group without zinc supplementation and 120 $mg.kg^{-1}$ and 40 $mg.kg^{-1}$, respectively. No signs of disorders such as loss of appetite, growth depression or abnormalities of the skin was appeared in chicks. It seems that reduced supplemented zinc levels from 120 to 20 $mg.kg^{-1}$ (total Zn 153.13 $mg.kg^{-1}$ to 45.28 $mg.kg^{-1}$ respectively) not influenced growth performance parameters of broilers fed corn-wheat-soybean meal diet.

Keywords: broiler; zinc level; zinc oxide; carcass yield; liver

INTRODUCTION

Zinc (Zn) is an essential trace mineral, cofactor of more than 200 enzymes **(Nair and Choudhury, 2013)** involved in protein synthesis, carbohydrate metabolism and many other biochemical reactions, affects all cellular functions, especially growth and development of organism **(Ao et al., 2011)**. In all species, zinc is necessary for growth, immune system and disease resistance. Deprivation of zinc is characterized by loss of appetite, growth depression, abnormalities of the skin or outgrowths (hair, wool, feathers, hoof, horn) and reproductive disorders **(Suttle, 2010)**. Deficiency of zinc in chicks can cause decreased growth, frizzled feathers, shortened and thickened legs or enlarged hocks **(Nielsen, 2012)**.

The National Research Council (1994) estimates the dietary zinc requirement for broilers as 40 $mg.kg^{-1}$. The requirement of nutrients is usually defined as the minimum dietary concentration required for animal performance. A diet much higher in zinc (60 – 100 $mg.kg^{-1}$) apparently is needed to prevent disorders such as frizzled feathers in poultry **(Underwood and Suttle, 1999)**.

Feeds are routinely supplemented with zinc, because feed materials are either too low in zinc or availability of zinc is inadequate to cover the requirements. Zinc is added to the diets in inorganic sources (usually zinc oxide, zinc sulphate, zinc chloride) or in organic forms complexed to amino acids, proteins, or carbohydrates. The nutritional value of mineral sources depends on the composition of the diet, concentration in the feed, interactions with other mineral elements, and the bioavailability of the element to the chicks **(Star et al., 2012)**. The most commonly used sources of zinc are the oxide (ZnO). Fear of zinc deprivation causes exceed NRC (1994) recommendation, but if the requirement is markedly exceeded, additional zinc is not absorbed or endogenously secreted, but passes the gut and ends up in the manure **(EFSA, 2014)**. Manure from broilers fed high zinc levels spread on fields may enrich soil and drainage water with zinc and zinc contamination can affect quantity and quality of humus and lead to reduced crop yields.

The potential problem of high zinc in manure leaded to a recommendation by the Scientific Committee for Animal Nutrition (SCAN) to reduce zinc levels in feeds, followed by Regulation (EC) No 1334/2003 to decrease the maximum total zinc contents in complete feed for all animals. Maximum authorised total zinc content for poultry is 150 $mg.kg^{-1}$ complete feed. In 2014, EFSA posted a study „Scientific Opinion on the potential reduction of the currently authorised maximum zinc content in complete feed“ and the FEEDAP Panel proposed new maximum content of total zinc in complete feed for poultry (except turkeys for fattening) at the level of 100 mg $Zn.kg^{-1}$. EFSA expected that the reduction of maximum zinc contents in complete feed (from 150 to

100 mg Zn.kg^{-1} for broiler chickens) ensure health, welfare and productivity of food-producing animals as well as reduction of zinc emissions from animal production of about 20% in case of the application in feeding practices without affect consumer safety. The reduction of currently authorised maximum total zinc content in feeds would decrease the zinc load in the environment, but it is necessary to check the effect of reduced zinc levels on animal health and performance.

MATERIAL AND METHODOLOGY

Experimental birds, diets and treatments

A total of 160 7-d-old broiler chicks (Ross 308) were allotted to 16 balance cages with 10 birds per cage. The chicks had free access to feed and water throughout feeding trial. The lighting regime was 18 hours light and 6 hours dark. Birds were marked by wing tags and housed in a room that had a temperature set according to Management Handbook for broilers Ross 308. Temperature and relative humidity was recorded every day.

The experiment started at 11 days of broiler age and chicks were fattened up to 35 days of age. It consisted of 4 dietary treatments with 4 replications per treatment. As shown in Table 1, the basal diet was formulated to meet or exceed NRC (1994) nutritional requirements except zinc, with using Zn-low mineral premix containing minimum amount of zinc, so basal diet contained 25.84 mg Zn.kg^{-1} and it was added 120 mg of zinc.kg^{-1} (Zn 120) to achieve overall 153 mg Zn.kg^{-1}, (150 mg Zn.kg^{-1} is currently the maximum authorised total zinc contents for poultry). In other groups was added 40 (Zn 40) and 20 (Zn 20) mg of zinc.kg^{-1} and one group was without zinc supplementation. The source of added zinc was zinc oxide (ZnO). Total content of zinc in the diets were analysed (Table 2).

Feed consumption was noticed every day. Body weight of each chicks was measured on the digital scales at the start of experiment (11 d of age), then twice a week in the morning before feeding and at the final day (35 d of age) before slaughter.

Table 1 Composition of the basal diet fed from d 11 of age to 35 d of age.

Ingredients	%
Maize	34
Wheat	31.5
Soybean meal	26
Sunflower oil	4
Vitamin-mineral premix [1]	2
Experimental Zn-premix[2]	2
Chromium oxide	0.5
Nutrient composition	
ME_N (MJ.kg^{-1})	12.69
Crude protein	20.66
Ether extract	5.89
Crude fibre	3.14
Ash	5.53
Lysine	1.19
Methionine	0.58
Calcium	0.97
Non-phytate P	0.30
Zinc (mg)	25.84

[1]Supplied per kilogram of premix: lysine 101.65 g, methionine 135.63 g, threonine 51.22 g, calcium 200 g, phosphorus 98.19 g, natrium 62.89 g, sulphur 0.39 g, chlorine 119.69 g, copper 752.5 mg, iron 3768.6 mg, zinc 44.73 mg, manganese 6046.07 mg, cobalt 11 mg, iodine 47.95 mg, selenium 8.96 mg, vitamin A 680000 IU, vitamin D 250000 IU, vitamin E 2250 mg, K_3 74.8 mg, B_1 206.44 mg, B_2 344 mg, B_6 300.44 mg, B_{12} 1999.2 mg, biotin 11 mg, niacinamid 1793.4 mg, calcium pantothenate 676.2mg, folic acid 82.8 mg, cholinechlorid 9000 mg.

[2]Content different levels of Zn according to the dietary treatments.

Table 2 Dietary treatments.

Group	Supplement level of zinc (mg.kg^{-1})	Total content of zinc in the diet (mg.kg^{-1})
Zn 120	120	153.13
Zn 40	40	71.96
Zn 20	20	45.28
non-supplement	0	25.84

Evaluation of carcass quality

At the end of the experiment (35 d of age), 96 broilers (24 birds from each treatment) were selected, weighed and slaughtered by cervical cutting. Carcasses and livers were weighed, breast and leg meat were cut, skinned and percentages of live body weight were calculated.

Statistical analysis

Data has been processed by Microsoft Excel (USA) and STATISTICA.CZ, version 12.0 (CZ). The results were expressed as mean ±standard deviation (SD). It was used one-way analysis (ANOVA). Sheffe´s test was applied to defined statistical differences and differences between groups were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The correct environment and brooding conditions should be managed to meet all nutritional and physiological requirements to support body-weight gain throughout the growing period **(Nevrkla et al., 2015)**. Zinc oxide is commonly used source of zinc added as a supplement to poultry diets. The advantage of inorganic zinc sources is lower price, so inorganic zinc sources are still preferred than organic ones.

The effects of supplemental zinc level on slaughter weight and carcass yield are shown in Table 3.

Against non-supplement diet, slaughter weight was improved by Zn supplement of 20 mg.kg^{-1}, total dietary Zn of 45.28 mg.kg^{-1}. That agree with recommendation by **NRC (1994)** that a total dietary Zn concentration of about 40 mg.kg^{-1} is necessary to achieve normal growth in chicks. However, these parameters decreased by increasing zinc contents to 120 mg.kg^{-1} (total Zn 153.13 mg.kg^{-1}). **Mohanna and Nys (1999, In: Huang et al., 2007)**

Table 3 Effects of supplemental zinc levels on slaughter weight (g) and carcass weight (g).

Group	Slaughter weight	Carcass weight
Zn 120	2052.7 ±275.2	1505.1 ±229.5
Zn 40	2041.0 ±330.3	1499.4 ±253.2
Zn 20	2126.5 ±328.2	1573.8 ±263.2
non-supplement	1972.1 ±270.6	1438.5 ±213.7

No significant differences at a level of p <0.05.

reported that body weight gain increased with the dietary Zn supplementation of 25 mg.kg^{-1} (45 mg.kg^{-1} total dietary Zn), when chicks were fed a diet supplemented with Zn (added as Zn sulfate) at 0, 10 or 25 mg.kg^{-1}.

Jahanian et al., (2008) observed that this parameter was not affected by added zinc during wk 1 to 5, but in contrast with our results, in their trial taken 42 days, daily feed intake and weight gains decreased by decreasing Zn level from 120 to 40 mg.kg^{-1}. On the other hand, weight gain could be influenced by many other factors **(Nevrkla et al., 2014)**. **Haščík et al., (2010)** noted average saughter weight 2086 g at the age of 40 d of broiler cockerels fed commercial feed mixture and carcass weight 1475.20 g. **Liptaiová et al., (2010)** attained an average slaughter weight of unsexed broiler chickens 1651 g at 38 days of age and carcass weight 1124.17 g.

Carcass yield parameters are expressed as a percentages of live body weight measured at the day of slaughter (35 day of age). Breast meat and leg meat was weighed without skin. As shown in Table 4, the best efficiency of carcass (73.9%) and its parts, breast meat (21.3%) and leg meat (19.4%) was found in group Zn 20 and these parameters decreased with increasing Zn levels up to 120 mg Zn.kg^{-1}.

Nevertheless reduced zinc levels had no significant effect on carcass yield. Similar to our opinion, **Jahanian et al., (2008)** referred no influence of dietary zinc supplementation on carcass parameters. In their study, the highest percentage of carcass (68.64%) and breast meat (21.00%) was noticed in broilers given diet with added 80 mg Zn.kg^{-1} and relative carcass weight decreased by 120, followed 40 mg Zn.kg^{-1}, whereas breast yield decreased by 40, followed 120 mg Zn.kg^{-1}.

Our results show that zinc supplementation did not affect carcass yield, but affected relative weight of livers. Relative liver weights (see Table 5) is expressed as a percentage of live body weight measured at the day of slaughter (35 day of age). There was significant difference (p <0.05) between group Zn 40 and chicks fed non-supplemented diet.

Table 4 Effects of varying supplemental zinc levels on carcass yield (% of live body weight).

Group	Carcass	Breast meat	Leg meat
Zn 120	73.2 ±0.4	20.7 ±0.5	19.1 ±0.2
Zn 40	73.4 ±0.3	21.0 ±0.3	19.1 ±0.2
Zn 20	73.9 ±0.4	21.3 ±0.4	19.4 ±0.2
non-supplement	72.8 ±0.3	21.3 ±0.3	19.2 ±0.2

No significant differences at a level of p <0.05.

Table 5 Effect of dietary zinc on livers.

Group	Relative liver weights (% of live body weight)	Zinc concentration (mg) in 1000g of liver
Zn 120	2.13 ±0.19 ^{ab}	24.4 ±2.2 ^{a}
Zn 40	1.96 ±0.26 ^{a}	26.9 ±2.2 ^{b}
Zn 20	1.99 ±0.26 ^{ab}	25.8 ±2.3 ^{ab}
non-supplement	2.19 ±0.31 ^{b}	25.14 ±3.3 ^{ab}

Different letters ^{a,b} in the columns indicate significant differences at a level of p <0.05.

The heaviest livers were assigned to chicks fed on diet supplemented by 120 mg Zn.kg^{-1} (43.69), but relative to live body weight, non-supplemented group was the highest value (2.19), followed group Zn 120 (2.13).

Zinc concentration in liver calculated to 1000 g of liver is shown in Table 5. Dietary Reference Values have been established for zinc as 7 – 11 mg.day^{-1} for adult males and 6 – 9 mg.kg^{-1} for adult females. Tissues and products of animal origin participate in about 40 – 50% of total zinc intake. Based on collected data by **EFSA (2014)**, reduction in dietary zinc from 150 mg Zn.kg^{-1} to requirements do not affect zinc concentration in animal tissues so expect no concern about consumers' safety.

CONCLUSION

In this experiment, reduced zinc levels were evaluated for their effects on the growth performance of broiler chicks from 11 days up to 35 days of their age. Dietary zinc level had no significant effect on body weight or carcass yield. Only relative liver weight and zinc concentration in liver, there were significant differences (p <0.05). No signs of disorders such as loss of appetite, growth depression or abnormalities of the skin was appeared in chicks. It seems that reduced supplemented zinc levels from 120 to 20 mg.kg^{-1} (total Zn 153.13 mg.kg^{-1} to 45.28 mg.kg^{-1} respectively) not influenced growth performance parameters of broilers fed corn-wheat-soybean meal diet and incline to the proposal by EFSA to reduce total maximum zinc content in complete feed for broiler chicken, even so it will be necessary to examine interaction with other minerals before formulating feed with reduced zinc content.

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Contact address:

Hana Štenclová, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 61300 Brno, Czech Republic,
E-mail: xstenclo@mendelu.cz.

Filip Karásek, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 61300 Brno, Czech Republic, E-mail: filip.karasek@mendelu.cz.

Ondřej Šťastník, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 61300 Brno, Czech Republic, E-mail: ondrej.stastnik@mendelu.cz.

Ladislav Zeman, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 61300 Brno, Czech Republic, E-mail: ladislav.zeman@mendelu.cz.

Eva Mrkvicová, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 61300 Brno, Czech Republic, E-mail: eva.mrkvicova@mendelu.cz.

Leoš Pavlata, Mendel University in Brno, Faculty of Agronomy, Department of Animal Nutrition and Forage Production, Zemědělská 1, 61300 Brno, Czech Republic, E-mail: leos.pavlata@mendelu.cz.

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QUALITY ASSESSMENT OF JUICE PREPARED FROM DIFFERENT VARIETIES OF CURRANT (*Ribes* L.)

Andrea Mendelová, Ľubomír Mendel, Tomáš Krajčovič, Peter Czako, Ján Mareček, Helena Frančáková

ABSTRACT

In the Slovak Republic currants are traditionally grown species of small fruits mainly in house gardens. Although currently their area is very small compared to the other types of fruit. We can see the importance of growing this genus (*Ribes* L.) in its good adaptability to climate conditions, in small growing demands and in stable production of nutritionally highly valuable fruit. Currant berries as well as fresh currant juice are characterized by the presence of whole complex of antioxidant active substances. The aim of this study was to evaluate the nutritional quality of currant juice prepared from various species and varieties of genus *Ribes* (L.). based on the content of their total polyphenols, anthocyanin dyes and antioxidant activity. In work we used varieties Blanka, Primus, Viktória, Heinemannova neskorá, Red Lake, Treny, Jonkheer van Tets, Fertödi, Titania, Triton and Öjebyn. Contents of evaluated components were assessed spectrophotometrically. Total polyphenol content of monitored samples determined by the Folin-Ciocalteu method reached values from 1897.43 mg GAE.dm^{-3} DM to 3712.21 mg GAE.dm^{-3} DM. The highest one was in juice from variety Primus and the lowest from variety Blanka. In white varieties of currant, the presence of anthocyanin dyes was immeasurable. In varieties of red and black currant anthocyanin dye content achieved values from 1947.64 mg.dm^{-3} DM (Jonkheer van Tets) to 4161.07 mg.dm^{-3} DM (Heinemannova neskorá). The antioxidant activity determined by the FOMO method reached values from 4130.42 mg AA.dm^{-3} DM to 6571.69 mg AA.dm^{-3} DM. We recorded the highest antioxidant activity in juice of variety Fertödi and the lowest of variety Primus.

Keywords: currant; juice; anthocyanin; total polyphenols; antioxidant activity

INTRODUCTION

Members of the genus *Ribes* L. are mostly bushes naturally occurring in wild or cultivated in gardens and orchards in different mild climate areas of the world. Fruits are a rich source of vitamin C and other health promoting substances such as organic acids, pectin, micronutrients and trace elements **(Mattila et al., 2011)**. Berry fruit is an important source of various biologically active compounds with interesting physiological effects. **Puuppone et al., (2015)** state that small fruit is rich in fiber, vitamins, minerals, anthocyanins, and especially in various phenolic compounds. **Szajdek and Borowska (2008)** present in their work that main representatives of biologically active compounds of berry fruit are particularly vitamin C and polyphenols, such as anthocyanins, phenolic acids, flavanols, flavonols and tannins. **Battino et al., (2009)** state that berry fruit is characterized by a high content and wide variety of phenolic compounds, which differ in structure and molecular weight. The phenolic compounds present in currants are benzoic acid and cinnamic acid derivatives, tannins, stilbenes and flavonoids such as anthocyanins, flavonols and flavanols catechins. Their concentration is usually higher in the skin and just beneath it, than in the central part of the fruit. **Pinto et al., (2007)** found that most represented phenolic acids in berry fruit are cinnamic and benzoic acid derivatives, which predominantly occur in the form of esters and glycosides. To the benzoic acid derivatives present in currants belong p-hydroxybenzoic acid, salicylic acid, gallic acid and ellagic acid. From the cinnamic acid derivatives, the authors confirmed the presence of coumaric acid, caffeic acid and ferulic acid in currants. **Szajdek and Borowska (2008)** state that in the black currant a high p-coumaric and caffeic acid content was detected.

Goleniowski et al., (2013) report positive effect of phenolic compounds in the prevention of many civilization diseases such as coronary heart disease, stroke and cancer. Anthocyanins belong to the most important flavonoids occurring in black and red currant. Anthocyanins in currants are in the form of mono-, di- or triglycosides, wherein the glycoside residues are typically substituted at C3, or less frequently at C5 or C7 position. The most predominant sugars in anthocyanin molecule are glucose, galactose, rhamnose, arabinose and rutinose. Anthocyanin glycoside residues are often acylated by acids, mostly by p-coumaric, caffeic or ferulic acid, and less often by p-hydroxybenzoic, malonic or acetic acid **(Sójka, Król, 2009)**. Anthocyanins have many biologically significant features and the main attention is paid to their antioxidant activity. It is well known that they play an important role in the prevention of degenerative neuronal disorders,

cardiovascular diseases, cancer and diabetes **(Lee et al., 2013)**.

From the stilbene group **Szajdek and Borowska (2008)** pointed to the presence of trans-resveratrol in red currant berries. Trans-resveratrol is a phenolic compound produced by plants in response to stress conditions, e.g. climate variability, exposure to ozone, sun radiation or heavy metals presence in soil. The pharmaceutical effects of trans-resveratrol include antioxidant and anti-inflammatory activity, as well as inhibition of LDL cholesterol oxidation, platelet aggregation and growth of various tumor cells **(Fei, 2015)**.

The chemical composition of berry fruit is highly variable depending on the variety, site of cultivation, on ripening, harvesting and storage conditions **(Talcott, 2007)**. **Battino et al., (2009)** state that the content of phenolic compounds in the berry fruits is determined by a number of factors such as species, cultivation method, region, weather conditions, maturity, harvesting, storage time and conditions. The authors further submit that fruits that grow in the cold northern climate with a short growing season, without fertilizers and pesticides, has higher polyphenol content than the same varieties grown in milder climate.

The aim of the work was to compare the quality of different types and varieties of currant processed to currant juice. We focused mainly on the determination of total polyphenols, antioxidant activity and anthocyanin dyes.

MATERIAL AND METHODOLOGY

In the work we evaluated 11 varieties of white, red and black currants. From the white group were varieties Blanka, Primus and Viktória, from the red group Heinemannova neskorá, Red Lake, Treny and Jonkheer van Tets and from the black group Fertödi, Titania, Triton and Öjebyn. The fruits were grown in Botanical garden of Slovak University of Agriculture in Nitra and collected at the stage of consumer maturity. Growing area, according to agro-climatic characteristics is included into very hot region and very dry sub-region with an average annual temperature of 9.5 °C and average annual rainfall of 584.5 mm. According to the soil characteristics, it is a heavy Gleyic Fluvisol formed on alluvial uncalcareous and calcareous sediments. In order to obtain the juice from currants, we used the screw press machine. There were used the whole currant bunches and during the pressing, peelings and seeds were removed.

Total polyphenol content was analyzed by the Folin-Ciocalteu method, whose principle is the reaction of Folin-Ciocalteu reagent with reducing substances to form a blue complex. The blue coloring intensity is proportional to the polyphenol content. We performed the evaluation with a spectrophotometer UV-VIS Jenway, at a wavelength of 700 nm and the content of total polyphenols is expressed as equivalent of gallic acid in mg GAE. dm^{-3} **(Singleton and Rossi, 1965)**.

Anthocyanin dye content was determined by spectrophotometry. The samples were extracted in ethanol with addition of 0.01% HCl. Repeated dye extraction until complete sample decolorization was carried out by heat. Anthocyanin dye content was investigated by measuring the absorbance on spectrophotometer UV-VIS Jenway at the wavelength selected by the dominant anthocyanin present in a given kind of fruit.

Antioxidant activity was determined by the FOMO method **(Prieto et al., 1999).** The principle of method is the reduction of Mo (VI) to Mo (V) by the action of reducing substances in the phosphorus presence. Coloring intensity of the resulting green phosphomolybdate complex is measured spectrophotometrically at a wavelength of 695 nm. Reducing ability of the compounds is expressed as the equivalent amount of ascorbic acid (AA), which is required to achieve the same reduction effect.

Results of analyzes were processed by statistical package Statistica 8.0 (StatSoft Inc., Tulsa, USA). Differences between the samples were monitored by Fisher's LSD test.

RESULTS AND DISCUSSION

High quality of black and red currant berries was for a long time evaluated only on the basis of the sugar, organic acids and vitamin C content. We know that the high nutritional quality of berries corresponds to the wide complex of compounds, often referred to as a phenol compounds, and these, together with vitamins, dyes and minerals, take part in forming the fruit antioxidant activity **(Nour et al., 2011)**.

In our work, we mainly focused on determination of total polyphenol content in samples of currant juices. We found out that the highest polyphenol content was in juice of red currant variety Titania with value 694.65 mg $GAE.dm^{-3}$ and the lowest content in juice of white currant variety Blanka with value 178.17 mg $GAE.dm^{-3}$. Total polyphenol content in currant juice samples was decreasing in the order Titania >Triton >Fertödi >Öjebyn >Primus >Treny >RedLake >Viktória >Jonkheer van Tets >Heinemannova neskorá >Blanka. After the total polyphenol content conversion to dry matter, the highest content showed juice of variety Primus (3712.21 mg $GAE.dm^{-3}$ DM) and the lowest of variety Blanka (1897.43 mg $GAE.dm^{-3}$ DM) (Table 1).

Table 1 Varietal differences in the total polyphenol content of currant juices.

Variety	mg $GAE.dm^{-3}$	mg $GAE.dm^{-3}$ DM
Blanka	178.17	1897.43^{a}
Heinemannova neskorá	189.03	1903.63^{a}
Viktória	225.360	1946.11^{b}
Jonkheer van Tets	220.12	2349.16^{c}
Red Lake	262.44	2688.92^{d}
Öjebyn	466.59	2903.29^{e}
Treny	282.66	3092.59^{f}
Triton	558.32	3184.93^{g}
Titania	694.65	3656.04^{h}
Fertödi	508.13	3658.25^{h}
Primus	390.15	3712.21^{i}

Note: $^{a-i}$ Means with the same letter are not significantly different from each other (Fisher's LSD test, $p > 0.05$); DM – dry matter.

By Fisher's LSD test we observed mutual differences between the currant juice samples in total polyphenol

content. Statistically significant (p <0.01) highest polyphenol content was detected in the juice of white currant variety Primus, which was followed by the black currant juice varieties of Fertödi, Triton and Titania. There was not detected any statistically significant difference (p >0.05) between the variety of Fertödi and Triton. From juices made from red currant, the highest polyphenol content was found in the variety Treny. The lowest polyphenol content from the black currant group was found in the juice from variety Öjebyn. Middle polyphenol content was found in the juice sample from red currant varieties Red Lake and Jonkheer van Tets and in the juice from white currant variety Victoria. The lowest polyphenol content was in the juice samples from red variety Heinemannova neskorá and in the juice of white variety Blanka.

Total polyphenol content in selected varieties of raspberries, blackberries and currants grown in Hungary was observed by **Dénes et al., (2011)**. The highest content of polyphenols with an average value of 533 mg.100g^{-1} recorded authors in the black currant and blackberries with value 379 mg.100g^{-1}. Average content of polyphenols detected in white currant varieties was 333 mg.100g^{-1} and 192 mg.100g^{-1} in red ones. Results of the above-mentioned authors also correspond with our findings. **Sójka a Król (2009)** used Folin-Ciocalteu method to determine total polyphenol content in the black currant marcs and reached values ranged from 2189.6 to 2285.6 mg.100g^{-1}. From that we can conclude that solid fruit components have higher content of polyphenolic substances than currant juice.

Nótin et al., (2011) investigated, what is the effect of drying temperature on the content of currant polyphenols. They used a black currant variety Titania. The samples were dried in a vacuum at 40, 50 and 60 °C, until moisture content below 10%. By exploring was found that the smallest changes in the polyphenol content were obtained by drying at 50 °C. Larger losses were observed when the black currants were dried at a temperature above 60 °C or below 40 °C, but for a longer time.

The aim of work of **Mitić et al., (2011)** was to assess the quality of dried red currants of Random variety grown in different regions of Serbia. The chemical composition can be highly variable depending on the growing region, what was also confirmed by this study. The authors found that the polyphenol content of red currants from the Beograd region with values from 3.96 to 12.68 mg GAE.g^{-1} was higher than in the currants from the Niska Banja region with values from 3.47 to 7.46 mg GAE.g^{-1}.

Anthocyanin dyes are responsible for a wide range of red to violet fruits and vegetables coloration. The obtained results have confirmed this statement too. In the juices from white currant varieties Blanka, Primus and Viktória was found an undetectable presence of anthocyanin dyes (Table 2). On the basis of detected values we can state that the highest content of anthocyanin dyes was in juices from black currant varieties and specifically in juice of variety Titania with value 599.13 mg.dm^{-3}. The lowest levels of anthocyanin dyes were found in the juice of red currant variety Jonkheer van Tets with value 182.49 mg.dm^{-3}. Samples of currant juices can be ordered by the decreasing anthocyanin dye levels in fresh mass as follows Titania >Triton >Öjebyn >Fertödi >Heinemannova neskorá >Red Lake >Treny >Jonkheer van Tets. After the conversion of anthocyanin content to dry matter was their highest content detected in juice of variety Heinemannova neskorá (4161.07 mg.dm^{-3} DM) and the lowest in juice of variety Jonkheer van Tets (1947.64 mg.dm^{-3} DM) (Table 2).

Table 2 Varietal differences in the anthocyanin dye content of currant juices.

Variety	mg.dm^{-3}	mg.dm^{-3} DM
Jonkheer van Tets	182.49	1947.64^{a}
Red Lake	244.47	2504.85^{a}
Öjebyn	444.18	2764.06^{bc}
Fertödi	423.52	3049.13^{cd}
Triton	537.15	3064.19^{cd}
Titania	599.13	3153.33^{d}
Treny	303.00	3315.20^{d}
Heinemannova neskorá	413.19	4161.07^{e}

Note: $^{a-e}$ Means with the same letter are not significantly different from each other (Fisher's LSD test, p >0.05); DM - dry matter.

Samples were mutually compared using Fisher's test. The highest content of dyes was found in juice of red variety Heinemannova neskorá, which statistically significantly differed (p <0.01) from the other currant juice samples. The second highest content was detected in juice of red currant variety Treny, which statistically significantly did not differ in anthocyanin content from juice sample prepared from black currant variety Titania. Black currant samples created 3 consecutive homogeneous groups from *d* to *bc*. The highest content was found in juice of black currant variety Titania, which statistically significantly did not differ from varieties Triton and Fertödi, but differed from Öjebyn sample, where we found the lowest content of anthocyanins. Juices from varieties Triton, Fertödi and Öjebyn statistically significantly did not differ in the anthocyanin content among themselves. We found the lowest dye content in juices of red currant varieties Jonkheer van Tets and Red Lake.

Koponen et al., (2008) investigated the content of anthocyanins in black currants and found out that their content in unprocessed currants is higher than the content after their processing. The total anthocyanin concentration in unprocessed currants was 3170 mg.kg^{-1} and after processing into currant juice concentration decreased to 2790 mg.kg^{-1}. Enzymatic modification of currant juice led to an increase in total anthocyanin concentration to values from 2870 to 3330 mg.kg^{-1}, which are similar to those in unprocessed currants.

Dénes et al., (2011) monitored the concentration of anthocyanins in selected varieties of currant, blackberries and raspberries grown in Hungary. The highest anthocyanin values were found in black currants at the level of 3540 mg.kg^{-1} DM, which is the value very similar to our results. Anthocyanin content at the level of 1450 mg.kg^{-1} DM found authors in blackberries and at level 4190 mg.kg^{-1} DM in a red currant variety, what is similar to our sample Heinemannova neskorá.

Mikkelsen and Poll (2002) state in their work that in the production process of black currant juice was maintained about 75% of anthocyanin content.

Rubinskien et al., (2005) were determining the anthocyanin content in the 9 varieties of black currant grown in Lithuania. The highest content of anthocyanins was recorded in juice produced from Kupoliniai variety with obtained value of 195.6 mg.L^{-1} and the lowest content was in Ben Lomond variety with a value of 119.9 mg.L^{-1}.

Määttä et al., (2001) investigated the content of anthocyanins in black (Öjebyn), red (Red Dutch) and white (White Dutch) currant varieties. Black currant had very high anthocyanin content up to 3011 mg.kg^{-1}, while the red currant variety only 1770 mg.kg^{-1}. The presence of anthocyanins was not confirmed in white currants. As mentioned earlier, anthocyanins are responsible for the typical black and red pigments of relevant currants, whereas white currants lack their presence.

Koponen et al., (2008) observed the effect of pectolytic enzymes addition on the content of anthocyanins in juice from blueberries and black currants. The authors discovered that by the use of pectolytic preparations, the anthocyanin content increased up to 83% in blueberry juice and to 58% in black currant juice compared to the control containing no enzymatic preparation.

Currants belong to the fruits with highly positive health effects, also due to the whole complex of substances with antioxidant effects.

Based on the obtained results, we can say that the highest antioxidant activity from our samples reached the juice of black currant variety Titania (1167.61 mg AA.dm^{-3}) and on the contrary, the lowest reached white currant variety Blanka (417.10 mg AA.dm^{-3}). On the basis of a decreasing antioxidant activity, the monitored juices can be ranked as follows Titania >Fertödi >Öjebyn >Triton >Jonkheer van Tets >Heinemannova neskorá >Red Lake >Viktória >Treny >Primus >Blanka. By the antioxidant activity content conversion to dry matter was the highest antioxidant activity detected in juice of Fertödi variety (6571.69 mg AA.dm^{-3} DM) and the lowest in juice of variety Primus (4130.42 mg AA.dm^{-3} DM).

When evaluating differences in antioxidant activity between the juices by a Fisher´s LSD test, we found that in the monitored quality indicators are the smallest relative differences among samples right in the juice antioxidant activity. Whereas the juice samples were divided into 9 homogeneous groups when evaluating the polyphenols and to 6 homogeneous groups (without white currants evaluation) when evaluating anthocyanins, they were divided into 5 homogeneous groups when assessing the antioxidant activity (Table 3).

Juices from varieties Titania and Fertödi had the highest statistically significant antioxidant activity, in which they did not differ. They statistically significantly differed only from the juices of white varieties Primus, Victoria and Blanka. Juices from white varieties had the lowest antioxidant activity, but they statistically significantly differed just from juices of varieties Titania, Fertödi a Jonkheer van Tets. To the balanced group of juices with similar antioxidant activity belong juices of red varieties Heinemannova neskorá, Red Lake and Treny and juices of black varieties Öjebyn and Triton, among which we did not find statistically significant differences in antioxidant activity.

Using the FRAP method, **Borges et al., (2010)** investigated the antioxidant capacity in samples of black and red currants, blueberries, raspberries and cranberries. Authors observed the highest measured values of antioxidant capacity in black currants 51.6 μmol $Fe^{2+}.g^{-1}$, then in blueberries 30.0 μmol $Fe^{2+}.g^{-1}$ and raspberries 27.7 μmol $Fe^{2+}.g^{-1}$. Lower antioxidant activity was found in red currants 24.6 μmol $Fe^{2+}.g^{-1}$ and cranberries 18.6 μmol $Fe^{2+}.g^{-1}$.

Table 3 Varietal differences in the antioxidant activity of currant juices.

Variety	mg AA.dm^{-3}	mg AA.dm^{-3} DM
Primus	434,11	4130,42^{a}
Viktória	502,98	4343,50^{a}
Blanka	417,10	4441,98^{ab}
Treny	458,00	5011,00^{abc}
Triton	879,48	5016,97^{abc}
Red Lake	517,33	5300,49^{abc}
Heinemannova neskorá	531,73	5354,83^{abc}
Öjebyn	891,34	5546,6^{abc}
Jonkheer van Tets	567,61	6057,74^{bc}
Titania	1167,61	6145,32^{c}
Fertödi	612,81	6571,69^{c}

Note: $^{a-c}$ Means with the same letter are not significantly different from each other (Fisher's LSD test, p >0.05); DM - dry matter.

Namiesnik et al., (2013) focused in their work on the determination of antioxidant capacity in selected types of berries, while they used gooseberries, cranberries and blueberries. The analysis was realized using the FRAP method. The highest values of total antioxidant capacity authors found in blueberries 94.10 μM TE.g^{-1}. The antioxidant capacity of cranberries and gooseberries was 26.97 μM TE.g^{-1} and 6.51 μM TE.g^{-1}.

Kendir and Köroğlu (2015) observed antioxidant activity of several species of the genus *Ribes* - *Ribes alpinum, R. anatolica, R. biebersteinii, R. multiflorum, R. nigrum, R. orientale, R. rubrum and R. uva-crispa*, growing wild in Turkey. The authors prepared water and methanol extracts of plant leaves and shoots and found that *Ribes orientale* reached the highest antioxidant activity among the assessed species.

Moyer et al., (2002) investigated correlation dependences between the antioxidant activity, the content of total polyphenols, and anthocyanin dyes in 107 genotypes of the genera *Vaccinium* L., *Rubus* L. a *Ribes* L., while found that the antioxidant activity correlates in the fruit more significantly with polyphenol content than with the anthocyanin dyes content.

CONCLUSION

Currant are an important raw material for the production of fruit juices, soft drinks and last but not least, of fruit wines. All these products are characterized by good organoleptic properties and beneficial effects on consumer health due to the presence of active antioxidant substances of different types. The aim of this work was to evaluate the content of total polyphenols, anthocyanin dyes and antioxidant activity in the juice from selected varieties of white, red and black currant. The value of total polyphenols in the currant juice ranged from 1897.43 mg. dm^{-3} DM (Blanka)

to 3712.21 mg. dm^{-3} DM (Primus). In the white varieties of currant was immeasurable amount of anthocyanin dyes. In the assessed juices from red and black currants was their content from 1947.64 mg. dm^{-3} DM (Jonkheer van Tets) to 4161.07 mg. dm^{-3} DM (Heinemannova neskorá). Among the evaluated indicators were the smallest differences between the samples in the indicator of antioxidant activity. The highest antioxidant activity reached juice sample of Fertödi variety (6571.69 mg AA.dm^{-3} DM) and the lowest sample of variety Primus (4130.62 mg AA.dm^{-3} DM).

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Contact address:
Ing. Andrea Mendelová, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: andrea.mendelova@uniag.sk.
Ing. Ľubomír Mendel, PhD., Plant Production Research Center Piešťany, Bratislavská 122, 921 68 Piešťany, Slovak Republic, E-mail: mendel@vurv.sk.
Ing. Tomáš Krajčovič, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, Slovak Republic, E-mail: krajcovic.thomas@googlemail.com.
Ing. Peter Czako, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, Slovak Republic, E-mail: peter.czako@uniag.sk.
doc. Ing. Ján Mareček, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, Slovak Republic, E-mail: jan.marecek@uniag.sk
doc. Ing. Helena Frančáková, CSc., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Plant Processing and Storage, A. Hlinku 2, 949 76 Nitra, Slovak Republic, Slovak Republic
E-mail: helena.francakova@uniag.sk.

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PREVALENCE OF PATHOGENIC *YERSINIA ENTEROCOLITICA* IN MINCED MEAT, PIG TONGUES AND HEARTS AT THE RETAIL LEVEL IN THE CZECH REPUBLIC DETECTED BY REAL TIME PCR

Alena Lorencova, Michal Slany

ABSTRACT

Yersiniosis is the third most frequently reported zoonosis in the European Union and *Yersinia enterocolitica* is the most common species causing human infections. Pigs are assumed to be the main reservoir of human pathogenic *Y. enterocolitica* with the presence of bacteria mainly in the tonsils and intestinal content. Undercooked pork and pork products have been suggested as the primary source of human yersiniosis. Nevertheless, data on the prevalence of pathogenic *Y. enterocolitica* in foodstuffs including pork products are very limited. A molecular based method (real time PCR) targeting the *ompF* gene (detection of *Yersinia* genus) and the *ail* gene (a chromosomally located virulence marker of *Y. enterocolitica*) was used to determine the prevalence of pathogenic *Y. enterocolitica* in minced meat and edible pork offal at the retail level in the Czech Republic. A total of 50 pig tongues, 50 pig hearts, and 93 samples of minced meat containing pork were purchased at nine retail outlets in Brno. High detection rates of *Yersinia* spp. were found in all types of samples (pig tongues, 80.0%; pig hearts, 40.0%; and minced meat, 55.9%). The highest prevalence of pathogenic *Y. enterocolitica* was found in pig tongues (40.0%), followed by pig hearts (18.0%) and minced meat samples (17.2%). Although from the point of view of food safety the merely molecular detection of DNA of the pathogenic bacteria could represent a false positive result, our results indicate the presence of pathogenic *Y. enterocolitica* in raw pork products at the retail level in the Czech Republic, which may pose a risk of consumer infection. Sufficient heat treatment and prevention of cross-contamination during preparation of food in the kitchen should be recommended.

Keywords: *Yersinia enterocolitica*; *ail* gene; *ompF* gene; real time PCR; pork products; retail; zoonosis

INTRODUCTION

In the European Union, yersiniosis was the third most frequently reported zoonosis in 2014, despite the significantly decreasing trend between 2008 and 2014. *Yersinia enterocolitica* was the most common species reported, having been isolated as the causative agent from 97.7% of the confirmed cases **(EFSA and ECDC, 2015)**. Clinical manifestations of human infection are usually fever, enterocolitis, pseudoappendicitis, and mesenteric lymphadenitis with diarrhoea, vomiting, and abdominal pain. Post-infection complications such as reactive arthritis or erythema nodosum can also emerge **(Galindo et al., 2011)**.

Pigs have been considered to be the primary reservoir for the pathogenic *Y. enterocolitica* that has been isolated especially from tonsils, tongues, or throats, and to a lower extent from faeces, which all can be a source of contamination for other parts of the carcasses during slaughter procedures **(Fredriksson-Ahomaa et al., 2000; Fredriksson-Ahomaa et al., 2001a; 2001b; Simonova et al., 2008; Van Damme et al., 2015)**. The European Food Safety Authority (EFSA) considers *Y. enterocolitica* as one of the most relevant biological hazards in the context of meat inspection of swine **(EFSA, 2011)**.

Eating of raw or undercooked pork and pork products (especially minced meat) has been strongly associated with human yersiniosis **(Tauxe et al., 1987; Grahek-Ogden et al., 2007)**. **Fosse et al., (2008)** estimated that 77.3% of clinical cases of yersiniosis in humans are connected with the consumption of pork and the same genotypes of *Y. enterocolitica* strains isolated from slaughterhouse environments, pork products in retail outlets and patients with yersiniosis support this hypothesis **(Fredriksson-Ahomaa et al., 2001a)**. Nevertheless, at present, there is no harmonised surveillance of pathogenic *Yersinia* in food and animals in the EU **(EFSA and ECDC, 2015)**.

Y. enterocolitica is a ubiquitous microorganism. However, not all strains recovered from food and environmental samples are pathogenic. From the point of view of health hazard, it is necessary to distinguish between pathogenic and non-pathogenic variants **(Fredriksson-Ahomaa and Korkeala, 2003; EFSA and ECDC, 2015)**. The use of traditional culture methods may lead to underestimation of pathogenic *Y. enterocolitica* in clinical, food and environmental samples. Pathogenic yersinia have seldom been isolated from pork or other foods except for edible pig offal, because of their usually small numbers in the samples, limited sensitivity of the culture media without the ability to distinguish between pathogenic and non-pathogenic strains and subsequent overgrowth of target organisms by background flora **(Fredriksson-Ahomaa and Korkeala, 2003;**

Laukkanen-Ninios et al., 2014; EFSA and ECDC, 2015).

More rapid, sensitive and specific DNA-based methods have provided a better estimation of the occurrence of pathogenic *Y. enterocolitica* in naturally contaminated samples even when the pathogen is initially present in low numbers **(Fredriksson-Ahomaa et al., 1999 and 2001c; Fredriksson-Ahomaa and Korkeala, 2003; Messelhäusser et al., 2011; Laukkanen-Ninios et al., 2014)**. However, subsequent isolation of *Y. enterocolitica* strains is needed for further strain characterization (especially for information on the biotype and serotype) and to assess its public health significance **(EFSA and ECDC, 2015)**.

The virulence of *Y. enterocolitica* results from a complex of plasmid- and chromosomally encoded genes. Because of easy loss of the virulence plasmid during laboratory handling, chromosomally located genes are more reliable target genes for PCR assays **(Fredriksson-Ahomaa and Korkeala, 2003; Galindo et al., 2011)**. The chromosomally located *ail* gene (attachment and invasion locus) is an essential virulence factor in strains of *Yersinia* spp. **(Miller et al., 1989)** and it is the most frequently used target to detect human pathogenic *Y. enterocolitica* **(Miller et al., 1989; Fredriksson-Ahomaa and Korkeala, 2003)**. An enrichment step prior to PCR is recommended to increase sensitivity and probability of detecting viable cells; false-positive results due to dead cells can be avoided **(Lambertz et al., 2007)**.

According to the EFSA report **(EFSA and ECDC, 2015)**, not enough information is available about the prevalence of human pathogenic *Y. enterocolitica* in foodstuffs at the retail level. The aim of this pilot study was to survey the prevalence of pathogenic *Y. enterocolitica* in edible pork offal (tongues and hearts) and minced meat at the retail level in the Czech Republic in order to estimate the risk of consumer infection via products of porcine origin.

MATERIAL AND METHODOLOGY

During the period of Juny 2014 to August 2015, a total of 50 pig tongues, 50 pig hearts, and 93 samples of individually packaged minced meat (50 pure pork and 43 mixed with 15 to 50% beef) were purchased from nine different butcher shops and supermarkets in Brno, Czech Republic. The samples were transported to the laboratory under refrigeration and were analysed immediately. A 25 g portion of each sample was cut into small pieces and homogenized in 225 mL PSB (phosphate buffered saline with sorbitol and bile salts, HiMedia, India) in a stomacher blender for 2 min and enriched at 25 °C for 18 – 20 h.

One millilitre of the enriched culture was centrifuged at 14,000 g for 5 min. The pellet was used for DNA isolation using the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) slightly modified to include mechanical homogenization with zirconia/silica beads (0.2 mm) in a MagNALyser instrument (Roche, Mannheim, Germany). An aliquot (5 μL) of extracted DNA was used as a template for home-made triplex real time PCR (qPCR) assay able to detect genus *Yersinia* (*ompF* gene) **(Stenkova et al. 2008**) and differentiate pathogenic *Y. enterocolitica* strains (*ail* gene) **(Lambertz et al., 2008)**. The previously published internal amplification control was used in the assay to eliminate false negative samples **(Slana et al., 2008)**.

RESULTS AND DISCUSSION

Based on a qualitative risk assessment of foodborne hazards associated with chilled pork carcasses, *Y. enterocolitica* was considered of high relevance **(EFSA, 2011)**. However, pigs are mostly asymptomatic carriers of pathogenic *Y. enterocolitica* without any signs of illness or macroscopic lesions. Thus, routine meat inspection practices cannot reveal infected pigs or contaminated carcasses and their products can enter the food chain.

In the Czech Republic, a growing number of cases of human yersiniosis were recorded in recent years **(Dr. Cestmir Benes, The National Institute of Public Health, Czech Republic, personal communication)**. However, no systematic monitoring of the occurrence of pathogenic *Yersinia* spp. in animals and in food is performed. In previous studies, pathogenic *Y. enterocolitica* has been recovered from smears from pig tongues (from 1.1 to 27%), tonsils (7.5%), rectal content (7.4%), and skin surface (2.8%) obtained from different slaughterhouses in the Czech Republic **(Aldová and Švandová, 1984; Aldová et al., 1990; Vázlerová and Steinhauserová, 2006; Simonova et al., 2008)**. However, no information is available about the presence of pathogenic *Y. enterocolitica* in pork products at the retail level.

In the present study, we have found a high prevalence of *Yersinia* spp. in all three types of collected samples (Figure 1) with the highest contamination level in pig tongues (80.0%), followed by minced meat (55.9%) and pig hearts (40.0%). However, as was shown previously, the majority of *Yersinia* isolates obtained from food and environmental samples are non-pathogenic without any clinical importance **(Fredriksson-Ahomaa and Korkeala, 2003)**.

This fact is in accordance with our results because the detection rate of pathogenic *Y. enterocolitica* in the examined samples was considerably lower compared with the contamination by *Yersinia* in general (Figure 1). The highest positivity was found in pig tongues (40.0%) and then in hearts (18.0%) and minced meat samples (17.2%). Using PCR methods, even higher contamination rates with pathogenic *Y. enterocolitica* in pig tongues at the retail level were detected in previous European studies: 44.9% in Bavaria (Germany) **(Messelhäusser et al., 2011)**, 83% **(Fredriksson-Ahomaa et al., 2001c)** and 92% **(Fredriksson-Ahomaa et al., 1999)** in Finland. Pathogenic *Y. enterocolitica* was found also in 50% of pig hearts from retail shops in Finland, however, only a small number of samples (n = 8) were investigated **(Fredriksson-Ahomaa et al., 2001c)**. In the abovementioned studies, the prevalence of pathogenic *Y. enterocolitica* was found to be higher with PCR than with culture methods, which indicates a higher sensitivity of the PCR method for detection of pathogenic *Y. enterocolitica* in naturally contaminated samples.

The high prevalence of *Y. enterocolitica* in pig tongues and other offal might be caused by cross-contamination by tonsil tissue during the slaughtering process **(Fredriksson-Ahomaa et al., 2000; 2001b; Messelhäusser et al., 2011)**. Preventing contamination completely is practically impossible because the tonsils are removed and hung together with tongue, liver, lung and heart on a hook after

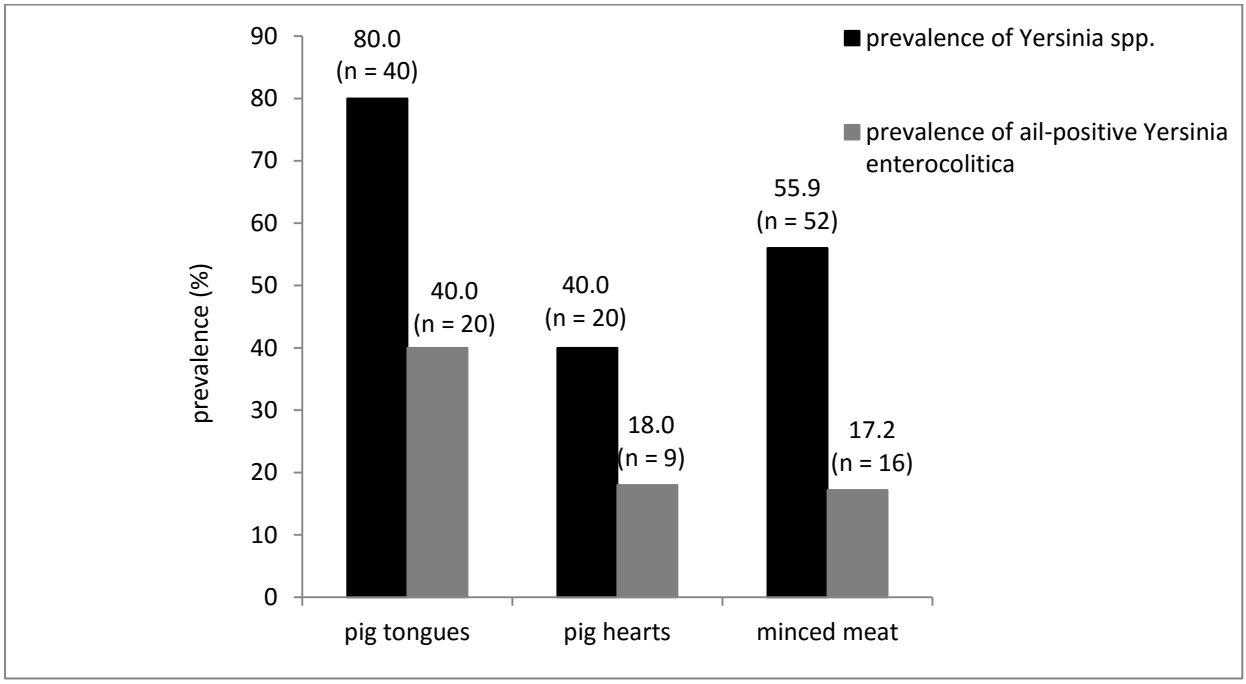

Figure 1 Prevalence of *Yersinia* spp. and pathogenic (*ail*-positive) *Yersinia enterocolitica* in pig tongues (n = 50), hearts (n = 50) and minced meat samples (n = 93) at the retail level using the real time PCR.

evisceration. The highest isolation rate (51%), in comparison to other raw pork products, of pathogenic *Y. enterocolitica* was also found in edible offal of slaughter pigs in southern Germany **(Bucher et al., 2008)**.

Van Damme et al., (2015) found that the initial presence of *Y. enterocolitica* in tonsils and/or in faeces of pigs at slaughter was significantly associated with carcass contamination and the findings of the same genotypes in tonsils, offal, and in minced pork support the assumption that tonsils are the primary source of contamination with pathogenic *Y. enterocolitica* at the slaughterhouse level **(Fredriksson-Ahomaa et al., 2000; Fredriksson-Ahomaa et al., 2001b)**. The blood of infected animals and rinse water can be another source of these bacteria for edible parts of the carcass and pathogenic strains were isolated also from the environment and from the air in a pig slaughterhouse **(Fredriksson-Ahomaa et al., 2000)**. Thus, the cross-contamination of carcases and offal of subsequently slaughtered non-infected pigs can also occur.

Minced meat represents another raw pork product with high risk of contamination by pathogenic *Y. enterocolitica*. Eating of raw ground pork and ground mixed meat was found to be strongly associated with human infection in Belgium **(Tauxe et al., 1987)**. In our study, 17.2% of individually packaged minced meat samples were found to contain pathogenic *Y. enterocolitica* using qPCR. Similarly, raw minced meat samples containing pork collected at the retail level in Finland **(Fredriksson-Ahomaa et al., 1999)** and in Sweden **(Lambertz et al., 2007)** were found to be relatively highly contaminated with pathogenic *Y. enterocolitica* using PCR methods (25% and 35% of samples, respectively). In the United States, 133 (38%) of 350 ground pork samples were found to be contaminated by pathogenic (*ail*-positive) *Y. enterocolitica* using qPCR assay. All samples investigated in that study were culture negative, which indicated only a low contamination level. Pathogenic *Y. enterocolitica* was also detected in 39% of cut meat samples (n = 155) collected at meat cutting plants before mincing in Finland studied with PCR **(Laukkanen–Ninios et al., 2014)**. On the other hand, in Germany, pathogenic *Y. enterocolitica* was detected only in 5 (4.9%) from 102 samples of pork minced meat using qPCR assay targeting the *ail* gene **(Messelhäusser et al., 2011)**.

In shops and then in kitchens *Y. enterocolitica* can easily contaminate other foods thorough direct contact with contaminated raw pork and edible offal or via contaminated hands or equipment during handling and preparation **(Fredriksson-Ahomaa et al., 2001a; 2001b)**. In our study, the detection of genomic equivalents of pathogenic *Y. enterocolitica* in pig tongues and/or hearts collected on the same day and in the same shop could be also the result of cross-contamination of offal stored together before sale. Moreover, because of the psychrotrophic character of *Y. enterocolitica*, these bacteria can persist and multiply in raw material and food during storage at refrigeration temperatures. This is of significant concern from the point of view of food safety and public health.

Sufficient heat treatment of raw meat and offal should eliminate *Y. enterocolitica*, but as was shown, bacteria can survive in core of the product, especially in foods high in fat content (e.g. some minced meat products) which can protect bacteria against the effect of high temperature **(Grahek-Ogden et al., 2007)**. Furthermore, subsequent cross-contamination of the heat-treated products can occur if good hygiene procedures are not followed.

The detection of only the DNA of the pathogenic bacteria in food could be dismissed as irrelevant with respect to food safety because of the possible presence of dead or damaged bacterial cells or free DNA alone **(Lambertz et al., 2007)**. However, if isolation of the pathogen is

difficult, as in the case of pathogenic *Y. enterocolitica*, positive PCR results indicate that it is present and this should be considered as a potential health hazard. In addition, an enrichment step prior to qPCR used in our study should increase the possibility of detecting only live bacteria **(Lambertz et al., 2007)**. Even if the bacteria were initially present in low numbers, their ability to survive and multiply in refrigerated meat and meat products increases the potential risk of infection to consumers.

CONCLUSION

Results of this pilot study indicate that raw pork products can be an important source of pathogenic *Y. enterocolitica* at retail level in the Czech Republic. Measures should be taken to prevent contamination of pig carcasses and edible offal during slaughter and good hygiene practices including proper cooking and prevention of cross-contamination at the household level should be adopted in order to minimize the spread of pathogenic yersiniae and risk of human infection. Further study is needed to obtain *Y. enterocolitica* isolates from the PCR-positive samples for their further characterisation in order to get important epidemiological information.

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Contact address:

Dr. Alena Lorencova, Ph.D., Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic, E-mail: lorencova@vri.cz

Dr. Michal Slany, Ph.D., Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic, E-mail: slany@vri.cz

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FLAX – EVALUATION OF COMPOSITE FLOUR AND USING IN CEREAL PRODUCTS

Marie Hrušková, Ivan Švec

ABSTRACT

Two types of yellow and brown linseed, differing in granulation, were tested in form of wheat flour composites (additions 2.5% and 5.0%) by using the Farinograph, the Extensigraph and the Rapid Visco Analyser (RVA) apparatuses. Additions of brown and yellow flax fibre significantly affected Falling Number and Zeleny test values. Curves of farinograph were differed according to flax fibre type – finer flax (better terminology) granulation meant somewhat stronger negative changes in dough stability and dough softening degree. Results of extensigraph test demonstrated changes in dough elasticity and extensibility due to lowering of gluten protein content. Appearance of the RVA profiles was verifiably different, reflecting diverse wheat and flax polysaccharides, added dietary fibre type and its granulation. Due to that, bread volume and shape was lowered up to one-half in case of golden flax composites. Similar tendencies with smaller negative influence caused the brown linseed. Fibre from flax is used for technical (textile) use, but linseed dietary fibre addition affected quality of laboratory prepared cut-off biscuits and dried pasta differently, showing a dependence on the fibre type, granulation as well as addition level. Sensory profiles of all mentioned product types were acceptable.

Keywords: brown and yellow linseed; granulation; rheology; bread; biscuits; pasta

INTRODUCTION

Flax (*Linum usitatissimum* L.) is old utility plant originated in Asia. Slim stem and light-blue flowers could distinguish the plant. Its fruit is boll containing tiny brown seeds. There exist linseed varieties (e.g. Amon, Raciol) with lighter seed, named as "yellow flax".

Fibre flax is appraised according to flax thread characteristics, which exists into two forms for industrial processing. Between world producers belong France, Belgium and Netherlands, in the Czech Republic is not planted from the year 2010.

Linseed is planted for seeds and oil production, for both food and industrial usage. According to Catalogue of oil plant varieties (ÚKZÚZ Brno, 2015), four linseed varieties sorted in groups with low, high and usual linoleic-to-alpha-linolenic acid ratio were tested. Between varieties with medium content of these acids belongs the Czech variety Raciol, registered in year 2011.

Flax seed is, with respect to chemical composition, recommended into curative diets. Between brown and yellow coloured seeds, nutrition difference is not verifiable, but consumers prefer yellow seeds owing to somewhat intensive nutty-butter by-taste. Seed composition is typical by high oil content (40%), dietary fibre (28%) and proteins (21%). Further known constituents are minerals (4%) and non-starch polysaccharides (6%) as lignans, hemicelluloses and phenolics (**Fitzpatric, 2008; Bernacchia et al., 2014**; **Ding et al., 2014; Nitrayová et al. 2014**). Flax oil is the most favourable nutrition component, rich in omega-3 unsaturated fatty acids with short chain. Also content of alpha-linolenic acid is substantial **(Cunnane et al., 1993)**. For commercial purposes, limited stability of these oil components is discussed, which have approved health benefits in lifestyle diseases prevention (**Denmark-Wahnefriend, 2006)**. Employing of flax seed in cereal products is limited by specific structure characteristic. Owing to hard cover layers, it could be used as decorative material for spreading of special bread types. In cases of wholemeal bread, golden flax seeds are used more frequently. When it is involved into bread recipe, technological process has to be adapted in terms of so-called wort, which owing to time period and water temperature ensures sufficient water sorption and seeds softening. For such purpose, brown flax is preferred, because of difference in crumb colour profile compared to product based on wheat flour only.

Flax dietary fibre is commercial food supplement gained as by-product during oil pressing or extraction, sold in a dry powder form. Walramcom company, flax fibre producer from the New Zeeland, present mean nutritional values for the supplement from brown and golden flax: saccharides 2.4%, proteins 32.0% and total fat 16.6% (of which 13% unsaturated fatty acids). According to production method, majority mass portion presents dietary fibre (TDF 45.2%, IDF 37.9% and SDF 7.3%). Proteins have a non-gluten nature, and thus they are safe for coeliac patients. Further, flax fibre is a good source of constituents with high anti-oxidant activity, especially lignans (e.g. secoisolariciresinol diglucoside) and vitamin E. For flax lignans, a specific function in prevention cancer diseases of breast and prostate. **Budwig (2011)** presented their

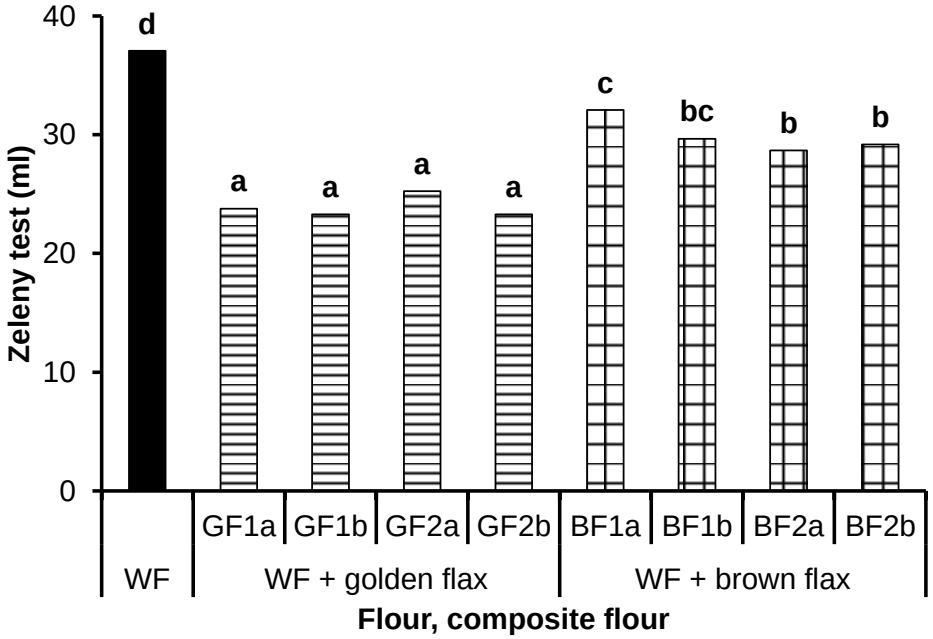

Figure 1 Zeleny test values for wheat flour (WF) and flour composites. For samples coding, see Table 1.

content 75 – 800 times higher compared to other vegetable and pulses. Owing to low presence of saccharides, flax fibre is appropriate for diabetics and sportsmen. Plant proteins may successfully enriched daily diet of vegetarians and vegans. Recommended consumption is equal to 13 g as additive into oat flakes, yogurts or soups. In terms of gluten-free material, it could serve as a base of bread, pancakes, pies etc. Flax fibre has a potential to be used as recipe component of sweet bread e.g. muffins **(Chetana et al., 2010)**, gluten-free products and pastas **(Kishk et al., 2011; Hrušková a Švec, 2013)**.

The pilot study was aimed at characteristics comparison of several commercial types of flax fibre, using them in form of composite flour during non-fermented and fermented dough properties testing. Verifying technological potential and consumer quality of these flour composites, leavened bread, cookies and pasta were manufactured in a laboratory scale.

MATERIAL AND METHODOLOGY

Preparation of flour composites

Semi-bright fine wheat flour (WF) was produced from wheat harvested in year 2015 by industrial mill Delta Prague. According to Falling number (392 s) and Zeleny sedimentation values (392 s and 37 ml, respectively), quality of the basic material is medium with lower amylases activity. The company Walramcom (New Zeeland) produced flax fibre (FF) samples, and they represent grounded flax seeds press cake after oil extraction (GF1, GF2 – golden flax fibre with granulation 0-300 μm and 500-700 μm, respectively; BF1, BF2 – brown flax fibre with granulation 300-500 μm and 500-700 μm, respectively). According to nutritional label, dietary fibre content is comparable in all tested ff samples. Flour composites involve 2.5% or 5.0% of ff on flour base (samples coding GF1a and GF1b, etc.).

Technological quality of flour composites

Technological features of wf and flour composites are described by Zeleny test (ČSN ISO 5529) and Falling number (ČSN ISO 3093). Non-fermented dough properties were determined by using of farinograph and extensigraph brabender (Germany), following the international norms (ČSN ISO 55 30-1, 55 30-2, respectively). Behaviour of suspension flour-water was recorded on the RVA 4500 equipment (Perten Instruments, Sweden; AACC method 76-21). According to internal procedures of the UCT Prague, rheological parameters of fermented dough was measured, using fermentograph SJA (Sweden),

Table 1 Falling number for wheat flour (WF) and flour composites.

Flour, flour composite	WF	GF1a	GF1b	GF2a	GF2b	BF1a	BF1b	BF2a	BF2b
Falling number* (s)	392 bc	349 ab	319 a	305 a	330 ab	442 c	438 c	426 c	456 c

GF1, GF2 - golden flax fibre with granulation 0-300 μm and 500-700 μm, respectively.
BF1, BF2 - brown flax fibre with granulation 300-500 μm and 500-700 μm, respectively.
Example of sample coding: GF1a, GF1b - flour composites containing 2.5% or 5.0% of golden flax fibre, respectively.
* a-c: row means described by the same letter are not significantly different (p = 95%).

Table 2 Farinograph characteristics of non-fermented dough from wheat flour (WF) and flour composites.

Flour, flour composite	Water absorption* (%)	Dough development time* (min)	Dough softening degree* (FU)
WF	67.8 a	3.15 bc	50 b
GF1a	70.3 b	2.00 a	30 ab
GF1b	72.5 d	2.50 ab	30 ab
GF2a	70.0 b	2.50 ab	20 a
GF2b	72.0 cd	4.00 cd	10 a
BF1a	71.0 bc	3.00 abc	30 ab
BF1b	71.9 cd	3.50 bcd	10 a
BF2a	70.3 b	3.50 bcd	15 a
BF2b	72.3 d	4.50 d	10 a

GF1, GF2 - golden flax fibre with granulation 0-300 μm and 500-700 μm, respectively.
BF1, BF2 - brown flax fibre with granulation 300-500 μm and 500-700 μm, respectively.
Example of sample coding: GF1a, GF1b - flour composites containing 2.5% or 5.0% of golden flax fibre, respectively.
FU – farinograph unit.
* a-d: column means described by the same letter are not significantly different (p = 95%).

maturograph and oven rise recorder (OTG) Brabender (Germany). From prepared wheat-flax fibre composites, bread, cut-off biscuits and pasta were manufactured, following further internal methods ended by quality evaluation.

Statistical evaluation of ff effect

Influence of FF type and dosage level was evaluated it terms of variation of selected dough rheological features and final products (Tukey test, p = 95%). Aimed on determination of quality features dominant for bread, biscuits and pasta, Principal Components Analysis (PCA) was used. In cases PCA of bread, biscuits and pasta, the datasets were analogous to ensure the comparability of the analysis findings – two analytical features, three farinograph and two extensigraph ones, a pair of the pasting characteristics and foursome of the product quality attributes immanent to the product type.

RESULTS AND DISCUSSION

Evaluation of technological quality of flour composites

According to Zeleny test values, bakery quality of proteins could be considered as lower compared to WF (Figure 1). Addition of GF caused a decrease in a higher extent (about 30%) in relation to BF influence (-18%) and WF control. As could be noticed in Figure 1, neither addition level nor FF granulation did not trigger significant differences.

In wheat flour sample, amylases activity as the Falling number was estimated about ca 25% lower than optimum for standard bakery processing is (250 s). With respect to variability in the FF type (GF vs. BF), granulation (2 types) as well as addition level (2.5% vs. 5.0%), flax fibre contributed to Falling number change softly. An insignificant lowering and increase was observed for GF and BF, respectively, with weak impact of addition level.

Evaluation of non-fermented dough features

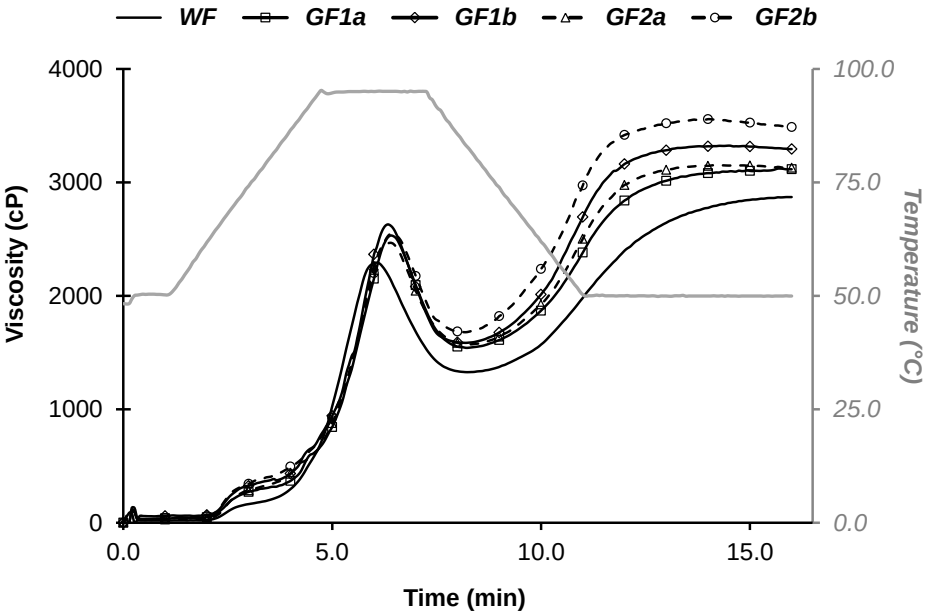

Figure 2 RVA profiles of wheat flour and selected flour composites. For samples coding, see Table 1.

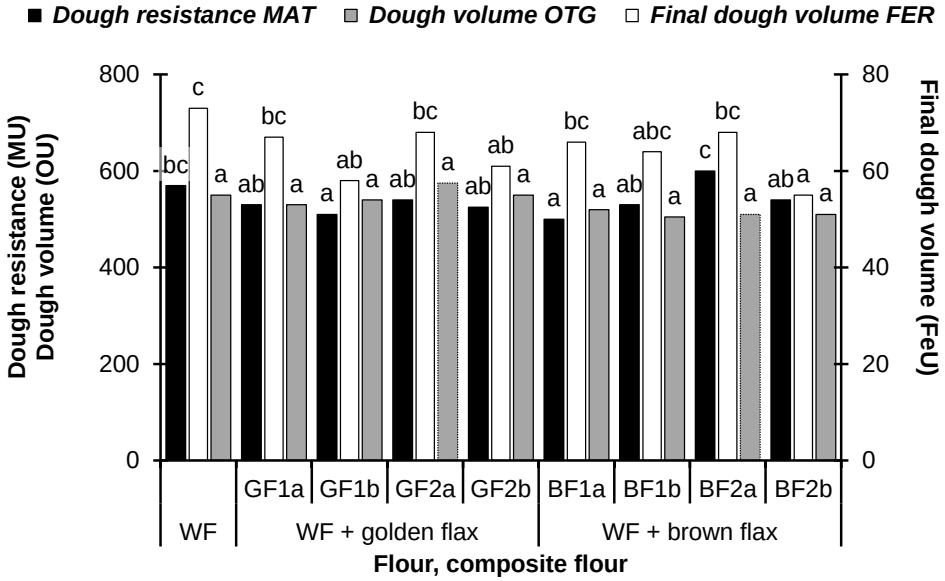

Figure 3 Characteristics of fermented dough from wheat flour and flour composites. For samples coding, see Table 1.

In case of standard farinograph proof, rheological properties of non-fermented dough prepared from flour composites are characterised by water absorption increase about 3% – 7%. Higher values were determined for 5% addition and BF of coarser granulation. Dough development time was shortened by the GF effect (from 3 min to 2 min), and reversal trend was observed for the BF (prolongation about 25%). Preparation of composite dough variants did not leave the line of standard process, and the values of dough softening degree point to higher resistance to overmixing (Table 2); on the farinograms, the second maxima were identified for more enhanced samples, which discussed **Xu et al. (2014)**.

According to extensigraph characteristics, change in dough viscoelastic properties implies the elasticity-to-extensibility ratio determined after 60 min of dough resting, which simulates II. phase of fermentation process during wheat bread manufacturing. The ratio increased from 2.18 (WF control) about 10% – 70% reflecting FF type and granulation of GF and BF (data not shown). In composite dough, diluted gluten protein contributed to lowering of bakery quality of tested dough samples; extensigraph energy decreased seriously by incorporation of finer GF (about 15%), while impact of coarser GF addition was softer (decrease ca 10%).

Both BF counterparts rather increased dough bakery quality, and effect of granulation was indefinite. RVA profile course describing viscous behaviour of wheat suspension could be designated as standard, values of the Peak Viscosity and Final Viscosity corresponds to presumed using for bread manufacturing. As illustrates Figure 2, suspensions of flour composites differed from the control in earlier gelatinisation beginning, reflecting the FF type and granulation. Between golden and brown flax, measured differences correspond to diverse representation of polysaccharide fractions, and they

Table 3 Features of bread prepared from wheat flour and flour composites.

Flour, flour composite	Specific bread volume** (ml/100g)	Bread shape*, ** (-)	Crumb penetration** (mm)
WF	334 d	0.61 a	14.3 f
GF1a	285 cd	0.54 ab	9.5 d
GF1b	242 abc	0.41 a	5.8 a
GF2a	283 cd	0.53 ab	10.4 e
GF2b	182 a	0.42 a	6.2 b
BF1a	205 ab	0.51 ab	6.2 ab
BF1b	268 bcd	0.55 ab	8.0 c
BF2a	197 ab	0.44 ab	6.6 b
BF2b	246 abc	0.56 ab	9.1 d

GF1, GF2 - golden flax fibre with granulation 0-300 μm and 500-700 μm, respectively.
BF1, BF2 - brown flax fibre with granulation 300-500 μm and 500-700 μm, respectively.
Example of sample coding: GF1a, GF1b - flour composites containing 2.5% or 5.0% of golden flax fibre, respectively.
* Height-to-diameter ratio.
** a-d: column means described by the same letter are not significantly different (p = 95%).

Table 4 Features of biscuits prepared from wheat flour and flour composites

Flour, flour composite	Specific biscuit volume** (ml/100 g)	Spread ratio*, ** (-)	Sensory profile** (-)
WF	165 ab	4.35 b	12.0 ab
GF1a	172 b	4.41 b	11.5 ab
GF1b	134 a	4.81 b	12.5 b
GF2a	167 ab	4.53 b	11.0 a
GF2b	147 ab	4.13 b	11.0 a
BF1a	143 ab	1.98 a	11.5 ab
BF1b	145 ab	1.73 a	11.5 ab
BF2a	149 ab	2.07 a	11.5 ab
BF2b	155 ab	2.05 a	11.5 ab

GF1, GF2 - golden flax fibre with granulation 0-300 μm and 500-700 μm, respectively.
BF1, BF2 - brown flax fibre with granulation 300-500 μm and 500-700 μm, respectively.
Example of sample coding: GF1a, GF1b - flour composites containing 2.5% or 5.0% of golden flax fibre, respectively.
* Diameter-to-height ratio.
** a-d: column means described by the same letter are not significantly different (p = 95%).

demonstrated a reversal tendency than published **Mueller et al. (2010)**. Influence of three factors on RVA features are discussed by **Švec and Hrušková (2016)**.

Evaluation of fermented dough features

With the help of three laboratory apparatuses, fermentograph, maturograph and OTG, three phases called fermentation, proofing and first stage of baking are simulated, allowing to describe fermented dough behaviour and estimation of technological potential of flour composites for operational application. Gained readings allow to evaluate 12 parameters, from which three are substantial for comparison with wheat dough – final dough volume (fermentograph), dough resistance (maturograph) and dough volume (OTG test). As shown Figure 3, type, granulation as well as FF type affected these features, but measurement accuracy did not allow distinguishing composite dough items from wheat control.

There could be noticed, that final dough volume depends more on FF addition level than its type. With exception of BF2a composite dough, dough resistance of other samples reached comparable level to wheat one. Bread volume in the third phase of fermentation predicts specific bread volume, and soft increase as result of GF enhancement was observed. Our results agree with effect of FF addition (6%) described by **Xu et al., (2014)**.

Evaluation of wheat bread features

Simulating a straight-dough process in a bakery, a baking trial was conducted according to the internal method (Babiaková, 2015). Quality assessment of laboratory prepared bread demonstrated a negative relationship between GF dosage and specific bread volume. For 5% addition of GF1 and GF2, a decrease reached almost one-third compared to control (242 ml.100 g^{-1} and 182 ml.100 g^{-1}, respectively, against 334 ml.100 g^{-1}). Buns vaulting became lower as FF portion in bread recipe increased; height-to-diameter ratio decrease was significant in cases of higher enhancement. Quantifying sensorial score, the change was confirmed, too – crumb of fortified bread samples were tougher and thus less tasty. By BF addition, consumer quality lowering of wheat bread

Table 5 Features of cooked pasta prepared from wheat flour and flour composites

Flour, flour composite	Absorption* (%)	Swelling index* (-)	Sediment* (ml)
WF	159.2 c	1.45 a	120 abcd
GF1a	134.0 ab	1.52 a	96 ab
GF1b	131.6 ab	1.58 a	96 ab
GF2a	147.2 bc	1.56 a	104 abc
GF2b	135.6 ab	1.58 a	80 a
BF1a	126.0 a	1.32 a	160 d
BF1b	135.2 ab	1.35 a	126 bcd
BF2a	136.8 ab	1.40 a	144 cd
BF2b	132.8 ab	1.50 a	80 a

GF1, GF2 - golden flax fibre with granulation 0-300 μm and 500-700 μm, respectively.
BF1, BF2 - brown flax fibre with granulation 300-500 μm and 500-700 μm, respectively.
Example of sample coding: GF1a, GF1b - flour composites containing 2.5% or 5.0% of golden flax fibre, respectively.
* a-b: column means described by the same letter are not significantly different (p = 95%).

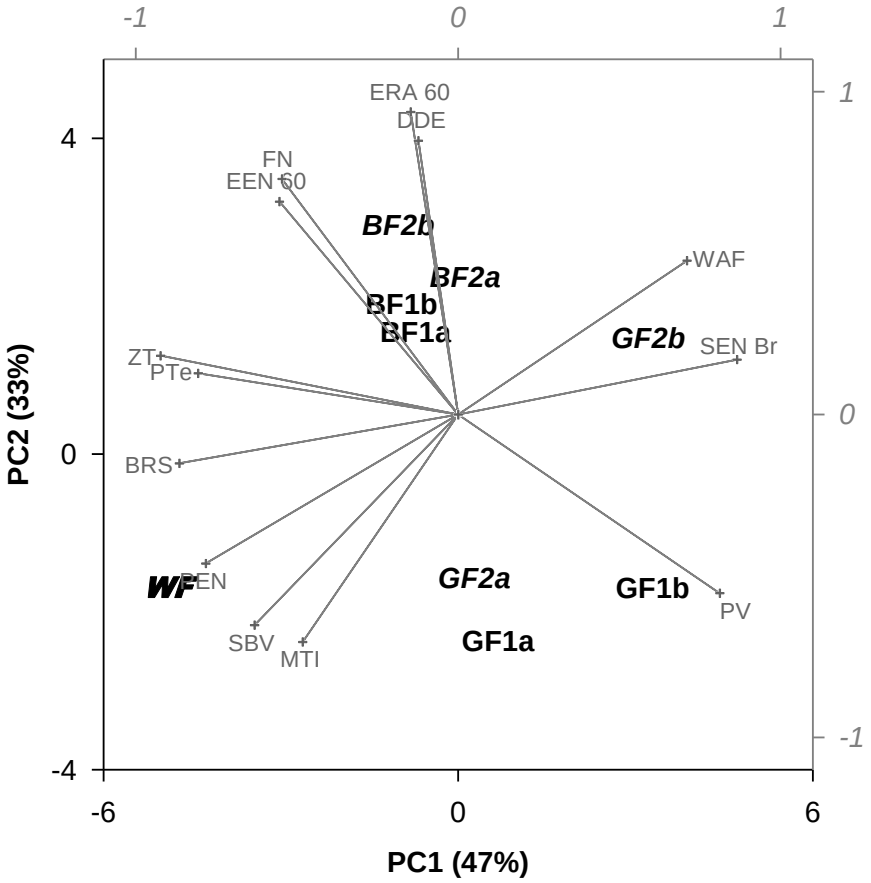

Figure 4 Principal component (PC) analysis of flax fibre effect on dough and bread technological quality. FN – Falling number, ZT – Zeleny test; WAB – water absorption, DDE – dough development time, MTI – mixing tolerance index (dough softening degree); ERA 60, EEN 60 – extensigraph elasticity-to-extensibility ratio and energy, respectively (dough resting 60 min); PTe – pasting temperature, PV – peak viscosity; SBV – specific bread volume, BRS – bread shape (height-to- diameter ratio, PEN – crumb penetration, SEN Br – bread sensory profile. For samples coding, see Table 1.

was not so dramatic, higher dosage of BF1 and BF2 (i.e. 5.0%) lowered bread volume to 268 ml.100 g^{-1} and 246 ml.100 g^{-1}, respectively; that finding correspond to conclusion in paper of **Marpalle et al. (2014)**. Samples containing 5.0% of both BF reached significantly higher volumes than their less enhanced counterparts did. Besides, shape and crumb texture of buns with BF obtained a score closer to the WF standard (Table 3).

Multivariate statistics explained 80% of data variability; 47% was covered by principal component PC1 and 33% by PC2 (Figure 4). Within PC1 x PC2 area, conjoining of observed dough and bread features as well as tested samples has a relation to their dependence rate on the FF effect. As was discussed, impact of GF and BF addition was verifiably different – golden flax fibre additions influenced bread quality less negatively. Within PC1 x PC2 area, position of GF1a, GF1b and GF2a samples are obviously closer to WF one, confirming lower level of the dough softening degree (MTI) and reversely higher values of the specific bread volume and crumb penetration. Within the biplot, there could be noticed a significant role of bread sensory score in bread recipe discrimination (explored from 75% by PC1, and from 3% by PC2).

Evaluation of cut-off biscuit features

Cut-off biscuits are characteristic by manufacturing technique, i.e. by cutting-off from dough plate of calibrated thickness, and within the Czech assortment of long-life confectionery, they represent ca 20%. The mentioned internal method (Hrušková and Švec, 2015) operates with seven quality characteristics, of which three (specific volume of baked biscuits, shape as spread ratio (diameter-to-height ratio) and sensory profile allow to compare different recipe variants. For biscuits containing GF, 2.5% such fibre had a positive influence on the evaluated features; the impact of GF granulation was less provable. Brown flax fibre caused a soft specific volume decrease (about approx. 10%), and biscuits shape spread into approx. a half scale (Table 4). Sensorial profiles of GF and BF biscuits did not deviate from the wheat control one. Yellow linseed contributed to attractive yellow shade of biscuit surface, and brownish one in case of BF. Coarser flax dietary fibre (granulation over 500 μm) was visually detectable as darker dots in biscuit crust, especially in case of biscuits containing brown seeds FF.

PCA results of biscuits quality, based on the analogous data set for composite flour and dough behaviour as in case of bread, shown comparable dependences as illustrated in Figure 4 (biplot not shown). The first two PC

explored also 80% of data variability, and the dominant features for biscuits recipe distinguishing maintained the Zeleny test, the extensigraph energy, both RVA pasting features and the specific volume together with the biscuit shape (spread ratio). The product sensory score depended mainly on the PC3 (80%), PC1-PC2 pair explained 18% of the scatter only.

Evaluation of dried pasta features

Employing a laboratory pasta line, dried (elbow shape) pasta was prepared, following the internal method of Vítová (2009). The link compose of pasta press Korgold TR 70 (Korngold, Austria), pre-drier Sun P+ and drying kiln Sun 405/2 (Mezos, Czech Republic), simulating process in factory. Prepared pasta is evaluated during pressing and in raw – dried – cooked forms by 12 characteristics in total. According to selected three ones (absorption, swelling index, sediment height), basic comparison of different pasta types could be carried out. Pressing of FF-fortified pasta passed off in standard way, pasta surface temperature did not overcome recommendedlevel 40 °C. Neither raw nor dried elbow pasta did not demonstrate an excessive shape deformation. After drying, pasta colour corresponded with FF type and addition level – GF contributed to lighter and BF to pleasant darker shade in surface colour of basic product. For all pasta variants, optimal cooking time reached a standard duration (8.00 min), but decrease in absorption, about 7.5% to 20.0% was evaluated. Swelling index, which characterises ability to absorb boiling water, was higher for samples enrichen by GF without impact of granulation and addition level. For pasta involving finer BF (300-500 μm), a lowering of the feature was observed. Sediment volume express a mass extracted during cooking into salt water, determined as a height of turbidity after 1 hour of standing. The feature has a relation to polysaccharides components in recipe, and measured values indicate a dependence on the FF addition level and its granulation.

Pasta quality features appointed to the dough one did not changed the data variation seriously – the PC1 explained 47% and the PC2 32% of the data scatter (Figure 5). Absorption of cooked pasta corresponds strongly with mechanical properties of proteins and polysaccharides in non-fermented dough, and swelling index with Peak viscosity on the RVA curve. Pasta samples containing either GF or BF were statistically different similarly to bread and biscuit items; within the biplot, pasta consumer quality belonged to the dominant quality features (57% and 29% of variation covered under PC1 and PC2, respectively).

CONCLUSION

Owing to chemical composition, flax seed and dietary fibre have a potential to be used in food industry. Dietary fibre gained from seeds of common flax is declared as

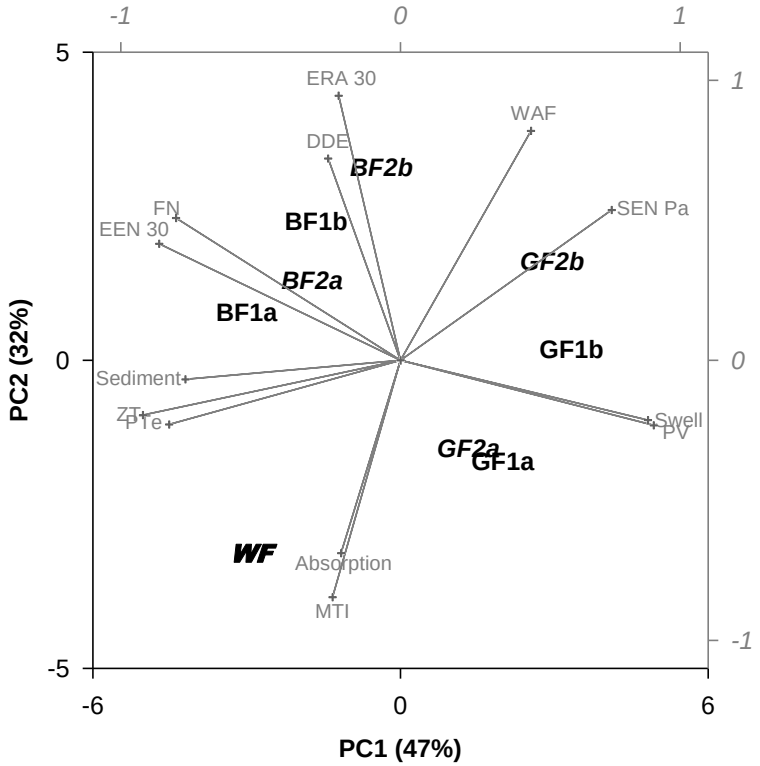

Figure 5 Principal component (PC) analysis of flax fibre effect on dough and pasta technological quality. FN – Falling number, ZT – Zeleny test; WAB – water absorption, DDE – dough development time, MTI – mixing tolerance index (dough softening degree); ERA 30, EEN 30 – extensigraph elasticity-to-extensibility ratio and energy, respectively (dough resting 30 min); PTe – pasting temperature, PV – peak viscosity; Absorption – amount of water absorbed by pasta, Swell – Swelling ratio, Sediment – height of sediment after pasta cooking, SEN Pa – pasta sensory profile. For samples coding, see Table 1.

source of plant proteins, lipids of valuable constitution and non-starch polysaccharides. Added into wheat flour, flax dietary fibre changed its technological and rheological behaviour in correspondence with flax fibre type (yellow/brown flax), fibre granulation as well as addition level. Compared to wheat flour, Zeleny test values demonstrated a lowering of protein quality and of Falling number soft increase, although the changes extent was significantly different between neither the fibre types nor both granulations. Wheat flour enrichment was verifiably reflected in water absorption rise and in the RVA features scatter; the recorded viscosity parameters differentiated flour composites containing yellow and brown flax dietary fibre. Rheological characteristics of fermented composite dough variants were less affected compared to wheat control as well as in relation to flax dietary fibre types, because observed differences were close to measurement accuracy of the internal methods. Bread buns containing flax dietary fibre was characterised by lower specific volume and less vaulted shape. According to objective quality features, shape and crumb of bread prepared from flour composite with fibre from yellow flax seeds were closer to wheat bread control characteristics. Towards to cut-off biscuit attributes, flax fibre from yellow seeds had stronger positive effect than the brown one. Enrichment of dried wheat pasta recipe did not affect its standard shape, both in raw and dried stage; after cooking, golden flax counterparts scored better. During sensory tests, all cereal product enhanced by flax fibre were described as acceptable for common consumers. In relation to tested recipe composition, a higher technological potential was observed for commercial fibre gained from yellow flax seeds, produced by the company Walramcom, the New Zeeland.

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Contact address:

Marie Hrušková, University of Chemistry and Technology Prague, Faculty of Food and Biochemical Technology, Department of Carbohydrates and Cereals, Technická 5, 166 28 Praha 6, Czech Republic, E-mail: Marie.Hruskova@vscht.cz.

Ivan Švec, University of Chemistry and Technology Prague, Faculty of Food and Biochemical Technology, Department of Carbohydrates ad Cereals, Technická 5, 166 28 Praha 6, Czech Republic, E-mail: Ivan.Svec@vscht.cz.

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EFFECT OF SPICES COMMERCIAL MIXTURE WITH GLUCONO-DELTA-LACTONE ON THE QUALITY OF FERMENTED DRY-CURED SAUSAGES

Miroslav Kročko, Margita Čanigová, Viera Ducková, Ondřej Bučko

ABSTRACT

The main fermented meat products are fermented sausages in which lactic acid bacteria (LAB) are the essential agents of the ripening process. Their application as starter organisms ensures the dominance of the starter during the whole ripening process. However, when no starter cultures are used, direct addition of acids like a glucono-delta-lactone (GdL) is preferred. The goal of this study was to determine the influence of commercial spices mixtur (containing GdL) on selected technological parameters of fermented dry-cured sausages – Danube sausage in comparison with currently available conventional spices. Comparison was evaluated also with addition of starter cultures. Determinations of technological (value of pH, water activity, color) and microbiological properties (count of *Lactobacillus* spp., *Enterobacteriaceae* family, yeasts and moulds) were realized after 24 hours, 5 and 30 days. The sensory analysis of sausages was carried out after 30 days of ripening process. In sausages with the addition of commercial spice mixture in combination with starter culture were determined the lowest values of pH and a_w at the end of ripening process (30 days). Bacteria of *Enterobacteriaceae* family were occurred in the samples with the addition of currently available conventional spices at the beginning of ripening, but after 5 days of ripening were bacteria of this family not detected. The counts of yeasts in analyzed samples were not detected. Counts of LAB at the end of ripening proccess (30 days) were lower in coparison with result obtained after 5 days; however their count was comparable with count determined at the beginig of the ripening. Our results show, that the combination of starter culture and commercial spice mixture containig GdL may cause excesive sour taste and sensory defect of dry fermented meat products.

Keywords: GdL; dry-fermented sausages; starter culture; colour; sensory evaluation

INTRODUCTION

Nowdays, fermented sausage production can be considered more than a method of preservation – as, instead, a process of transformation, diversification which is strongly linked to culture and tradition of individual countries **(Fernández el al., 2000, Liu et al., 2011)**.

Fermented meat is produced with the addition of microbes when different condiments are mixed together with meat. The microbiota involved in the fermenting process is diverse and complex, and closely related to the ripening technique. Lactic acid bacteria (LAB) are usually present in high hygienic quality raw meat at low amounts and dominate the fermentation later **(Tu, et al., 2010)**. Their presence effectively prevents harmful bacteria growth and controls the fermentation processes. During the fermentation, acids and alcohols are produced, leading to a decrease of pH **(Xu, et al., 2008)**. *Lactobacillus* species are the most prevalent microorganisms in dry fermented sausages, and their use as starter cultures is widespread **(Hammes et al., 1990)**. Even though lactic acid bacteria are known as weak lipolytic and proteolytic organisms **(Johansson et al., 1994).**

In many cases, particularly when no starter cultures are used, direct addition of acids is preferred in order to assure pH lowering within a very short time. Common organic acids are used for this purpose, mainly lactic and citric acids, as well as their sodium and potassium salts, which show much less ability to lower pH values. Besides these, an acid-related molecule may also be used: glucono-delta-lactone (GdL) **(Toldrá, 2007)**.

GdL is a Generally Recognized as Safe (GRAS) substance and is a weak acid, which converts to gluconic acid in water and slowly dissociates into hydrogen ions with time **(Chang et al., 2009)**. After all, GdL slowly hydrolyzes to gluconic acid with a resulting reduction in pH, which finally causes the residual nitrite reduction **(Juncher et al., 2000)**. However, GdL does not control the indigenous flora, and consequently, using only GdL might result in post-acidification giving fault fermentation and sensory drawbacks **(Andersen and Cislaghi, 2007)**. Using a combination of starter culture and spices has resulted in changes in certain microbial properties, change in free fatty acids and effectively in product lowers the pH and a_w **(Zhao et al., 2011)**. Therefore, the aim of this study was to evaluate the effect of commercial spice mixture containing GdL in combination with starter culture on physical, microbiological and sensory characteristics of fermented sausages during ripening.

MATERIAL AND METHODOLOGY

Occurrence of GdL in spice mixture was determined by electrophoretic analyzer (Villa Labeco, Slovakia)

Pork and beef lean meat in ratio 2 : 1 in combination with back pork fat (30%) were trimmed and cured (2.0% salt and 0.01% nitrite). Then the cured trimmed meat mix was divided into four equal parts. Currently available conventional spices paprika (100 $g.kg^{-1}$), pepper (4 $g.kg^{-1}$), caraway (4 $g.kg^{-1}$), garlic (10 $g.kg^{-1}$), spicy paprika (4 $g.kg^{-1}$) were added to the first (C) and second parts (CC) of sausage mixture. Commercial spice mixture designed for meat processing plants contain GdL, was added to the third (M) and fourth part (MC) of sausage mixture. Added conventional spices and commercial spice mixture came from the same manufacture company and were sold under the same brand. Furthermore, second part (CC) and fourth part (MC) of sausage mixture contains starter culture Lyocarni SHI-59 (Clerici Sacco, Italy) in amount 0.2 $g.kg^{-1}$. Each part of sausage mixture was separately minced (4mm blade) and filled into 34 mm natural sausage casings, smoked and ripened in climatic chamber for 30 days.

Determinations of technological and microbiological properties were realized after 24 hours, 5 and 30 days.

Determination of pH value:

The pH value of Danube sausages was measured using a Gryf 209 (Gryf HB, Czech Republic) apparatus during whole period of ripening.

Determination of color: Color spaces L*, a*, b* was determined by CM 2600D spectrophotometer (Konica Minolta, Germany) after homogenization of samples. Color on the surface of homogenized sausages was measured with SCE (Specular Component Excluded).

Determination of a_w was determined by Testo 645 (Germany).

Microbioological examination

The samples of sausages (5 g) were taken after specified storage periods and homogenized in saline for 30 second by apparatus Heidolph DIAX 900 (*Heidolph*, Germany). The samples for enumeration of mesophilic bacteria (MBC) were cultured on selective diagnostic plate count agar (*Biokar Diagnostic*, France) at temperature 30 ±1 °C for 72 hours. The samples without addition of starter cultures (M and C) for enumeration of indigenous lactic acid bacteria count (LAB) were cultured on MRS agar (*Himedia*, India) at temperature 30 ±1 °C for 5 days.

The samples with addition of starter cultures (CC and MC) for enumeration of lactic acid bacteria count of genus *Lactobacillus* (LAB) were cultured on MRS agar (*Himedia*, India) at temperature 37 ±1 °C for 5 days in anaerobic conditions (Anaerogen, Oxoid UK). Count of *Enterobacteriaceae* family (ETB) was determined on VRBG agar (*Himedia*, India) at temperature 37 °C after 24 hours of cultivation. Count of yeasts and moulds were determined on DRBC and DG18 agar (Merck, Germany) at temperature 25 °C after 5 days.

Sensory evaluation

The sensory analysis of sausages was carried out after 30 days of ripening process. Samples of sausages before and after heat treatment (heating in 80°C water, while in core of sausages reached the temperature 70 °C for 10 minutes) were evaluated by a 6-member semi-trained panel of laboratory co-workers. Panelists evaluate, color, aroma, taste on 5-point hedonic scale where 1 (the worst) and 5 (the best) were the extremes of each characteristic.

RESULTS AND DISCUSSION

The following 4 types of sausages were evaluated:

- sample C – sausages with addition of conventional spices,
- sample M – sausages with addition of commercial spice mixture (with GdL),
-sample CC – sausages with addition of conventional spices and starter culture,
-sample MC – sausages with addition of commercial spice mixture (with GdL) and starter culture.

The lowest values of pH in samples of fermented sausages were determined after 5 days of ripening. The lowest pH values (5.25) after 30 days of ripening were determined in the samples of M and MC fermented sausages (Figure 1). According to Slovak decree no. 1895/2004-100, the both samples containing commercial spices mixture (M and MC) may be included into subgroups with pH value below 5.5, and mark them as fermented products. The products C and MC were classified according to pH and a_w value to subgroups dried (pH 5.5 to 6.2). The higher pH value of the sample C may be due to indigenous lactobacilli with the low acidifying ability **(Casaburi et al., 2007)**.

Continuous decline of the water activity was observed during whole ripening process. The most significant decrease of water activity was found in products M. The water activity after 24 hours in all samples was

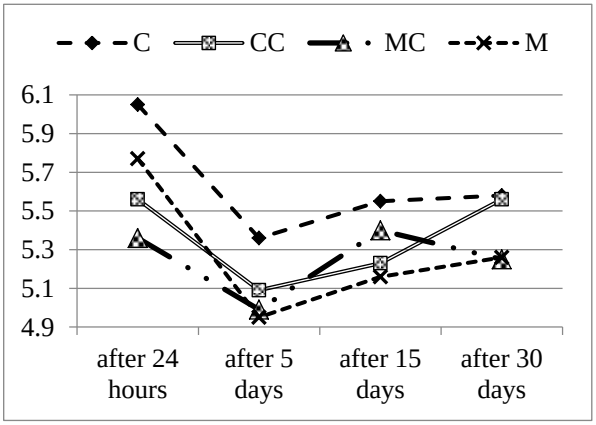

Figure 1 Changes of pH values during ripening of Danube sausages.

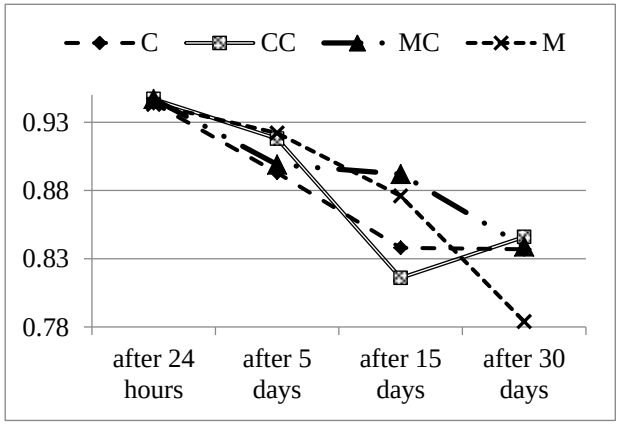

Figure 2 Changes of a_w values during ripening of Danube sausages.

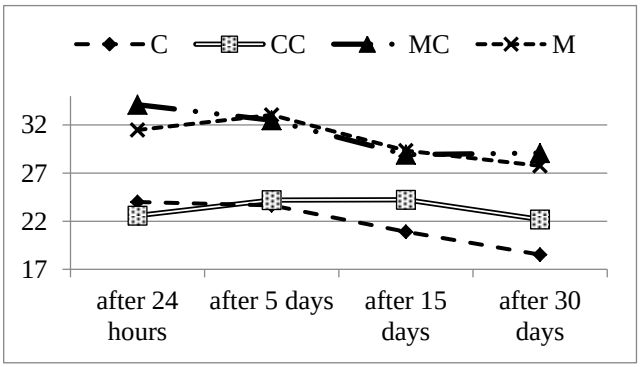

Figure 3 Intensity of red color (a*) during ripening of fermented sausages.

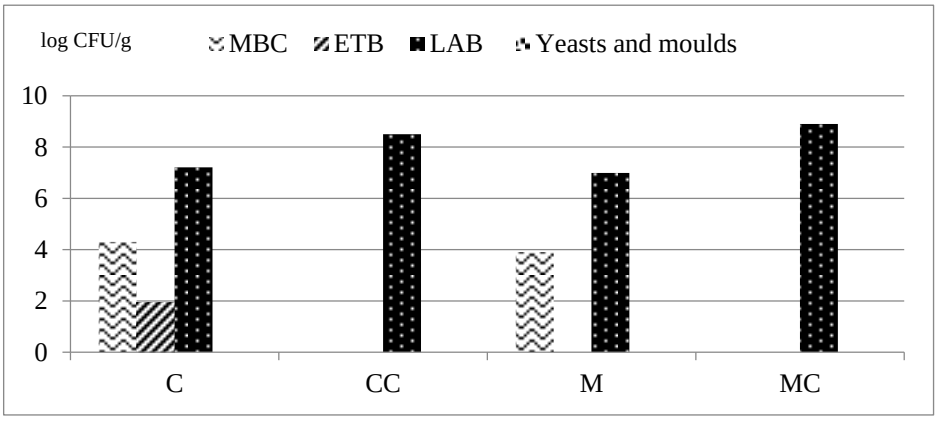

Figure 4 Occurrence of microorganisms in fermented sausages after 24 hours of ripening.

characterized by values close to the level which is an average value of 0.945 (Figure 2).

Intensity of red color (a *) during the whole period of ripening was determined in fermented sausages with the addition of commercial spice mixture, regardless of the addition of starter culture (CM and MC). These results are in contrary with previous studies **(Hong et al., 2008; Hong and Chin 2010)** noted increasing the GdL level significantly decreased the a* value (redness) of the fermented meat products.

The cause is probably due to the addition of colorant cochineal also called carmine (E 120) in a commercial spice mixture.

The addition of starter culture to the products with the addition of currently available conventional spices (CC) also increased the intensity of the red color (Figure 3).

In products with only the addition of currently available conventional spice (C) was found after 24 hours the number of microorganisms of the family *Enterobacteriaceae* 1.94 log CFU.g^{-1} (Figure 4). During the next period of maturation were not bacteria of this family detected. The reason for the occurrence of the family *Enterobacteriaceae* could be contamination of traditional spices used in manufacture.

In the all samples of fermented sausages count of LAB up to 5 days of ripening increased. Counts of LAB at the end of ripening proccess (30 days) were lower in coparison with result obtained after 5 days; however their count was comparable with count determined at the beginig of the ripening. Addition of commercial spice mixture which contains GdL had no effect on the count of LAB in the samples of fermented sausages during whole period of ripening proccess.

The rapid growth of LAB in the initial stage of fermentation is beneficial in reducing the pH of the fermented product and inhibits undesirable bacteria such as microorganisms of the family *Enterobacteriaceae* **(Essid and Hassouna, 2013)**.

Occurrences of yeasts in fermented meat products were not detected. According to **Fernández-López et al. (2008)** the number of yeast in fermented meat products stored 30 days is usual. Yeasts are characterized by lipolithic activity of secondary importance and can also contribute to the formation of an organoleptic profile of the final products. The sensory evaluation of fermented sausages was in raw state (uncooked), which was supposed to evoke the same possibilities for evaluating as consumers in stores. The highest values for appearance were after 30 days of ripening assigned to both samples of fermented sausages with addition of starter cultures (CC, MC). The products with added conventional spices (C) and commercial spice mixtures (M) without addition of starter cultures were characterized by strongly wrinkled surface and yellowish fat grains. The characterized intensity of red color in sliced samples was determined in sausages with added commercial spice mixtures (M, MC). Fermented sausages produced with currently available conventional spices (C), were characterized by a weaker shade color after red paprika. In the final assessment of raw sausages without heat treatment were the highest values for overall

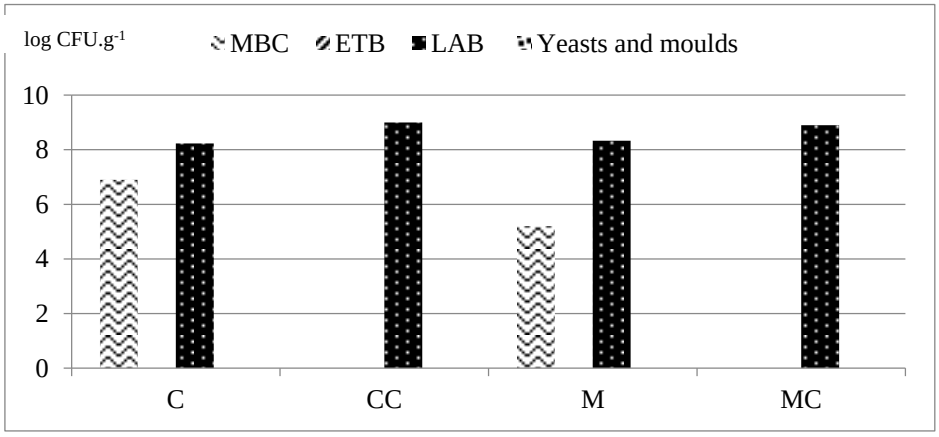

Figure 5 Occurrence of microorganisms in fermented sausages after 5 days of ripening.

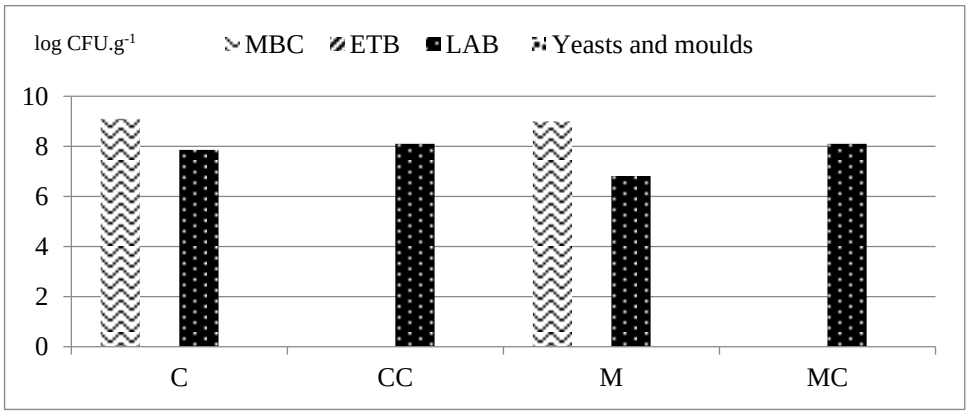

Figure 6 Occurrence of microorganisms in fermented sausages after 30 days of ripening.

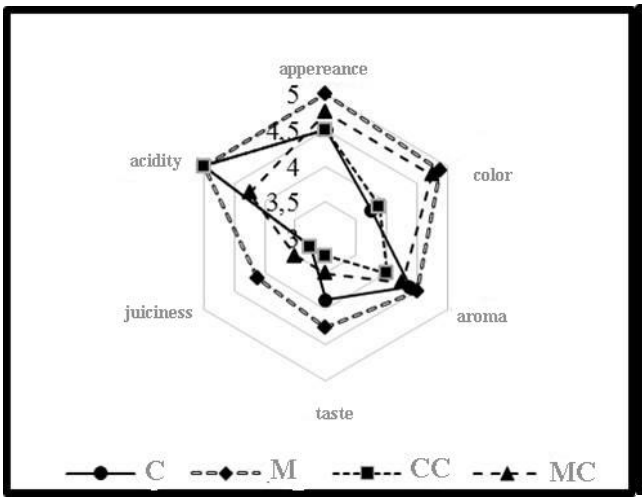

Figure 7 Sensory analyses of fermented sausages before heat treatment.

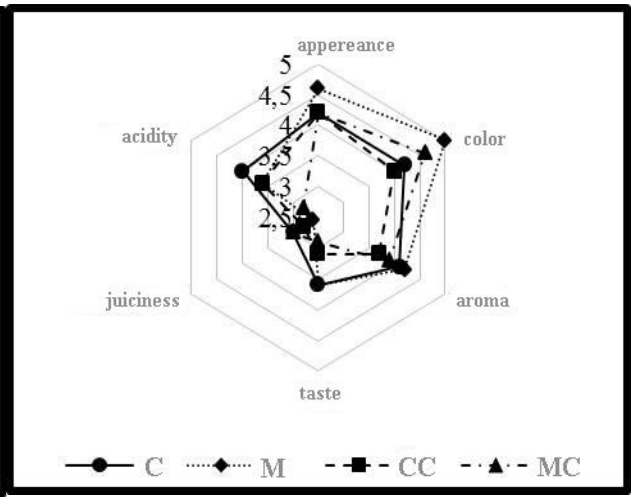

Figure 8 Sensory analyses of fermented sausages after heat treatment.

acceptability assigned to the samples with the addition of commercial spice mixture with combination of starter culture (MC).

The highest intensity of characteristic odor evaluated after heat treatment was found in products using currently available conventional spices (C) and commercial spice mixtures (M) without addition of starter cultures. On the other hand sausages with commercial spice mixture and starter culture (MC) due to sour taste reached the lowest values for overall taste acceptability. Increased sour taste of these products was probably caused by combining of commercial spice mixture containing GdL and starter culture. Also **Schillinger and Luecke, (1989)** and **Feiner (2006)** reported that application of GdL might negatively influence the taste (metallic off-flavour and bitterness), texture (grittiness), and color (paleness) of the salami relative to the amount of GdL added. Furthermore, high levels of GdL might promote growth of peroxide-forming lactobacilli resulting in rancidity and further color problems.

CONCLUSION

Composition of commercial spice mixtures designated for the manufacture of meat products is currently considered as know-how of manufacturing companies. Therefore, the packaging of these spice products does not provide the exact composition. It is necessary to label on the package of commercial spice mixtures the presence and quantity of GdL, especially in production where is probably posibility to use starter cultures. Our results show, that the combination of starter culture and commercial spice mixture containig GdL may cause excesive sour taste and sensory defect of dry fermented meat products.

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Contact address:

Miroslav Kročko, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department for Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: mirokrocko@yahoo.com.

Margita Čanigová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department for Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: margita.canigova@yahoo.com.

Viera Ducková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department for Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: viera.duckova@uniag.sk.

Ondřej Bučko, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Department of Animal Husbandry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: ondrej.bucko@uniag.sk.

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QUERCETIN-INDUCED CHANGES IN FEMORAL BONE MICROSTRUCTURE OF ADULT MALE RABBITS

Ramona Babosová, Radoslav Omelka, Hana Ďúranová, Veronika Kováčová, Martina Lukáčová, Marcela Capcarová, Monika Martiniaková

ABSTRACT

Flavonoids are a group of plant metabolites with antioxidant effects. One of the most abundant flavonoids in the human diet is quercetin. It is found widely in fruits, vegetables and has a lot of beneficial effects on human health. Quercetin has a positive pharmacological effect on bone metabolism and it prevents the organism against bone loss. However, its impact on the size of basic structural units of the compact bone is still unknown. Therefore, the aim of present study was to investigate the impact of the quercetin on femoral bone microstructure in 5-month-old male rabbits. Five rabbits of Californian broiler line were randomly divided into two groups. In the experimental group (E group; n=3), animals were intramuscularly injected with quercetin at dose 1000 $\mu g.kg^{-1}$ body weight (bw) for 90 days, 3 times per week. Two rabbits without quercetin administration served as a control group (C group). According to our results, intramuscular application of quercetin had an insignificant effect on cortical bone thickness in male rabbits. In these rabbits, changes in qualitative histological characteristics were present in the middle part of the compacta, where primary vascular longitudinal bone tissue was present and expanded there from the periosteum. Also, a lower number of secondary osteons was found in these animals. From the histomorphometrical point of view, significantly decreased sizes of primary osteons' vascular canals and secondary osteons (p <0.05) were found in rabbits administered by quercetin. Our findings indicate that subchronic administration of quercetin at the dose used in our study had considerable impact on both qualitative and quantitative histological characteristics of the compact bone in adult male rabbits.

Keywords: quercetin; femoral bone; histomorphometry; rabbit

INTRODUCTION

Flavonoids are a group of natural polyphenolic substances which consists of two aromatic rings linked by 3 carbons, usually in an oxygenated heterocycle ring **(Liu, 2004)**. These aromatic secondary plant metabolites have been recognized as important bioactive compounds due to their physiological and pharmacological role, and their health benefits **(Hooper and Cassidy, 2006)**. Fruits and vegetables, tea, and cocoa are rich natural sources of flavonoids **(Chen et al., 2010; Egert and Rimbach, 2011; Sekeroğlu and Sekeroğlu, 2012)**. In human diet, one of the most important vegetable with rich content of antioxidant polyphenols is onion. The results by **Kavalcová et al. (2015)** showed that a higher content of polyphenols and thus, a higher antioxidant activity have more colorful varieties of onions. According to **Danihelová and Šturdík (2011)**, average daily intake of flavonoids is strongly dependent on individual, country and culture usages. It is approximately in the range of 150 to 300 mg/day

In nature, flavonoids are most frequently found as conjugates in glycosylated or esterified forms; however, they can occur as aglycones, especially as a result of the effects of food processing **(Aggarwal and Heber, 2014)**. Many flavonoids are shown to have antioxidative activity, free-radical scavenging capacity, coronary heart disease prevention, and anticancer activity **(Yao et al., 2004)**. Their antioxidant capacity is associated with the presence of series of structural characteristics (most probably related to the phenolic hydroxyl groups attached to the ring structure) that allow them to quelate ions of transition metals such as Fe^{2+}, Cu^{2+}, or Zn^{2+} and to catalyze the electron transport **(Braun et al., 2011)**. Moreover, they are able to inhibit lipid peroxidation and platelet aggregation and improve increased capillary permeability and fragility **(Hubbard et al., 2004; Cirico and Omaye, 2006)**.

In the recent past, dietary supplements of flavonoids, as their alternative sources, have become increasingly popular. However, it is important to point out that natural sources of flavonoids contain a complex mixture of secondary plant metabolites and not only flavonoids *per se* **(Crassidy et al., 2011)**. This complex mixture cannot be simply exchanged by single purified substances as dietary supplements. Therefore, it is very essential to evaluate possible adverse effects of purified flavonoids as dietary supplements on human health. Indeed, there is growing evidence that purified flavonoids given in high doses may affect trace element, folate, and vitamin C status. Also, they can exhibit antithyroid and goitrogenic activities **(Egert and Rimbach, 2011)**.

One of the most widely distributed flavonoid in plants is quercetin (3, 3', 4', 5, 7-pentahydroxyflavone; **Liang et al., 2011**). Quercetin is found in many common foods including apples, tea, onions, nuts, berries, cauliflower, cabbage and many other foods **(Lakhanpal and Rai, 2007)**. The normal intake of quercetin is less than 5-40 mg/day. However, people who eat the peel of food with high amounts of quercetin may consume 200-500 mg/day **(Harwood et al., 2007)**. Only 30-50% of ingested quercetin is absorbed, the rest passes through gastro-intestinal tract **(Ross and Kasum, 2002)**.

Quercetin has a broad range of significant health promoting properties **(Agullo et al. 1997; Verhoeyen et al., 2002; Boots et al., 2007)**. According to several authors **(Formica and Regelson, 1995; Manach et al., 1996; Boik, 2001; Satyanarayana et al., 2001; Brookes et al., 2002; Davis et al., 2009; Wein et al., 2013; Wu et al., 2014; Forte et al., 2016)** quercetin has cardioprotective, anticarcinogenogenic, antioxidant, anti-inflammatory, antibacterial and antiapoptic properties. It facilitates apoptosis of tumor cells, in part through depression of an endogenous cytoprotective molecule, heat shock protein 70 **(Hosokawa et al., 1990)**. As well, quercetin may inhibit apoptosis in some nontumorigenic cells. For example, quercetin inhibits hydrogen peroxide (H_2O_2) induced apoptosis of mesangial cells, fibroblasts and epithelial cells **(Ishikawa and Kitamura, 2000)**.

This flavonoid also disposes reactive oxygen species (ROS) and reactive nitrogen species (RNS) scavenging activity **(Heijnen et al., 2001; Nickel et al., 2011; Dehghan and Khoshkam, 2012)** under *in vitro* and *in vivo* conditions **(Choi et al., 2001; Nabavi et al., 2012)**. Therefore, it has often been associated with the reduced risk of oxidative-stress related chronic diseases such as coronary heart disease, stroke and diabetes **(Skibola and Smith, 2000)**.

On the other hand, quercetin has potentially toxic effects, including its mutagenicity, prooxidant activity, mitochondrial toxicity, and inhibition of key enzymes involved in hormone metabolism **(Okamoto, 2005; Zhang et al., 2009)**. **Dunnick and Hailey (1992)** reported that high doses of quercetin over several years might result in the formation of tumors in the kidney of rats. The results by **Rise et al. (2006)** showed that quercetin can modulate ovarian functions by interfering with cell steroidogenic activity and angiogenic activity. Quercetin can also be a potential neurotoxic substance **(Jakubowicz-Gil et al., 2008)**. According to **Robaszkiewicz et al. (2007)**, quercetin-induced antioxidant or prooxidant effects are largely relates to its dose given to biological system. At concentrations > 50 μM, quercetin is able to participate in the oxidation of NADPH in liver cells, shifting the cellular conditions to a more oxidized states **(Buss et al., 2005).**

Regarding the bone, quercetin has a positive pharmacological effect on bone metabolism and it prevents the organism against bone loss **(Boots et al., 2007; Sharan et al., 2011)**. It inhibits mRNA expression of osteoclast-related genes and osteoclast differentiation, thereby reduces bone resorption **(Guo et al., 2012)**.

The studies by **Notoya et al. (2004)** and **Wattel et al. (2004)** revealed that inhibitory potential of quercetin on *in vitro* osteoclastic differentiation is connected via a mechanism involving NF kappa B and activator protein 1 (AP-1). Also, increased alkaline phosphatase activity in MG-63 osteoblasts followed by quercetin application was demonstrated **(Robaszkiewicz et al., 2007)**. **Zhou and Lin (2014)** reported that quercetin could enhance the osteogenic differentiation of adipose-derived stem cells (ASCs) and osteoblastic MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells. Moreover, it could stimulate Osterix (Osx), BMP-2, Runx2, OCN, OPN, COL1 and ALP gene expression in ASCs, and increase bone sialoprotein (BSP) and OCN gene expression in osteoblastic MC3T3-E1 cells **(Kim et al., 2006; Satué et al., 2013)**. However, the effect of quercetin on osteoblast function is contradictory **(Yamaguchi and Weitzmann, 2011)**. According to **Prouillet et al. (2004)** it stimulates proliferation and differentiation of rat calvarial osteoblasts and MG-63 osteoblast-like cells. **Braun et al. (2011)** have found protective effect of quercetin on primary human osteoblasts against the toxic influence of cigarette smoke. This fact indicates that a dietary supplementation with quercetin could improve bone structure, skeletal integrity, and even fracture healing in smokers. On the contrary to above findings, **Kanno et al. (2004)** mention that quercetin induces apoptosis of MC3T3-E1 mouse calvarial osteoblasts. **Notoya et al. (2003)** found that it inhibited not only the proliferation but also the differentiation and mineralization of of rat calvarial osteoblast-like cells (ROB cells; **Hagiwara et al., 1996; Partridge et al., 1981)**. Quercetin-induced apoptosis (through a mitochondria-dependent mechanism involving ERK activation) and inhibition of migration (through activation of ERK and p38 pathways) of osteoblasts were also showed in the research by **Nam et al. (2008)**.

The impact of quercetin on histomorphometry of basic structural units of the compact bone is still unknown. Therefore, the aim of our study was to investigate the effect of intramuscular application of quercetin on femoral bone microstructure in adult male rabbits.

MATERIAL AND METHODOLOGY

Our research was carried out on five male rabbits of meat line M91, maternal albinotic line (crossbreed New Zealand White, Buskat rabbit, French Silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big Light Silver) of approximately 5 months of age, with a body weight 4.00 ±0.5 kg. Animals were obtained from an experimental farm of the Animal Production Research Centre in Nitra (Slovak Republic) and were housed in individual flat-deck wire cages. The animals were maintained under constant conditions of light (12-h light/12-h dark), temperature (20-24 °C) and humidity (55% ±10%), with access to food (feed mixture) and drinking water *ad libitum*. The rabbits were randomly assigned into two groups. In the first group (E group; n=3), quercetin was applied intramuscularly in the concentration of 1000 $\mu g.kg^{-1}$bw 3 times per week, for 90 days. The dose of quercetin (reflecting the constant exposure of animals to quercetin in rabbit feed) was chosen based on the literature data **(Choi and Li, 2005; Knab et al., 2011; Lesniak-Walentyn et al., 2013)**. Two rabbits without quercetin application served as a control group (C group). Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures were approved by the State Veterinary and Food Institute

of Slovak Republic, no. 3398/11-221/3 and ethics committee.

At the end of experimental period (after 90 days), all rabbits were killed and their femora were used for histological analyses. Thin sections from femora were prepared according to the methodology of **Martiniaková et al. (2008)**. The qualitative histological characteristics of compact bone were determined according to the internationally accepted classification systems of **Enlow and Brown (1956) and de Ricqlés et al. (1991),** who classified bone tissue into three broad categories: primary vascular tissue, non-vascular tissue and Haversian bone tissue. 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 minimum and maximum diameters of primary osteons' vascular canals, Haversian canals, and secondary osteons in all fields (i.e., anterior, posterior, medial and lateral) of the thin sections. The 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.

Statistical analysis was performed using SPSS 8.0 software. All data were expressed as mean ±standard deviation (SD). The unpaired t-test was used for establishing statistical significance (p <0.05) between both groups of rabbits.

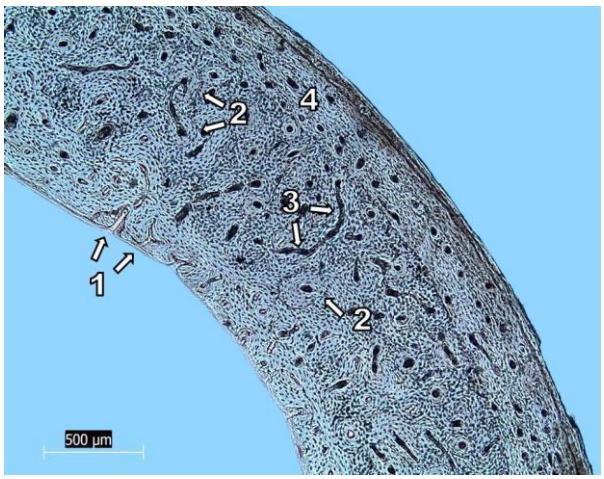
RESULTS AND DISCUSSION

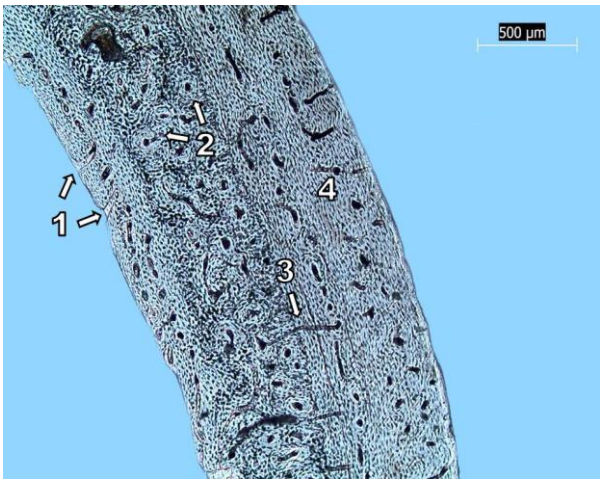
Our results showed an insignificant effect of quercetin on cortical bone thickness in male rabbits (1035.56 ±159.42 μm and 1025.06 ±209.09 μm in rabbits from E and C groups, respectively).

Compact bone microstructure in rabbits from C group (Fig.1) was formed near endosteal bone surfaces by primary vascular radial, irregular Haversian and/or dense Haversian bone tissues. The middle part of the compact bone considered of a layer of irregular and/or dense Haversian bone tissues. Secondary osteons were often connected with Volkmann's canals. The periosteal bone surface mostly consisted of primary vascular longitudinal bone tissue; irregular Haversian bone tissue was observed only in anterior side. These findings are consistent with the results of several authors **(Enlow and Brown, 1958; Martiniaková et al., 2003; Martiniaková et al., 2006)**.

In rabbits from E group, endosteal bone surface was composted by primary vascular radial and irregular Haversian bone tissues. Primary vascular longitudinal bone tissue was in some areas (anterior and posterior) near endosteal surface completely resorbed. The rabbits intramuscularly administered by quercetin had fewer secondary osteons in the middle part of *substantia compacta* because primary vascular longitudinal bone tissue expanded into this part of bone from periosteum. The periosteal border was formed only by primary vascular longitudinal bone tissue (Fig. 2).

Intramuscular administration of quercetin caused evident alterations in femoral bone microstructure of male rabbits. A lower number of secondary osteons in the middle parts of the *substantia compacta* could be associated with accelerated bone resorption. ROS have been reported to play a crucial role in the process of bone resorption **(Halliwell et al., 1992; Yang et al., 1998)**. During this process, osteoclasts produce large amounts of ROS, and their excessive accumulation inhibits bone formation and stimulates further bone resorption **(Baek et al., 2010)**. Quercetin has been described as the protector against ROS RNS **(Nickel et al., 2011; Kovacevic and Matulic, 2013)**. However, quercetin has the potential to produce ROS at high doses **(Rahman et al., 1992)**. In the process of scavenging reactive species, quercetin may be converted into potentially harmful oxidation products or subjected to *in vitro* oxidative degradation resulting in the formation of an ortho-quinone and the subsequent production of ROS (i.e., superoxide and hydrogen peroxide; **Boots et al., 2003**). The resultant prooxidant properties of quercetin are responsible for its mutagenic and cytotoxic effects **(Sahu**





1 - primary vascular radial bone tissue, 2 - several secondary osteons form irregular Haversian bone tissue,
3 - Volkmann's canals, 4 - primary vascular longitudinal bone tissue

Figure 1 Microstructure of femoral bone in rabbits from control (C) group.

Figure 2 Microstructure of femoral bone in rabbits from experimental (E) group.

Table 1 Data on vascular canals of primary osteons in male rabbits from C and E groups.

Rabbit's group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
C	80	344.68 ±50.07	66.43 ±4.78	11.48 ±0.99	9.60 ±0.98
E	120	317.35 ±51.82	63.80 ±5.11	11.10 ±1.00	9.13 ±1.00
t-test		***p* <0.05**	***p* <0.05**	**NS**	***p* <0.05**

Note: N: number of measured structures; NS: non-significant changes.

Table 2 Data on Haversian canals in male rabbits from C and E groups.

Rabbit's group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
C	80	322.15 ±65.07	64.25 ±6.53	11.13 ±1.35	9.20 ±1.19
E	120	301.32 ±56.49	62.20 ±6.06	10.82 ±1.37	8.90 ±0.98
t-test		**NS**	**NS**	**NS**	**NS**

Note: N: number of measured structures; NS: non-significant changes.

Table 3 Data on secondary osteons in male rabbits from C and E groups.

Rabbit's group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
C	80	5979.63 ±2816.19	273.19 ±60.51	47.97 ±11.30	38.12 ±9.18
E	120	4629.72 ±1888.92	244.67 ±45.93	43.81 ±8.79	32.86 ±8.00
t-test		***p* <0.05**	***p* <0.05**	**NS**	***p* <0.05**

Note: N: number of measured structures; NS: non-significant changes.

and Washington, 1991; Soria et al., 2010). So, it can indicate toxicity in that case, such as an induction of apoptosis of osteoblasts **(Notoya et al., 2003; Spencer et al., 2003; Son et al., 2008)**.

From histomorphometrical point of view, 200 vascular canals of primary osteons, 200 Haversian canals, and 200 secondary osteons were measured in rabbits from E and C groups. The results are summarized in Tables 1, 2 and 3. The values for measured parameters (area, perimeter, maximum and minimum diameters) of primary osteons' vascular canals and secondary osteons (except for their maximum diameters) were significantly lower (p <0.05) in rabbits from E group. The values from parameters of Haversian canals did not differ between both groups of rabbits. The primary osteons' vascular canals constriction in rabbits from E group can be related to the adverse effect ofquercetin on blood vessels, which provide bone nutrition **(Greenlee and Dunnell, 2010)**. According to **Pries et al. (2005)**, blood vessels are able to adapt its structure (vascular remodeling) as a response to continuous functional changes. *In vitro* studies **(Leikert et al., 2002; Huisman et al., 2004; Wallerath et al., 2005; Jackson and Venema, 2006; Schmitt and Dirsch, 2009)** suggest an adverse effect of quercetin on the enzyme nitric oxidesynthase (eNOS) expression and endothelial nitric oxide (NO) production. NO acts as a potential vasodilator,and itcontributes to the migration and growth of endothelial cells necessary for initiation of angiogenesis *in vivo* **(Carmeliet, 2000; Jackson and Venema, 2006)**. We suppose that alterations in the size of primary osteons' vascular canals in rabbits from E and C groups are connected with an adverse effect of quercetin on the expression of eNOS.

We assume that significant differences (p <0.05) in the size of secondary osteons between rabbits from E and C groups may be associated with the destruction of collagen fibers which are present in the osteons **(Dylevský, 2007)**. **Kang et al. (2001)** found that quercetin significantly inhibited collagens I and III expression and had a growth-inhibitory effect on keloid-derived fibroblasts. The adverse impact of various concentrations of quercetin (20, 40, and 80 $\mu mol.l^{-1}$) on human fibroblasts examined **Stipcevic et al. (2006).** According to these authors, the administration of the highest dose of quercetin leads to significantly decreased collagen concentration (more than 50%) in fibroblasts. We supports that similar effect could also been observed in osteoblasts.

CONCLUSION

The study indicates that intramuscular application of quercetin at dose 1000 $\mu g.kg^{-1}$ bw for 90 days, 3 times per week caused significant changes in qualitative and quantitative histological characteristics of the compact bone tissue in male rabbits. Rabbits exposed to quercetin had a lower number of secondary osteons in the middle part of the *substantia compacta*, and disposed thicker layer of primary vascular longitudinal bone tissue (periost and middle part of the bone). Histomorphometrical evaluations

showed significantly decreased sizes of primary osteons' vascular canals and secondary osteons in males from the E group. Our article provides initial information of the impact of quercetin on femoral bone microstructure in experimental animals.

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Contact address:

Ramona Babosová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra, Slovakia, E-mail: ramona.babosova@gmail.com.

Radoslav Omelka, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Botany and Genetics, 949 74 Nitra, Slovakia, E-mail: romelka@ukf.sk.

Hana Ďúranová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra, Slovakia, E-mail: hduranova@ukf.sk.

Veronika Kováčová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra, Slovakia, E-mail: veronika.kovacova@ukf.sk.

Martina Lukáčová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Botany and Genetics, 949 74 Nitra, Slovakia, E-mail: martina.tkacova@ukf.sk.

Marcela Capcarová, Slovak University of Agriculture in Nitra, Department of Animal Physiology, 949 76 Nitra, Slovakia, E-mail: marcela.capcarova@uniag.sk.

Monika Martiniaková, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra, Slovakia, E-mail: mmartiniakova@ukf.sk.

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ASSESSMENT OF DNA QUALITY IN PROCESSED TUNA MUSCLE TISSUES

Zora Piskatá, Eliška Pospíšilová

ABSTRACT

Authentication of tuna fish products is necessary to assure consumers of accurate labelling of food products. The quality of species specific DNA crucially affects the efficiency of amplification during the subsequent PCR. The problem in DNA detection in canned products lies in the possibility of the fragmentation of DNA during the processing technologies and the use of ingredients (oil, salt, spice), that may inhibit the PCR reaction. In this study three DNA extraction methods were compared: DNeasy Blood and Tissue Kit, DNeasy mericon Food Kit and Chemagic DNA tissue 10 Kit. The quantity and quality of DNA were evaluated by measuring DNA concentration and ratios A260/A280. Several parameters were estimated: the effect of whole and mechanically treated muscle, sterilization procedure used in canned process (high temperature in combination with high pressure) and addition of raw materials. The highest DNA concentrations were observed in non-processed muscle that is not influenced by the sterilization process. Canned whole muscle demonstrated lower DNA yield, and furthermore, the mechanical treatment (canned ground) resulted in lower values of DNA concentration that was registered by using all three types of DNA extraction kits. DNeasy mericon Food Kit produced DNA of higher concentration in non-processed sample, Chemagic DNA tissue 10 Kit delivered higher DNA yields than kits DNeasy Blood and Tissue Kit and DNeasy mericon Food Kit in canned samples, although the purity was lower, but still within the range 1.7 – 2.0. DNA was considered to be satisfactorily pure in all three types of samples and using all three types of DNA isolation. In case of the samples enriched of ingredients and treated with sterilization process as whole or ground muscle Chemagic DNA tissue 10 Kit produced in all samples (whole and ground muscle) the highest values of DNA concentration, but almost all values of A260/A280 were lower than 1.7. Therefore DNeasy mericon Food Kit appears to be a favorite one, in all samples with whole muscle gives higher values of DNA concentrations than DNeasy Blood and Tissue Kit. Addition of ingredients influenced the DNA yield in terms of decreasing in samples containing vinegar and lemon, but some of the ingredients resulted surprisingly in higher yield of DNA. This was not consistent in whole and ground muscle, and the differences were described also among particular kits. The impact of ingredients was not conclusively approved and their importance to the suitability of extracted DNA for PCR amplification is needed to be discussed in further analysis.

Keywords: canned product; fish food; DNA extraction; PCR; *Thunnus albacares*

INTRODUCTION

Fish species identification gains attention due to the commercialization of fish through filleted, salted, smoked or canned fish products. Tuna fish belong among the most economically important fishery resources because are typically used to manufacture canned products, the main format for marketing of these species **(Espineira et al., 2009)**. Different quality and price of tuna species can lead the manufacturers to the tendency to highlight the quality of fish products. From that reason the substitution or mixing of valuable fish by less valuable ones may occur. The Council Regulation (EC) **No. 1536/92** laying down common marketing standards for preserved tuna and bonito states specific rules for the tuna marketing. The species belonging to tuna and bonito are named in the annex of this Regulation. Below is determined, that the trade description on the prepackaging of preserved tuna or bonito shall state the type of fish (tuna or bonito). The identification of tuna and bonito species according to their morphological features is possible only in whole or lightly processed fish. In processed products such as filleted or canned fish the morphological characteristics are removed, hence analytical methods as an important tool for species identification must be used. Analytical methods are focused mainly on protein or DNA molecule, which are extracted from the fish tissues. Due to the protein denaturation caused by heating or canning (high temperature in combination with high pressure) process **(Mackie et al., 1999)**, DNA is more suitable molecular marker for fish species authentication, because it is more resistant to the thermal treatment. Indeed DNA is also degraded into smaller fragments during the thermal process but these are still detectable. **Ram et al. (1996)** claim, that the canning process degrades DNA to fewer than 123 bp in length. Moreover DNA is largely independent of tissue source, age, or sample damage **(Bossier, 1999; Lockley and Bardsley, 2000)**. Nevertheless the fragment size is limited factor for the

subsequent PCR reaction that is based on the selective amplification of specific region of DNA using oligonucleotides **(Lockley and Bardsley, 2000)**. PCR **(Bartlett and Davidson, 1991; Bottero et al., 1997; Dalmasso et al., 2006)** and its modification – PCR-RFLP **(Takeyama et al., 2001, Pardo and Pérez-Villareal, 2004, Lin et al., 2005, Lin and Hwang, 2007)**, PCR-SSCP **(Rehbein et al., 1999; Colombo et al., 2005)**, real-time PCR **(Lopez and Pardo, 2005)** or PCR-ELISA **(Santaclara et al., 2015)** represents crucial approaches available for tuna fish species identification. PCR analysis comprises of DNA extraction from the sample, PCR and electrophoresis, or alternatively other detection system for the final results evaluation. The critical step is extraction of high quality DNA in great enough quantities from the heterogeneous food matrices. In view of the fact, that raw material for the final product manufacture comes under different effect during the manufacturing process (high temperature, high pressure, addition of ingredient, etc.), which considerable influences the quality of DNA **(Chapela et al., 2007, Besbes et al., 2011, Cawthorn et al., 2011)**, it is required for every type of food products to apply and optimize particular DNA isolation procedure. In addition ingredients and other substances presented in food products may work as PCR inhibitors, substances that may negative affect the sensitivity of PCR reaction. Or in another case, the DNA may be stimulated due to the ingredients.

Primary requirement of this study is to find out, how far is DNA influenced by the technological processes using in food industry (mechanical treatment, high temperature, high pressure, addition of ingredients) in model canned samples from the muscle tissue of yellowfin tuna (*Thunnus albacares*) and how the subsequent sample preparation and DNA extraction procedure can affect its qualitative and quantitative parameters.

MATERIAL AND METHODOLOGY

Samples preparation

The samples of tuna fish were prepared from the muscle tissue of yellowfin tuna (*Thunnus albacares*), that was purchased in the Czech market as frozen steak. Its species identity was confirmed via sequencing of the partial sequence of cytochrome *b* gene (Seqme, Hradec Kralove, Czech Republic). Besides non-processed muscle tissue two types of tuna samples that were made under similar conditions used in cans production were prepared - canning of solid piece of muscle (whole) and canning of

Table 1 List of ingredients.

	Raw food	[g]	Whole muscle / Mechanically modified muscle 27 g	
1	Raw muscle	-	42	
2	Sunflower oil	15	42	
3	Olive oil	15	42	
4	Soy sauce	15	42	
5	Brine	5%	42	
6	Alcohol vinegar	10	37	
7	Wine vinegar	10	37	
8	Apple cider vinegar	10	37	
9	Lemon + juice	4,5	31,5	
10	Tomato puree	20	47	Total [g]
11	Chili spice	1	28	
12	Oregano	0,5	27,5	
13	Fresh garlic	2	29	
14	Garlic spice	1	28	
15	Onion	5	32	
16	Corn	10	37	
17	Pea	10	37	
18	Bean	15	42	
19	Carrot	10	37	
20	Tomatoes	10	37	
21	White + green pepper	5+5	37	
22	Black olives	10	37	
23	Fresh chili pepper	5	32	

mechanically processed muscle (ground). Mechanic treatment was provided using the cutter setting in two rotations. Furthermore the sets of the samples comprising whole/ground muscle enriched of the selective ingredients were mixed thoroughly and placed into the autoclavable glass vessels with caps – the amount and composition is described in Table 1. The proportions were assessed according to the real composition described on the packaging of tuna fish products occurring on the commercial market. The samples were subjected to the sterilization in autoclave (Systec V95); sterilization conditions included the temperature 121 °C and pressure 200 kPa for 15 min. These samples were prepared in laboratories of the Department of Meat Hygiene and Technology (University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic).

DNA isolation

The DNA was extracted in duplicate using three commercial available kits based on the column system (DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) - kit A and DNeasy mericon Food (Qiagen) – kit B) and magnetic separation using magnetic particles (Chemagic DNA Tissue 10 Kit (Chemagen, Baesweiler, Germany) – kit C). Pretreatment of the samples 2 - 8 (Table 1) was performed according to **Chapela et al. (2007)**; oil, lipids or other substances were removed from canned muscle by soaking it in the mixture of chloroform/methanol/water (1:2:0.8) overnight. The extraction procedures were performed according to the protocol supplied by the manufacturer. Sample weight was 10 mg in kit A and C, and 200 mg in kit B, proteolysis was carried out overnight in all types of the extraction protocols.

The assessment of DNA quality

The quality of extracted DNA was compared by measurement the concentration and purity using a UV spectrophotometer (NanoDrop™ 1000, Thermo Scientific). DNA extracts were quantified by measuring the absorbance at 260 nm (A260). DNA purities were estimated by calculating the A260/A280 ratios. Samples calculated to have A260/A280 ratios of 1.7 – 2.0 were assumed to be pure samples, free from protein and other contamination. Every sample was measured three times. The instrument calibration was performed using the Elution Buffer. Measurement was done at room temperature and sufficient mixing of all samples.

Species identification via sequencing of cytochrome b gene

For the species identification of yellowfin tuna (*Thunnus albacares*) in frozen fish the amplification of 569 bp fragment of cytochrome *b* gene using primer pair L14735 and BRmod (Espineira et al., 2009) was used. The PCR protocol consisted of initial denaturation step at 95 °C/3 min, following by 35 cycles including denaturation at 95 °C/30 s, annealing at 60 °C/30 s and extension at 72 °C/30 s, and terminated by final extension at 72 °C/3 min.

RESULTS AND DISCUSSION

In canned products DNA is considered to be damaged, exposure to heat, physical or chemical treatment that can affect the quality and quantity of DNA, presumably the fragmentation of DNA molecule. To choose an optimal extraction procedure several factors have to be taken into account. DNA should contain as little as possible proteins, RNA, organic compounds or any other PCR inhibitors. The DNA concentration and purity were determined spectrophotometrically by measuring the DNA absorbance and A260/A280 ratios. The DNA was considered to be satisfactorily pure when the ratios of the A260 to A280 were within the range of 1.7 – 2.0. Contamination of DNA with proteins usually reduces the A260 to A280 ratio to values lower than 1.7 **(Cawthorn et al., 2011)**. High 260/280 purity ratios are not necessarily indicative of a problem. Residual impurities carried over from the DNA extraction procedure, such as phenol or ethanol, are also reported to reduce the A260 to A280 ratio. Furthermore residual chemical contamination from nucleic acid extraction procedures may result in an overestimation of the nucleic acid concentration.

The main task was to find out whether non-processed and processed muscle tissue (from *Thunnus albacares*) has the difference between the concentration and purity of DNA. Another parameter was to follow up the effect of the addition of ingredients mainly used in canned tuna products. And also to evaluate the efficiency of the three commercial kits used for the DNA isolation. The first group of analyzed samples include sample prepared from non-processed muscle without any further technological processes (frozen muscle), sample prepared from whole muscle undergoing the sterilization process and sample prepared from mechanically treated (ground) muscle undergoing the sterilization process. The comparison of the DNA concentration and DNA purity is shown in Figure 1. The highest DNA concentrations were observed in non-processed muscle that is not influenced by the sterilization process. The sample with canned whole muscle demonstrated lower DNA yield, and furthermore, the mechanical treatment resulted in even lower values of DNA concentration that was registered by using all three

Table 2 Group rate of values A260/A280.

Ratio A260/A280	Kit A	Kit A	Kit B	Kit B	Kit C	Kit C
	W	G	W	G	W	G
<1.7	1	4	11	4	22	22
1.7 – 2.0	11	13	12	17	1	1
>2	11	6	0	2	0	0

Note: W – whole, G – ground.

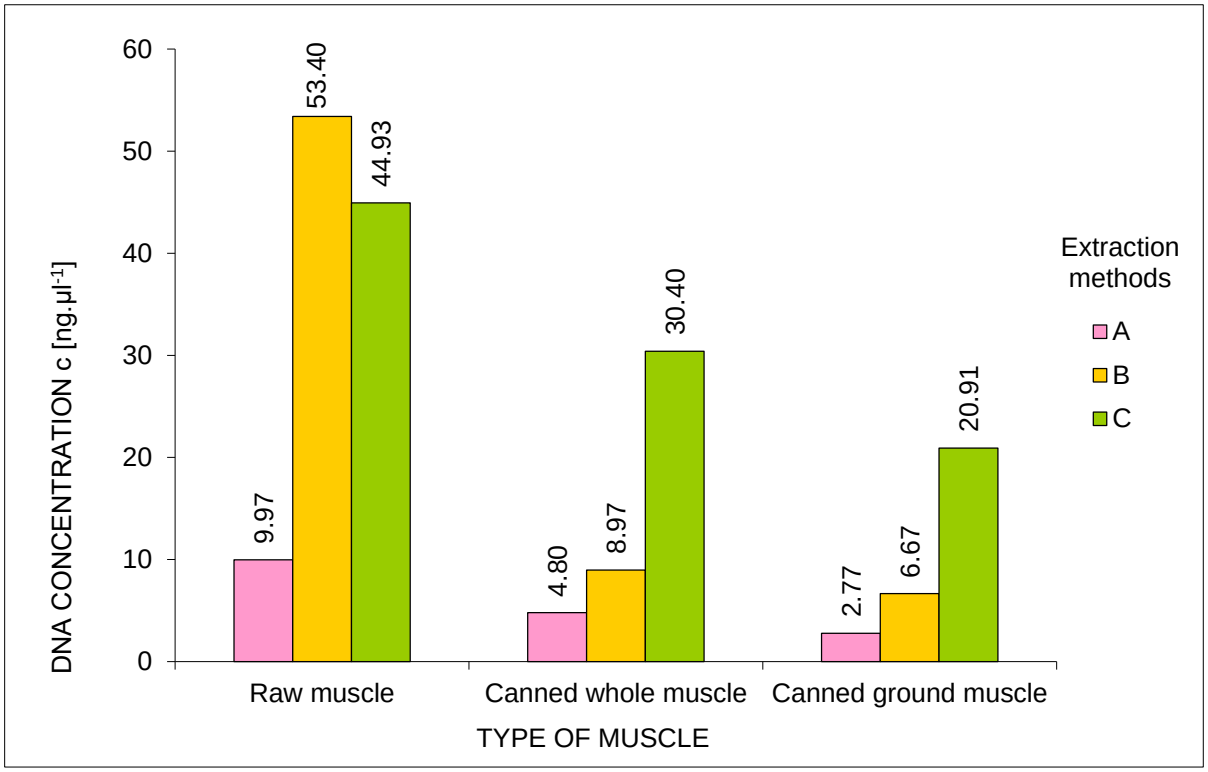

Figure 1 Determination of DNA concentration (type of muscle).

types of DNA extraction kits. Kit B produced DNA of higher concentration in non-processed sample, kit C delivered higher DNA yields than kit A and B, although the purity was lower, but still within the range 1.7 – 2.0. In the case of DNA purities, DNA was considered to be satisfactorily pure in all three types of samples and using all three types of DNA isolation.

The second group consisted of 23 samples prepared from the whole or ground muscle tissue and enriched with the ingredients (22 with ingredients and 1 muscle without ingredients). For the comparison of the samples of whole and ground canned muscle tissue (regardless of the effect of the ingredients) the Wilcoxon matched-pairs signed rank test was used and for the comparison of the efficiency of the particular extraction kits Friedman test + Dunn post-hoc test (non-parametric ANOVA) was used. The frequency values of A260/A280 in groups $A < 1.7$ / $1.7 \leq A \leq 2.0$ / $A > 2.0$ were estimated with χ^2 independence test and Fisher exact test.

Statistically significant differences in DNA concentration between the whole and ground muscle were found in the case of kits A (p <0.01; Wilcoxon test) and B (p <0.05; Wilcoxon test). While in kit A the values of DNA concentrations in most of the samples with whole muscle were lower than in the samples with ground muscle, in kit B it was conversely. In kit C statistically significant difference between whole and ground muscle was not proved. In kit A probably the chemical substances used during the extraction procedure could cause more efficient permeation to the ground muscle in comparison with whole muscle, but this was not observed in sample 1 (whole and ground muscle without ingredients). In case of the samples with whole muscle we managed to prove that among the kits there is statistically significant difference (p <0.01; Friedman test) in DNA concentration. Following testing demonstrated that statistically significant difference is evident between all pairs of kits (p <0.01; Dunn test), while the highest values of DNA concentration is presented with kit C, the lowest in kit A. In case of the samples with ground muscle we managed to prove that among the kits there is statistically significant difference (p <0.01; Friedman test). Following testing showed up that statistically significant difference is only between kit A and C, B and C (p <0.01; Dunn test), while the highest values of DNA concentration is produced by the kit C. Between kit A and B the statistically significant difference was not observed.

Statistically significant difference of A260/A280 between whole and ground muscle was observed only in kit B (p <0.01; Wilcoxon test), while highest values of A260/A280 was reached in samples with ground muscle. In case of the samples with whole muscle we managed to prove that among the kits there is statistically significant difference (p <0.01; Friedman test). Following testing demonstrated that statistically significant difference is evident between all pairs of kits (p <0.01; Dunn test), while the highest values of A260/A280 is presented by kit A, the lowest in kit C (except the samples 1 always under the limit 1.7). In case of the samples with ground muscle we managed to prove that among the kits there is statistically significant difference (p <0.01; Friedman test). Following testing showed up that statistically significant difference is only between kit A and C, B and C (p <0.01; Dunn test), while the lowest values A260/A280 is produced by the kit C. Between kit A and B the statistically significant difference was not observed.

χ^2 independence test confirms that there is association (p <0.01) between the distribution of A260/A280 ratios and kit resp. the type of the sample (whole/ground muscle). In case of the type of the sample the highest statistically significant difference (p <0.05) was detected in kit B (Table 2).

The effect of ingredients mixed together with muscle reveal the differences among particular kits and also among whole and ground muscle. According **to Chapela et al. (2007)** lower amount of DNA can be caused by the presence of brine, this finding could be explain by a washing out effect used in the extraction procedure. The decreasing effect of brine on DNA yield was observed only in kit C. In kits A and B the concentrations of DNA were even higher in comparison with the sample without brine. Other ingredients vinegar and lemon are substances

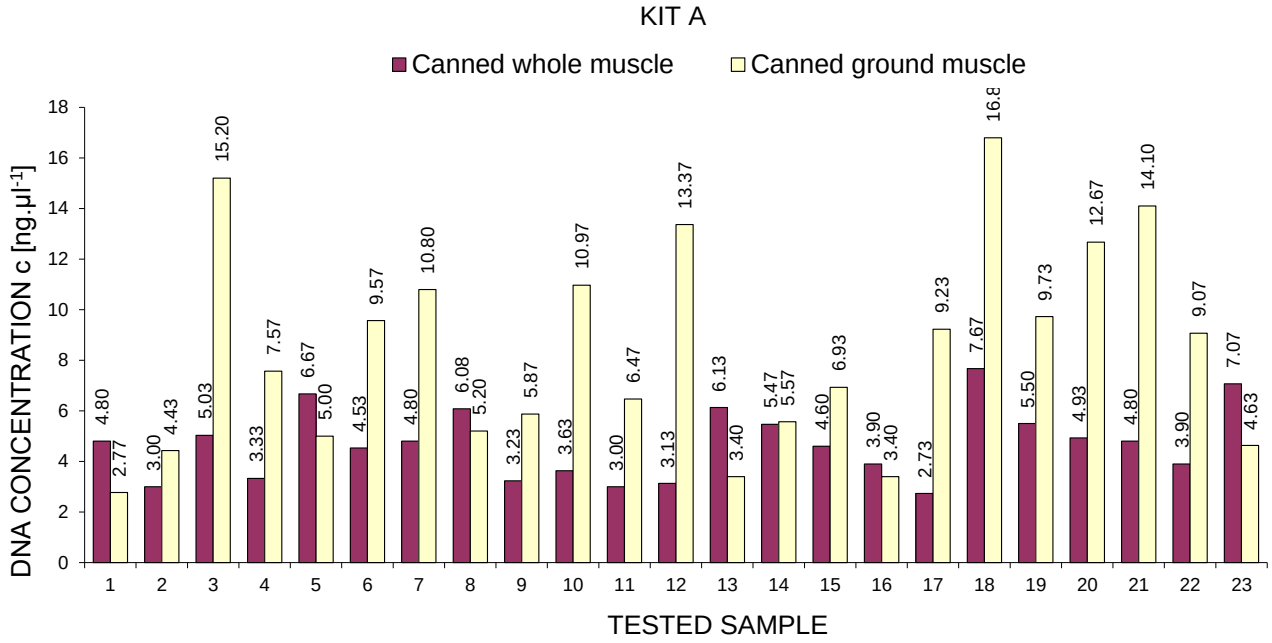

Figure 2 Comparison of DNA concentration of whole and ground muscle determined by kit A.

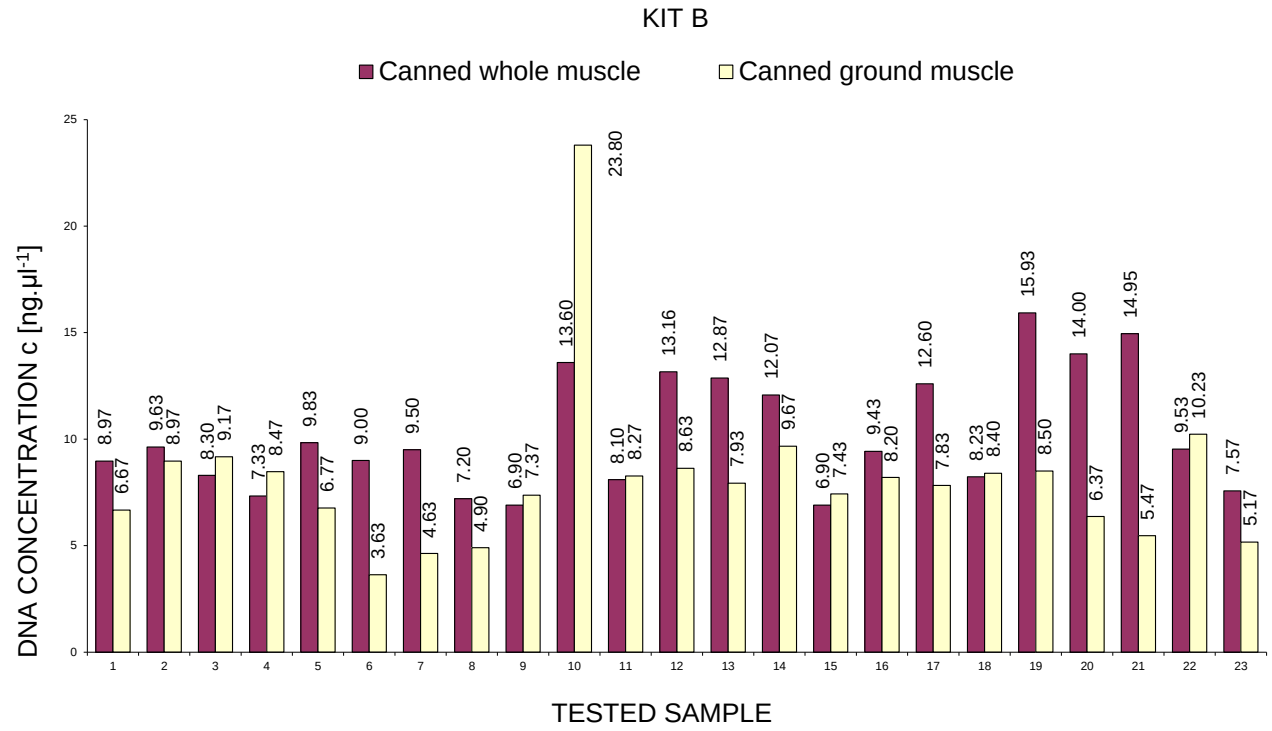

Figure 3 Comparison of DNA concentration of whole and ground muscle determined by kit B.

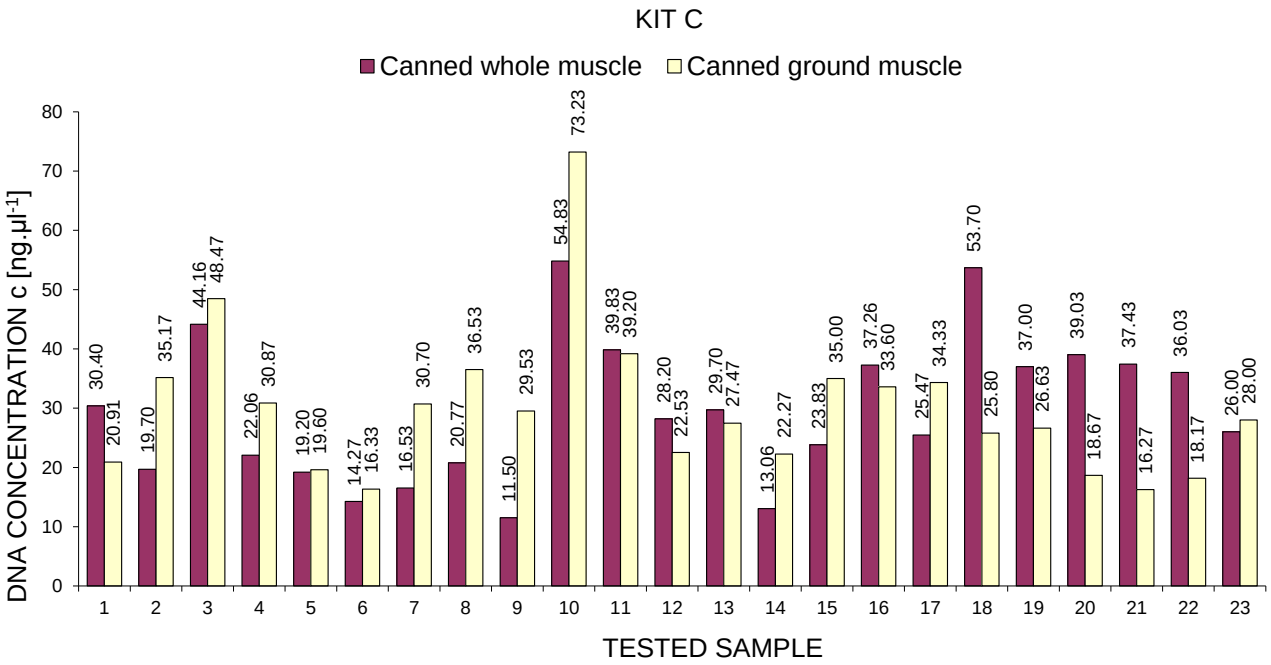

Figure 4 Comparison of DNA concentration of whole and ground muscle determined by kit C.

that are known as a low pH media which could be the reason of DNA degradation **(Bauer et al., 2003)**. In both kit B and C the decreasing effect on DNA yield caused by the presence of vinegar and lemon was observed. In kit A this was observed only in lemon and in case of ground muscle this effect was not demonstrated (Figures 2 – 4). Onion contains quercetin that belongs to the flavonoids and that can inhibit the protein kinase C activity (PKC) and mitogen activated protein kinase 1 (MEK). Therefore it appears to decrease the DNA yield in the samples containing onion **(Lee et al., 2008).** This was observed in whole muscle samples containing onion consistently in all three types of kits. In contrary the presence of ingredients in some samples resulted in better DNA yield. The color extracted from the samples containing particular ingredients (carrots in all three kits, tomato puree, chili, oregano, tomatoes, green pepper or black olives in kit B or C) could cause the higher values of absorbance which could misinterpret obtained results **(Chapela et al., 2007)**. Unexpectedly in kit A the lowest DNA yield was estimated in sample containing pea, the highest value of DNA concentration was assessed in sample containing bean. Although both are legumes their effect was completely contradictory. The quality assessed by the ratios A260/A280 were decreased (A260/A280 <1.7) in samples containing brine and vinegar in both kits A and B, in kit C every sample resulted in ratios lower than 1.7. Although purity ratios are important indicators of sample quality, the best indicator is functionality in the following PCR amplification. There are occasions when the purity ratios are within expected limits, but there is a problem with the sample. Accordingly the presence of ingredients may negative influence the subsequent PCR amplification, when they could inhibit the DNA polymerase activity in PCR **(Di Pinto et al., 2007)** and decrease its sensitivity. The impact of ingredients was not conclusively approved and their connotation to the suitability of extracted DNA for PCR amplification is needed to be discussed in further analysis.

CONCLUSION

The quality of DNA affect the efficiency of amplification during the subsequent PCR reaction. The results of this analysis revealed variability of particular extraction procedures in assessment of DNA quality and quantity in tuna muscle tissue treated with different modifications. The highest DNA concentrations were observed in non-processed muscle, whole canned muscle demonstrated lower DNA yield, and canned ground muscle resulted in even lower values of DNA concentration that was registered by using all three types of DNA extraction kits. Kit B produced DNA of higher concentration in non-processed sample, kit C delivered higher DNA yields in canned whole and ground muscle than kit A and B, although the purity was lower, but still within the range 1.7 – 2.0. In the case of DNA purities, DNA was considered to be satisfactorily pure in all three types of samples and using all three types of DNA isolation. Comparing the parameters of whole and ground canned muscle tissue with the content of ingredients, kit C produced in all samples with whole and ground muscle the highest values of DNA concentration, but almost all values of A260/A280 were lower than 1.7. Kit B in all samples with whole muscle gives higher values of DNA concentrations than kit A, in samples with ground muscle this assumed in almost all samples, so it appears to be a good choice for the DNA isolation from canned whole muscle with ingredients. The effect of ingredients mixed together with muscle reveal the differences in terms of

decreasing but also raising the DNA yield among particular kits and also among whole and ground muscle. Nevertheless the presence of ingredients may negative affect the subsequent PCR amplification, which will be the subject of further comparative analysis.

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Contact address:
MVDr. Zora Piskatá, Ph.D., Veterinary Research Institute Brno, Department of Food and Feed Safety, Hudcova 70, 621 00 Brno, Czech Republic, E-mail: piskata@vri.cz, Telephone: +420 533 331 913.
Mgr. Eliška Pospíšilová, Veterinary Research Institute Brno, Department of Food and Feed Safety, Hudcova 70, 621 00 Brno, Czech Republic, E-mail: pospisilova@vri.cz, Telephone: +420 533 331 913. Department of Meat Hygiene and Technology, University of Veterinary and Pharmaceutical Sciences, Palackeho trida 1/1946, 612 42 Brno, Czech Republic.

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THE COMPARISON OF BIOLOGICAL ACTIVITY OF CHOCOLATES MADE BY DIFFERENT TECHNOLOGICAL PROCEDURES

Lucia Godočiková, Eva Ivanišová, Július Árvay, Jana Petrová, Miroslava Kačániová

ABSTRACT

Chocolate is one of the most consumed delicacies in the world. Nowadays, raw chocolates without vanilla or allergens are getting more attention. The aim of this study was to evaluate and compare the biological activity of different types of chocolate – cold processed chocolate and chocolate made by traditional way. Total content of fat, crude fibre, polyphenols, flavonoids, phenolic acids and metylxantines – theobromine and caffeine was evaluated. The antioxidant activity was determined by a method using DPPH radical, reducing power method and phosphomolybdenum method. Both evaluated chocolates had similar content of fat and crude fibre, but sample of chocolate made by traditional way probably due to the higher content of cocoa mass had almost two times higher content of total polyphenols, flavonoids and phenolic acids as cold processed chocolate. Also the content of theobromine and caffeine was slightly higher in chocolate made by traditional way. This sample had the highest antioxidant activity – 93.68 mg TEAC.g^{-1} determined by phosphomolybdenum method, while in the sample of chocolate made by cold processed way was measeured value 50.82 mg TEAC.g^{-1}. Similarly, reducing power of chocolate made by traditional way was almost two times higher, but antioxidant activity determined with DPPH method was similar in both samples (3.58 and 3.62 mg TEAC.g^{-1}). The antioxidants and metylxantines in chocolates determine its potential to be a significant source of biologicaly active compounds with favorable effects to human health. It can be concluded in this study, that chocolate produced by conventional production technology can have more health-promoting ingredients reserved, but more extensive researches are still needed.

Keywords: chocolate; antioxidant activity; polyphenols; flavonoids; theobromine; caffeine

INTRODUCTION

In recent decades, nutrition research has focused on the investigation of bioactive dietary flavonoids, widely found in many plant-based foods and beverages, in order to elucidate their beneficial properties to human health. Cocoa (*Theobroma cacao* L.) and chocolate products appear to be one of the most promising foods due to their high polyphenol content, which evidently highlights the link with health-promoting properties **(Alañón et al., 2016)**.

Worldwide consumption of chocolate and cocoa-containing products increased by 10% from 2002 to 2010, which might be attributed to consumer economic enhancement and increasing knowledge of potential health benefits derived from cocoa constituents. Chocolate and cocoa-containing products are a good source of non-nutrient bioactive polyphenols with potential health benefits including reduced risk of cardiovascular disease and prebiotic activity **(Hu et al., 2016)**.

Chocolate and cocoa products are a rich source of flavonoids. Flavanoids, naturally occurring polyphenolic compounds present in plant-based foods, represent up to 20% of the compounds present in cocoa beans. Flavanols, and in particular epicatechin, are a subgroup of flavonoids, and are the most common cocoa flavonoids. High levels of flavanols are also found in tea, red wine, and fruits such as grapes and apples. In addition to cocoa flavonols, other psychoactive components of chocolate include the methylxanthines (caffeine and theobromine), both of which have been associated with improving alertness and cognitive function. One hundred grams of dark chocolate contain approximately 100 mg of flavanols, while 100 g of unsweetened cocoa powder without meythlxanthines can contain up to 250 mg of flavanols **(Crichton et al., 2016)**.

Chocolate represents functional properties due to its high level of flavonoid content, namely catechins and procyanidins, and beneficial impacts of chocolate consumption on human health. However, consumers are becoming more demanding in food market and they would like to have more options to choose from than ever before. Therefore, manufacturers desire to broaden their product ranges such as having organic chocolate, high-cocoa polyphenol-rich chocolate, probiotic chocolate, and prebiotic chocolate rather than ordinary chocolate. It was also showed that dark chocolate ensured a high probiotic survival rate **(Gültekin-Özgüven et al., 2016)**.

Therefore the aim of this study was to determine the biological activity of selected types of commerially available chocolates and evaluate their antioxidant activity, amount of total polyphenols, flavonoids, phenolic acids and main methylxantines – theobromine and caffeine.

MATERIAL AND METHODOLOGY

Biological material

The chocolates evaluated in this study were purchased from local market and signed as sample 1 and 2. Sample 1 was italian raw chocolate named „*Cioccolatino biologico 75% cacao con zucchero da fiori di palma da cocco*“, produced in town Modica, in Sicily (Italy). It contains 75% of cooca solids and the temperature throughout the process never exceeds 45 °C. Sample 2 was slovakian traditionally processed chocolate named „*Bean to Bar Dark 78%*“, with 78% cocoa solids. Both samples were made only from cocoa mass and coconut flower sugar and both were organic and distributed by same company.

Chemicals

All chemicals were analytical grade and were purchased from CentralChem (Slovakia) and Sigma Aldrich (USA).

Sample preparation

Samples were homogenized in a mortar and then defatted with petroleum ether. Defatted sample – 0.25 g of was extracted with 20 mL of 80% ethanol for 2 hours in a shaker (GFL 3005, Germany). After centrifugation at 4000 RPM (Rotofix 32A, Hettich, Germany) for 10 min, the supernatant was used for measurement (antioxidant activity, total polyphenols, flavonoids, phenolic acids, theobromine and caffeine – each analysis was carried out in ten replicates).

Determination of fat content

Fat content was determined by Fat extractor Ancom XT15 (ANKOM Technology, New York, USA) with the methodology recommended by the producer. The sample (1.5 g, W_1) was weighted to special filter bag (XT4, Ancom, USA) and dried for 3 hours in an oven (WTB, Binder, Germany) at 102 °C to remove moisture prior to the extraction. Samples were placed in a desiccant pouch for 15 minutes and after re-weighted (W_2) and extracted 60 minutes at 90 °C with petroleum ether. After process samples were removed and dried in an oven at 102 °C for 30 minutes, placed in desiccant pouch and re-weighted (W_3). The analysis was carried out in duplicate. Fat content was calculated by following formula:

$$\text{Fat content (\%)} = \frac{100 \times (W2 - W3)}{W1}$$

Determination of crude fiber content

Dietary fiber content was determinated with Ancom 200 Fiber analyzer (ANKOM Technology, New York, USA), with methodology recommended by the producer. One gram (W_2) of the sample was weighted to special filter bag (W_1 – bag tare weight; F57, Ancom, USA). Samples were defatted with petroleum ether, air-dried and placed to analyzer. 2000 mL of 0.1 M sulphuric acid was added and samples were hydrolyzed 45 minutes at 100 °C, after this process samples were washed with hot distilled water 3 times. Then 2000 mL of 0.1 M potassium hydroxide was added and samples were hydrolyzed 45 minutes at 100 °C, after this process were washed with hot distilled water 3 times. Water was gently pressed from the bags and bags were soaked in acetone for 5 minutes, removed, air-dried and placed in the oven at 102 °C (WTB, Binder, Germany) for 2 hours. After cooled to room temperature, bags were re-weigted and ashed in pre-weighetd crucibles for 2 hours at 550 °C for 2 hours. Ashed crucibles were weighted to calculate the loss of weight of organic matter (W_3). The analysis was carried out in duplicate. Crude fiber content was calculated by following formula:

$$\text{Crude fiber (\%)} = \frac{100 \times [W3 - (W1 \times C1)]}{W2}$$

C_1 – ash corrected blank bag factor (running average of loss of weight on ignition of blank bag/original blank bag)

Total polyphenol content

Total polyphenol content of chocolate extracts was measured by the method of **Singleton and Rossi, (1965)** using Folin-Ciocalteu reagent. A 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 minutes in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25 – 250 $mg.L^{-1}$; $r^2 = 0.9978$) was used as the standard and the results were expressed in $mg.g^{-1}$ gallic acid equivalents.

Total flavonoid content

Total flavonoids were determined using the modified method of **Willett, (2002)**. A 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M sodium acetate and 4.3 mL of distilled water. After 30 minutes in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (0.01 – 0.5 $mg.L^{-1}$; $r^2 = 0.9977$) was used as the standard and the results were expressed in $mg.g^{-1}$ quercetin equivalents.

Total phenolic acids content

Total phenolic acids content was determined using method of **Farmakopea Polska, (1999)**. A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10% $NaNO_2$ + 10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1-200 $mg.L^{-1}$, r^2=0.9996) was used as a standard and the results were expressed in $mg.g^{-1}$ caffeic acid equivalents.

Antioxidant activity

Radical scavenging activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(Sánchéz-Moreno et al., 1998)**. The extract (0.4 mL) was mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). After 10 minutes in darkness, absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 $mg.L^{-1}$; $r^2 = 0.9881$) was used

as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Reducing power

Reducing power of samples was determined by the method of **Oyaizu, (1986)**. One mililiter of sample extract was mixed with 5 mL PBS (phosphate buffer with pH 6.6) and 5 mL of 1% potassium ferricyanide (w/v) in the test tube. Mixture was stirred thoroughly and heated in water bath for 20 minutes at 50 °C. After cooling, 5 mL of 10% trichloroacetic acid was added. 5 mL of mixture was pipetted into the test tube and mixed with 5 mL of distilled water and 1 mL of 0.1% (w/v) ferric chloride solution. Absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Reducing power was expressed in $mg.g^{-1}$ Trolox equivalents, using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 $mg.L^{-1}$; $r^2 = 0.9974$) as the standard and results were expressed in $mg.g^{-1}$ Trolox equivalents.

Phosphomolybdenum method

Phosphomolybdic method was determined by a method of **Prieto et al. (1999)**. Monopotassium phosphate (2.8 mL, 0.1 M, w/v) was mixed with sulfuric acid (6 mL, 1 M), ammonium molybdate (0.4 mL, 0.1 M, w/v), distilled water (0.8 mL) and 1 mL of sample extract. Test tubes were mixed thoroughly and heated in water bath for 120 minutes at 90 °C. After cooling, absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Antioxidant activity was expressed in $mg.g^{-1}$ Trolox equivalents (10 – 1000 $mg.L^{-1}$, r^2=0.9975).

Methyxantines content determination by HPLC-DAD method

Theobromine and caffeine content was determined using separation gradient method RP-HPLC/UV-DAD by Agilent 1260 Infinity high performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a Purosphere reverse phase C18 column (4 mm × 250 mm × 5 μm) (Merck, KGaA, Darmstadt, Germany). The mobile phase consisted of HPLC methanol (B) and 0.1% formic acid in HPLC water (C). The following gradient program was employed: 0 – 2 min. isocratic elution (20% B and 80% C), 2 – 15 min. linear gradient elution (40% B and 60% C), and 40% B and 60% C 15 – 20 min. The flow rate was 1 $mL.min^{-1}$. Column oven temperature was set up to 25 °C and the samples were kept at 4 °C in the Peltier sample manager. The DAD signal was conducted at 210 – 400 nm with preferred wavelength 330 nm for quantitative purposes with data acquisition rate of 5 Hz.

Statistical analysis

The basic statistical analyzes were realized in SAS programming packages (THE SAS SYSTEM V 9.2.). Correlation coefficients were calculated by CORR analysis and it was also used t-test **(SAS, 2009)**.

RESULTS AND DISCUSSION

Fat content

Dark chocolate can be considered as products with an important nutritional density, because of their richness in carbohydrates and fats. Cocoa butter is considered the most important cocoa by-product, due to its physical (rheology and texture) and chemical characteristics, and also organoleptic qualities. The prevalence of saturated fatty acids over unsaturated fatty acids is considered to be negative from the nutritional point of view. For many years, saturated fatty acids whose chain length is C12:0 – C16:0 have been considered to provoke atherosclerosis, and to be associated with cardiovascular disease. Thus, because of its high SFA content, chocolate is often postulated as having a hypercholesterolemic effect. However, it has been suggested in recent clinical trials that stearic acid (C18:0), a non-cholesterolemic and atherogenic type of dietary saturated fat, is neutral. These trials have shown that chocolate consumption has neutral effects on serum total cholesterol and LDL-cholesterol, as neither lowers HDL-cholesterol **(Torres-Moreno et al., 2015)**.

Fat content of both evaluated samples was similar. Cold processed chocolate contained 35.24 ±0.01% of fat, while chocolate made by traditional way contained 37.60 ±1.47% of fat. The manufacturer of the chocolate made by traditional way maintains on the package that the chocolate contains 41 grams of fat per 100 g of chocolate, which correlates with our results. On the package of cold processed chocolate there was no data about fat content. **Rezende at al., (2015)** showed that increasing the cocoa butter content enhanced the acceptance score of chocolates. This relationship is widely accepted in chocolate production, as cocoa butter provides rheological properties that promote proper hardness, mouth feel and swallow.

Although evidence in the literature suggests that chocolate consumption may have beneficial effects on health, it must be noted that chocolate has a high total fat and sugar content; in consequence, daily consumption of large amounts of chocolate may increase weight in the long term. That is why scientific evidence suggests that chocolate consumption should be considered in the context of a healthy diet, and dark chocolate must be consumed in moderate amounts (20 – 25 g per day) **(Machálková et al., 2016; Torres-Moreno et al., 2015)**.

Crude fiber content

The health benefits of dietary fiber have long been appreciated. Higher intakes of dietary fiber are linked to less cardiovascular disease and fiber plays a role in gut health. Higher intakes of fiber are linked to lower body weights **(Slavin, 2013)**. The recommended dietary reference intake of total fiber is 25 g per day for young women and 38 g pred day for young men; however, a usual intake of dietary fiber in the US is only about 15 g per day **(Jurasová et al., 2011; Brownawell et al., 2012)**.

Content of cruder fiber was in both samples on the similar level – 0.561 ±0.01% for cold precessed chocolate and 0.535 ±0.02% for chocolate made by traditional way. Also **Torres-Moreno et al., (2015)** reported that chocolate contains less than 2% of fibre. Thus, chocolate

Table 1 Content of biologically active compounds in chocolate samples.

	TPC (mgGAE.g^{-1})	TFC (mgQE.g^{-1})	TPA (mgCAE.g^{-1})	DPPH (mgTEAC.g^{-1})	RP (mgTEAC.g^{-1})
Sample 1	8.14 ±0.98	0.34 ±0.08	9.47 ±0.91	3.62 ±0.09	31.39 ±1.14
Sample 2	16.37 ±2.07	0.74 ±0.02	16.16 ±0.63	3.58 ±0.03	55.91 ±0.95

TPC – total polyphenol content; TFC – total flavonoid content; TPA – total phenolic acid content; RP – reducing power; GAE – gallic acid equivalent; QE – quercetin equivalent; CAE – caffeic acid equivalent; TEAC – Trolox equivalent antioxidant capacity; ±standard deviation; sample 1 – cold processed chocolate; sample 2 – chocolated meda by traditional way.

consumption does not contribute significantly to protein and dietary fibre intake.

Erdem et al., (2014) showed that dietary fiber addition didn't show negative effects, such as off-flavor, unwanted aroma or taste, on color and organoleptic properties of chocolate samples. Among fibers, maltodextrin and lemon fiber addition had positive effects on the sensory characteristics.

Total polyphenol content

Latest studies have shown that chocolate was not only a simple blend of fat and sugar, but also a rich source of flavonoids and polyphenols which shows high antioxidant activities **(Erdem et al., 2014)**. Main groups of polyphenols are the catechins (37%), procyanidins (58%) and anthocyanins (4%). The polyphenols in cocoa beans contribute to about 12 – 18% of the dry weight of the whole bean **(Bordiga et al., 2015)**. Furthermore, these non-nutrient bioactive compounds have potential health benefits including reduced risk of cardiovascular disease and prebiotic activity **(Hu et al., 2016)**.

Total polyphenol content (TPC) of samples is shown in Table 1. Chocolate made by traditional way had almost double polyphenol content compared to cold processed chocolated. Similar results for dark chocolates produced in Serbia reported **Todorovic et al., (2015)**, where TPC varied from 7.21 ±0.49 to 12.65 ±0.45 mg GAE.g^{-1} for chocolates with 60 – 75% of cooca content.

Our results are also with accordance to **Hu et al., (2015)** results, which determined TPC 27.34 ±0.20 mg GAE.g^{-1} in defatted sample of 70% Peru origin chocolate, while our 78% Peru origin chocolate made by traditional way had similar amount – 26.23 ±2.07 mg GAE.g^{-1} in defatted sample.

Bordiga et al., (2015) reported that both processing conditions (fermentation and drying) and the chocolate-making practices influence the polyphenolic content in the final products. **Hu et al., (2015)** explained that manufacturers use a variety of cacao cultivars as well as processing and storage parameters, and all of these impact antioxidant capacity and phenolic profiles of the final products.

Total flavonoid content

Flavonoids are an important class of plant pigments, naturally found in fruit and vegetables. This class of naturally occurring polyphenolic compounds which cannot be synthesized by humans possesses a series of biological properties, acting on biological systems as antioxidants. They act as antiviral, antiinflammatory, and antitumoral agents, affecting capillary permeability and acting as exogenous antioxidants. Flavonoids present in the diet are directly linked to the prevention of arthrosclerosis, as various studies show that the reduction of total blood cholesterol levels and the antioxidant effect lead to lower risks of arthrosclerosis, teratogenicity, and coronary disease **(Calado et al., 2015)**.

Total flavonoid content (TFC) of chocolate made by traditional way was again much higher than cold processed chocolate (Table 1). **Calado et al., (2015)** showed, that bitter chocolate can have higher flavonoid content compared to some kinds of cooked vegetables and advises, that people who don't like foods like broccoli or eggplant (daily dose between 20 and 26 g) could eat more chocolate (daily dose only 8 g).

The cocoa bean is one of the richest sources of flavanols, but the art is to preserve these wholesome components as much as possible in the final consumable products. The negative impact of the manufacturing process of chocolate and cocoa powder products on the flavanol content should not be underestimated **(Paoletti et al., 2012)**.

Total phenolic acids content

Phenolic acids are secondary plant metabolites widely spread throughout the plant kingdom. Chemically, phenolic acids are hydroxylated derivatives of benzoic, cinnamic, phenylacetic and phenylpropanoic acids. In nature, hundreds of phenolic acids have been identified. They are most abundant in coffee, tea and especially in berries. Recent interest in phenolic acids stems from their potential protective role against oxidative stress, inflammation, diabetes and cancer in experimental studies **(Zamora-Ros et al., 2013)**.

Protocatechuic acid is a hydroxybenzoic acid that can be found in many foods and it is also the most important phenolic acid (69.16%) found in cocoa liquor. It has been reported to have several physiological functions including antioxidant, antibacterial activity, antimutagenic activity, antitumour activity, and anticancer effects. Coumaric acids, hydroxy derivatives of cinnamic acid, are another important group of phenolic compounds found in cocoa (2.65%) with antioxidant and antigenotoxic properties **(Zhou et al., 2015)**. Content of total phenolic acids (TPA) in chocolate samples is shown in Table 1. Chocolate made by traditional way again showed almost two times higher value with compare to cold processed chocolate. To date, limited data exist on the quantitative intake of phenolic acids **(Zamora-Ros et al., 2013)**.

Antioxidant activity

Radical scavenging activity

The antioxidant activity of cocoa powder is well known, but changes occur during the lifetime of the bean, and activity often depends on its processing. The content of polyphenols can vary greatly depending on the source of beans, primary and secondary processing conditions, and packaging and processing of chocolate making. Due to these factors, the ratio and types of polyphenols found in cocoa beans, as well as their activity, are unlikely to be the same as those found in the finished products **(Vertuani et al., 2014)**.

Cold processed chocolate has higher only radical scavenging activity determined using DPPH radical (Table 1). But if the product contains more cocoa butter, antioxidant capacity values would be lower than products with the same cacao content but more cacao solids. Because of the complexity of chocolate, many components, other than phenols are present that could influence the results in different ways **(Hu et al., 2016)**.

High antioxidant activity also reported **Vertuani et al., (2014)**, who showed that it is not only the amount of cocoa used that is important, but also the quality and provenience affect the product properties. In general, for organic chocolates, by increasing the percentage of cocoa, the amount of total polyphenols enhances and the antioxidant capacity of the product increases in proportion.

Reducing power

Antioxidant properties are known to be concomitant with the development of reducing power. Reductones can react directly with peroxides and can also prevent peroxide formation by reacting with certain precursors **(Kim et al., 2013)**.

Values for reducing power (RP) expressed as trolox equivalent antioxidant capacity (TEAC) are shown in Table 1. To compare it with results of other authors, RP of samples was expressed in absorbance at 700 nm (A_{700}), too. A_{700} of cold processed chocolate (1.04 ±0.03 nm) is almost 3 times higher than A_{700} of broccoli (0.30 ±0.00 nm) determined by **Kim et al., (2013)**. A_{700} of chocolate made by traditional way (1.85 ±0.03 nm) is higher than broccoli or cold processed chocolate or even leafy green vegetable *Aralia elata* (A_{700} 1.20 ±0.00 nm) consumed in Asia **(Kim et al., 2013)**. Reducing power and total flavonoids in evaluated chocolates showed a high correlation (r^2 = 0.972, p <0.05), similarly like reducing power and total phenolic acid content (r^2 = 0.982, p <0.05), implying that the phenolic compounds were major contributors to the observed antioxidant activity.

Phosphomolybdenum method

In the presence of antioxidant compounds, Mo (VI) is reduced to Mo (V) and forms a green colored complex of phosphomolybdenum (V) that gives absorbance at 700 nm. The method is based upon the spectrophotometric quantitation of total antioxidant capacity **(Prieto et al., 1999)**. Antioxidant capacity of cold processed chocolate was 50.82 ±3.69 mg TEAC.g^{-1}, while chocolate made by traditional way showed almost double the reducing power – 93.68 ±10.41 mg TEAC.g^{-1}. **Ibrić and Ćavar, (2014)** determined total antioxidant activity using molybdenum reduction method with catechin standard as IC_{50}, which is the concentration of extract to reduce 50% of molybdenum cation. IC_{50} of dark chocolate was 2.54 ±0.23 mg.mL^{-1}. There are also no previously published data concerning evaluation of antioxidant activity of cocoa products using this method.

Similarly like in reducing power, phosphomolybdenum method and total flavonoids and phenolic acids in evaluated chocolates showed a high correlation (r^2 = 0.964; r^2 = 0.979, p <0.05).

Methylxanthines content

Cocoa is the major natural source of the theobromine. Compared with coffee and tea, cocoa and chocolate products have much lower content of caffeine and only traces of theophylline. The level of methylxanthines in cocoa beans depends on varietal type and is influenced by the fermentation process. Besides psycho-pharmacological effects, methylxanthines of cocoa products particularly theobromine, and to a lesser extent caffeine, may have a role in lowering plasma glucose **(Todorovic et al., 2015)**.

As expected, theobromine was the predominant compound among methylxanthines. It content in cold processed chocolate was 22.095 ±0.058 mg.g^{-1} and slightly higher in chocolate made by traditional way (27.763 ±0.009 mg.g^{-1}). Content of caffeine was much lower, 1.326 ±0.002 mg.g^{-1} in cold processed chocolate and 1.758 ±0.005 mg.g^{-1} in chocolate made by traditional way, respectively. Theobromine/caffeine ratio was 16:1 for both samples.

Bordiga et al., (2015) and **Todorovic et al., (2015)** determined lower amount of these metylxantines in dark chocolates containing 40 – 75% cocoa solids. Theobromine/caffeine ratio was lower in chocolates containing more cocoa solids. The content of methylxanthines and the theobromine/caffeine ratio vary depending on the cocoa genotype **(Aprotosoaie et al., 2016)**.

Obtained values for methylxantines in cocoa products show that potential physiological effects of chocolate mainly come from theobromine, with only small contribution of caffeine. From literature is know that 50 g of dark chocolate can have sufficient quantity of theobromine to produce neurophysiological effects **(Todorovic et al., 2015)**. The main pharmacological activities include: central nervous system stimulation, cardiovascular and metabolic effects, bronhodilatation, diuresis, gastric secretion stimulation, and, in high doses, the stimulation of skeletal muscles Methylxanthines, mainly caffeine, enhance physical and intellectual performance, mitigate fatigue, and cause a feeling of alertness. Cocoa products represent only a small part of the human diet and the concentration of methylxanthine is low, so these products do not normally pose a risk to human health. Furthermore, theobromine appears to be even safer for humans than caffeine **(Aprotosoaie et al., 2016)**.

CONCLUSION

On the basis of the above findings we can conclude, that high percentage dark chocolate is in general very good source of biologicaly active compounds, but not only

antioxidants, but also fats, crude fiber or psychologically active compounds. Our results confirmed that traditional process of chocolate making probably due to the higher content of cocoa mass can preserve more nutritionally significant components than processes with low temperatures. Even if high quality, organic chocolates have interesting biological characteristic, much higher caloric value of this delicacies should not be forgotten. Since there is lack of data for this issue, especially raw chocolates, it is necessary to expand the array of samples and performed analyzes to confirm or refute our conclusions. Results in this work can be an important tool for next scientific works and for food producers.

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Contact address:
Lucia Godočiková, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Plant Storage and Processing, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xgodocikova@uniag.sk.

Eva Ivanišová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Plant Storage and Processing, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: eva.ivanisova@uniag.sk.

Július Árvay, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: julius.arvay@gmail.com.

Jana Petrová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.petrova@uniag.sk.

Miroslava Kačániová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@uniag.sk.

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CELIAC DISEASE: THE SITUATION ON THE SLOVAK MARKET

Ľudmila Nagyová, Ingrida Košičiarová, Jana Rybanská, Mária Holienčinová

ABSTRACT

Celiac disease, also known as celiac sprue, non-tropical sprue, idiopathic sprue, idiopathic steatorrhoea and gluten-sensitive enteropathy, is a serious genetic autoimmune disease, which damages the villi of the small intestine and interferes with absorption of nutrients from food. The latest researches show that while in the 1970s the prevalence of celiac disease in the world was 0.03%, in the present years the estimated prevalence is 1%. In average, the prevalence of celiac disease in the Western countries is close to 1:100. The celiac disease occurs more often in the case of women than of men, at a ratio of 2.8:1. The aim of the present paper was to bring few information about the celiac disease, highlight the increasing number of celiacs, as well as to determine the Slovak celiacs opinion about the situation on Slovak market and their consumer behaviour on the market of gluten free products. As research methods, there have been used the methods of survey and structured questionnaire consisting of 22 questions. The total number of respondents was 130 randomly selected celiacs from all over the Slovak republic. For a deeper analysis of the obtained results, there have been set out four assumptions and ten hypotheses, which have been tested with the use of Pearson´s chi-square test, Mann-Whitney U-Test and Cramer´s contingency coefficient. The results of the present paper show, that despite the fact that few of our findings are pleasing – almost 52% of our respondents stay that the labelling of gluten free products is sufficient, over 74% of respondents think that they have enough information about the availability of gluten free products and more than 89% of respondents think that the present scope of range of gluten free products is better as before; there are still some shortcomings, which has to be reduced or eliminated – only less than 7% of respondents think that the price of gluten free products is adequate, over 45% of respondents use this possibility of granting a monetary contribution for compensation of increased expenses on a special diet, almost 65% of respondents think that the scope of range of gluten free products is in the Slovak market insufficient, 53% of respondents think that the availability of gluten free products in the Slovak market is inadequate and only 48% of respondents prefer the domestic producers of gluten free products.

Keywords: celiac disease; gluten free product; gluten free diet; consumer behaviour; Slovak republic

INTRODUCTION

Celiac disease is a life-long gluten sensitive autoimmune disease of the small intestine affecting genetically susceptible individuals **(Gujral et al., 2012)**. In general we can say, that it can affect both – adults but also children and that it is not **(URL 1, 2015)**:

- simply a food allergy (IgE) – wheat allergies are rare among adults; in children, wheat allergies affect 0.04 – 0.05% of population,
- an idiosyncratic reaction to food proteins (mediated by IgE);
- typified by a rapid histamine-type reaction (such as bronchospasm, urticaria, etc.);
- an intolerance which is a non-immune system response to food.

*„When people with celiac disease eat foods or use products containing gluten, their immune system responds by damaging or destroying villi – the tiny, fingerlike projections on the inner lining of the small intestine. Villi normally absorb nutrients from food and pass the nutrients through the walls of the small intestine and into the bloodstream. Without healthy villi, people can become malnourished, no matter how much food they eat.“***(URL 2, 2015)**.

The first description of the disease derives from the 2nd century BC, when Aretaeus from Cappadocia described a patient with chronic diarrhea and failure to thrive and called it koiliakos, which was in 1856, translated by Francis Adams into English as “coeliac” **(Villanaci et al, 2011)**. The more detailed description was given by Samuel Gee (a paediatrician at the London Hospital of Saint Bartolommeo) in 1880 **(Ciclitira et al., 2005; Walker et al., 2010)** and the link between the consumption of gluten and the symptoms of the illness was demonstrated during the World War II **(Abdulkarim et al., 2003)**.

The classical symptoms of the disease are diarrhea, abdominal pain and weight loss **(Olén et al., 2011)**. Besides the classic symptoms there are appearing also new once, which are the result of insufficient absorption of important nutritional components in the digestion process. They are represented by osteoporosis, joint pain, chronic fatigue, skin lesions, anemia etc. **(Fasano, 2009)**.

Facts about celiac disease (National Foundation for Celiac Awareness, 2015; URL 3, 2015; URL 4, 2011)

- celiac disease is not a food allergy, it is an autoimmune disease, which damages the villi of the small intestine and interferes with absorption of nutrients from food , and which can never be "outgrown",
- celiac disease is a hereditary condition, which means it is passed through families – if one family member has celiac disease, other family members, especially 1st degree relatives (parents, brothers and sisters, or the children of people who have been diagnosed), should always be tested,
- it occurs in 3.9 – 12.3% of people with Diabetes Type 1, in 5 – 12% of people with Down syndrome, Turner syndrome and other auto-immune conditions, in 20% of people with collagenous colitis, in 4.5% of first degree relatives of people with the same disease,
- gluten is essentially toxic to people with celiac disease and gluten sensitivity,
- an estimated 1 in 133 Americans, or about 1% of the population, has celiac disease,
- celiac disease can affect men and women across all ages and races,
- it is estimated that 83% of Americans who have this disease are undiagnosed or misdiagnosed with other conditions,
- 6 – 10 years is the average time a person waits to be correctly diagnosed,
- 5 – 22% of celiacs have an immediate family member (1st degree relative) who also has celiac disease,
- celiac disease can lead to a number of other disorders including infertility, reduced bone density, neurological disorders, some cancers, and other autoimmune diseases
- the cause of disease is not known,
- over 90% of celiacs are undiagnosed or misdiagnosed,
- celiac disease declined during the bread shortages of the Second World War but climbed again after the war,
- half of all people with celiac do not show any symptoms,
- September 13th is a Celiac Awareness Day,
- some products, like lipstick, toothpaste and vitamins, use gluten for processing but because it is not food, does not need to be labelled,
- alcohol made with gluten-containing grains is gluten-free because the distillation process removes the gluten protein.

MATERIAL AND METHODOLOGY

The aim of the present paper was to bring few information about the celiac disease, highlight the increasing number of celiacs, as well as to determine the Slovak celiacs opinion about the situation on Slovak market and their consumer behaviour on the market of gluten free products.

In order to achieve the aim, as research methods, there have been used the methods of survey and structured questionnaire consisting of 22 questions formulated as closed, so that respondents (total number of respondents was 130 randomly selected celiacs, Table 1) had to choose one, alternatively several options.

Table 1 Characteristics of respondents.

Category of respondents	**Number**
Male	10
Female	120
Place of residence	**Number**
City	86
Village	44
Age structure	**Number**
15 – 19 years	11
20 – 25 years	22
26 – 35 years	47
36 – 49 years	38
50 and more years	12
Education structure	**Number**
Primary education	4
Secondary education without A level	9
Secondary education with A level	59
Higher professional education	7
Higher education	51
Net family income	**Number**
Up to 500 €	23
501 − 1.000 €	56
1.001 – 1.500 €	31
1.501 € and more	20
Region	**Number**
BanskáBystrica	20
Bratislava	34
Košice	4
Nitra	22
Prešov	4
Trenčín	18
Trnava	9
Žilina	19

Note: Source: Results of the research.

In consideration of lack of information about the exact number of celiacs living in Slovak republic (the estimated number of celiacs living in Slovak republic in the year 2014 was between 0.5 and 1% of the total population of Slovak republic **(URL 5, 2014)**, we tried to ensure the representativeness of the results by the random selection and geographic diversification of our respondents (celiacs).

The questionnaire was evaluated with the use of contingency tables, which were prepared by Excel, under which they were subsequently developed graphic representations.

For a deeper analysis of the obtained results, there have been set out the following assumptions:

1. Assumption no. 1 – we assume that most of our respondents are women.

2. Assumption no. 2 – we assume that most of our respondents have celiac disease diagnosed from 0 to 5 years.
3. Assumption no. 3 – we assume that most of our respondents use a so called possibility of granting a monetary contribution for compensation of increased expenses on a special diet.
4. Assumption no. 4 – we assume that most of our respondents think that the scope of range of gluten free products in the Slovak market is insufficient.

And the following and hypothesis:

1. H_{01} – there does not exist the dependence between the frequency of purchase of gluten free products and the respondent´s place of living.
   H_{11} – there exists the dependence between the frequency of purchase of gluten free products and the respondent´s place of living.
2. H_{02} – there does not exist the dependence between the place of purchase of gluten free products and the respondent´s age.
   H_{12} – there exists the dependence between the place of purchase of gluten free products and the respondent´s age.
3. H_{03} – there does not exist the dependence between the decisive criteria in the purchase of gluten free products and the respondent´s age.
   H_{13} – there exists the dependence between the decisive criteria in the purchase of gluten free products and the respondent´s age.
4. H_{04} – there does not exist the dependence between the decisive criteria in the purchase of gluten free products and the respondent´s level of education.
   H_{14} – there exists the dependence between the decisive criteria in the purchase of gluten free products and the respondent´s level of education.
5. H_{05} – there does not exist the dependence between the respondent´s opinion on the adequacy of available information and the region from which the respondent comes from.
   H_{15} – there exists the dependence between the respondent´s opinion on the adequacy of available information and the region from which the respondent comes from.
6. H_{06} – there does not exist the dependence between the respondent´s opinion on the scope of gluten free products on the Slovak market and the region from which the respondent comes from.
   H_{16} – there exists the dependence between the respondent´s opinion on the scope of gluten free products on the Slovak market and the region from which the respondent comes from.
7. H_{07} – there does not exist the dependence between the respondent´s opinion on the scope of gluten free products in his region and the region from which the respondent comes from.
   H_{17} – there exists the dependence between the respondent´s opinion on the scope of gluten free products in his region and the region from which the respondent comes from.
8. H_{08} – there does not exist the dependence between the respondent´s opinion on the availability of gluten free products on the Slovak market and the region from which the respondent comes from.
   H_{18} – there exists the dependence between the respondent´s opinion on the availability of gluten free products on the Slovak market and the region from which the respondent comes from.
9. H_{09} – there does not exist the dependence between the respondent´s opinion on the availability of gluten free products in his region and the region from which the respondent comes from.
   H_{19} – there exists the dependence between the respondent´s opinion on the availability of gluten free products in his region and the region from which the respondent comes from.
10. H_{010} – there does not exist the dependence between buying gluten free products abroad and the region from which the respondent comes from.
    H_{110} – there exists the dependence between buying gluten free products abroad and the region from which the respondent comes from.

To test the dependence respectively the independence between the tested variables there were used the tests of Pearson´s chi-square test, Mann-Whitney U-Test and Cramer´s contingency coefficient.

RESULTS AND DISCUSSION

As it was mentioned before, celiac disease is an autoimmune disease affecting mainly the small intestine, induced by an intolerance of proteins of wheat, barley, rye and oats. The intolerance refers to the mixture of proteins of cereal grain, which are commonly named and known as *gluten* – gluten is a protein found in wheat, rye, and barley, but it can be found also in other products like medicines, vitamins and supplements, lip balm, and even the glue on stamps and envelopes **(URL 6, 2015)**.

*"Someoftheglutenquantitiescouldbehazardousforsensitivepeopleasceliatics and allergic to gluten"***(Mati, et al., 2012)**. The causes of the intolerance can be several **(Bergendiová, 2012)**:

- the abnormal activation of own immune system to the presence of gluten in the food (so called hypersensitivity),
- the disorder of metabolism and of the activity of enzymes degrading the gluten,
- genetic factors.

The *symptoms* of celiac disease are a broad nature theme and therefore we have to remember that even in the case of celiac disease, each human is an individual case and example. There are patients in whom the celiac disease was manifested by other gastroenterology or other health problems and complications, which other patients may not have. It is also important to note that celiac disease may also occur in other than gastroenterology problems **(Hes et al., 2014)**.

Nevertheless the fact, that the *prognosis* of the disease is very good **(Frič, 2008)**, there still does not exist a test that could be universally accepted as a standard for the diagnosis. The early diagnosis and lifelong adherence to a so called *gluten free diet* causes that the complications caused by the illness are scarce and the life expectancy of celiacs does not significantly differ to the other populations **(Košičiarová et al., 2015)**.

Celiac disease diagnosis,prevalanceandtreatment

Pekárková et al., (2009) indicate four basic forms of celiac disease diagnosis:

1. *laboratory diagnosis* – the American College of Gastroenterology recommends, that antibody testing, especially immunoglobulin A anti-tissue transglutaminase antibody (IgA TTG), is the best first test for the diagnosis of celiac disease **(Goebel et al, 2015)**. The first step in the diagnosis is the investigation of patient´s blood count and prothrombin time when it comes to the quest for anemia, thrombocytosis, and coagulation disorders. Then, the serological tests are done, where the serological markers of celiac disease are investigated,
2. *endoscopy* – the procedure takes a little less than 30 minutes and is used for adults, sedatives and local anesthetic. Children are usually putted under general anaesthesia. During the biopsy, the gastroenterologist inserts a small tube with a camera through the digestive tract to the small intestine **(URL 7, 2015)**,
3. *histological examination* – samples sent for histological examination shall be assessed by histological scoring, in which is used so called Marsh classification,
4. *radio diagnostic methods* – in celiac disease are mainly used in the differential diagnosis, and therefore the exclusion of other diseases, such as maldigestive syndrome (characteristic for example with inflammation, cancer or cirrhosis) and malabsorption syndrome (important is to distinguish between the primary and secondary malabsorption syndrome).

Despite the fact, that the technology is still developing, scientists and doctors know more about the illness and that there are few possibilities how to diagnose the disease, the number of people sensitive on gluten is still increasing. While in the 1970s the *prevalence* of celiac disease in the world was 0.03% **(Lohi et al, 2007)**, in the present years the estimated prevalence is about 1% **(Košičiarová et al., 2015)**. In average, the prevalence of celiac disease in the Western countries is close to 1 :100 **(Gujral et al., 2012)**. The celiac disease occurs more often in the case of women than of men, at a ratio of 2.8 :1 **(Thomas et al., 2009)**. Heritability of celiac disease is autosomaly dominant with incomplete penetration. In the case of first degree relatives the celiac disease occurs in 8 – 18%, in the case of identical twins at 70% **(Prokopová, 2008)**.

The exact and estimated prevalence of celiac disease in USA, Europe and Slovak republic in the year 2014 is alarming. While in the year 2012 the prevalence of celiac dinase in USA was 0.71% (1 in 141) **(Rubio-Tapia et al, 2012)**, in the year 2014 the prevalence was 0.75% (1 in 133) **(URL 8, 2014)**, what represents an increase in 0.04%. Unfortunately, the situation in the case of Europe and Slovak republic, is wronger – while in the year 2010 and 2012 the prevalence of celiac disease in the Europe and in Slovak republic was for about 0.5% **(Mustalahti et al., 2010)** and 0.2% (1 in 404) **(Kabátová, 2014)** in the year 2014 it is estimated that the prevalence in Europe was exactly 1% **(URL 9, 2015)** and in Slovak republic between 0.5 and 1% **(URL 5, 2014)**, what represents an increase for about 0.5 – 0.8% in only two or four years. However, it must be also mentioned that lots of celiacs are still not diagnosed, what means, that the situation in the field of celiac disease can be much more wrong.

The most commonly used illustration of celiac disease forms is so called *celiac iceberg*, which is also shown on the Figure 1 and which can be very briefly described as follows – the tip of the iceberg is represented by the relatively small number of the world's population whose gross presentation of clinical symptoms often leads to the diagnosis of celiac disease. This is the classical case of celiac disease characterized by – gastrointestinal symptoms, malabsorption and malnourishment (it is confirmed with the "gold standard" of an intestinal biopsy).The middle part of the iceberg is largely invisible to classical clinical diagnosis, but not to modern serological screening methods in the form of antibody testing. This middle part is composed of asymptomatic and latent celiac disease as well as "out of the intestine" varieties of wheat intolerance. The base of the iceberg represents approximately 20 – 30% of the world's population (those who have been found to carry the HLA-DQ locus of genetic susceptibility to celiac disease on chromosome 6) **(Sayer, 2005)**.

The only possibility how to treat the disease is a so called *gluten free diet*, which means to exclude all the food, which contains wheat, rye and barley **(Suchá at al., 2015)**.

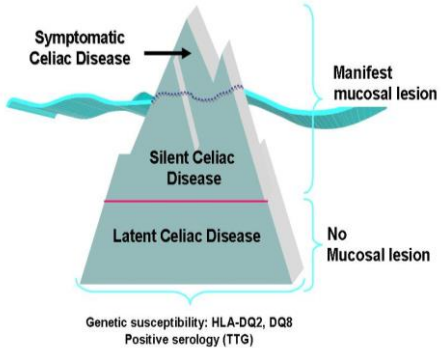

Figure 1 Iceberg image**.**

Source: Guandalini. Exploring the Iceberg.2009, p. 1.

The principles of gluten free diet can be described as follows **(URL 10, 2015)**:

1. avoid all the foods made from wheat, rye and barley,
2. avoid oats – soma celiacs can tolerate them, but the long term safety of oats in celiacs is unknown and some oat preparations can be contamined with wheat, thus it is better to avoid oats,
3. pay attention to processed food, which may contain gluten (e.g. canned soups, salad dressing, ice cream, candy bars, instant coffee, luncheon meats and processed or canned meats, ketchup and mustard, yogurt, pasta),
4. beware of tablets, capsules and vitamin preparations, which contain gluten,
5. avoid beer, but wine, brandy, whiskey and other alcohols without barley are good in moderation,
6. avoid milk and other dairy products, which contain lactose – with successful treatment, dairy products can be often reintroduced slowly into the diet later,

7. consult dietitians and national celiac disease societies for lists of gluten free food; read the food and product labels before buying or consuming any product,
8. because of the fact, that celiacs who have severe malabsorption can develop vitamin and mineral deficiencies, vitamin and mineral supplements are important.

Significant histological changes will, in keeping with the gluten-free diet, appear in three months and in two years of diet the celiacs will become practically asymptomatic **(Anderson, 2008)**. The main problem in the treatment is the non-compliance with the diet, which occurs in 50 – 80% of patients. Patients still continue in eating the food containing gluten due to lack of motivation or information. The key is the motivation of the patient, the doctor's approach and the cooperation with gastroenterologist or a registered dietitian who have the expertise in the gluten-free diet **(Hybenová at al., 2013)**.

Among the new treatments, which are nowadays tested and the research is concerned on them, are included the *genetic modification of the wheat* by which the gluten has to be removed, as well as *new medicine and vaccines*, which could prevent the damage to the intestine by the gluten **(URL 11, 2012)**.

Results of own research

With the aim to determine the Slovak celiacs opinion about the situation on Slovak market and their consumer behaviour on the market of gluten free products a structurized questionnaire survey was conducted in November 2015. The total number of respondents was 130 randomly selected celiacs from all over the Slovak republic.

From the Table 1 is clear, that the main groups of respondents were represented by women (92% of respondents – the assumption no. 1 was true), people living in the city (66% of respondents), people with the age between 26 and 35 years (36% of respondents), people with secondary education with A level (46% of respondents), people with net family income between 501 and 1.000 € and people from the Bratislava region (36% of respondents).

Up to the results of our own research we can say that most of our respondents have the celiac disease diagnosed from 0 to 5 years (68.46% of respondents – the assumption no. 2 was true), the most often symptoms up to which they have realized, that they are celiacs are diarrhea, abdominal pain, anemia, and allergy (rash and redness of the meal), which are also the typical symptoms of celiac disease **(URL 12, 2014),** most of our respondents did not have problems with the transition to a gluten free food (79.23% of respondents) and those who had the problems mentioned, that these problems were exactly – sadness, financial side of the illness, aversion to gluten free meals, need to learn how to cook without gluten, need to read all the information on the product and lack of choice (between the available products).

Despite the fact, that a few of our respondents have mentioned, that the situation with gluten free products and information about the celiac disease, resp. gluten free products on the Slovak market was before 10 to 15 years not very good, nowadays the situation is much more better – most of our respondents stay that the labelling of gluten free products is sufficient (51.54% of respondents), they have enough information about the availability of gluten free products (74.62% of respondents) and they think that the present scope of range of gluten free products is better as before (89.23% of respondents).

Unfortunately, there is still one drawback of gluten free products and that is their price – 93.08% of respondents mentioned that up to their opinion, the price of gluten free products is inadequate (Figure 2).

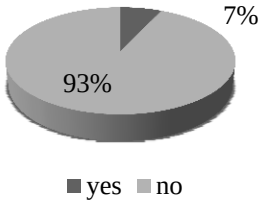

Figure 2 Adequacy of the price**.**
Source: Results of the research.

Because the Government of the Slovak Republic realizes, that this is a very huge problem, they offer (cross the Office of Labour, Social Affairs and Family) a so called possibility of granting a monetary contribution for compensation of increased expenses on a special diet. *"Health insurance companies are covering gluten-free products approximately from 60% of the price (flour, pasta, raw material) to 5 – 30% (ready baked bread, additional gluten free cookies)"* **(Rimarova, 2013)**. In spite of that, that most of our respondents, exactly 82.31% of respondents, know about this possibility, only 45.38% of them also use it (the assumption no. 3 was not true) and 46.15% of them are not satisfied with its height.

Because of the need to realize how are Slovak celiacs satisfied with the scope of range of gluten free products, as well as with their availability, in the questionnaire, there were formulated also the questions connected to these issues. Up to their evaluation we can say, that in these questions the Slovak market has still some reserves – most of our respondents (64.62% of them and 57.69% of them – the assumption no. 4 was true) think that the scope of range of gluten free products in the Slovak market, as well as in their region is insufficient and most of them also think (53.08% and 57.69% of respondents) that their availability in the Slovak market, as well as in their region is inadequate.

As it can be seen from the figure above, the most important criterion in the purchase of gluten free products is their quality (51.54% of respondents). This is, why we have been interested not just in the detection of the frequency of the gluten free product´s purchase, but also in

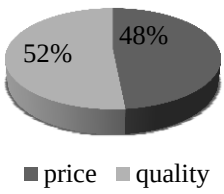

Figure 3 The decisive criterion in the purchase of gluten free products.
Source: Results of the research.

the place of their purchase (Figure 4), preference of domestic producers of gluten free products, mostly preferred labels of them, usage of the possibility to buy them abroad, as well as the reasons leading to their purchase abroad.

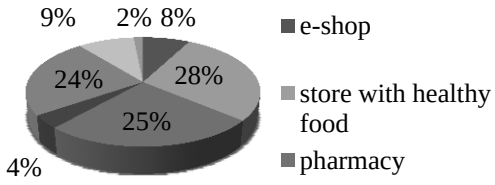

Figure 4 Place of purchase of gluten free products.
Source: Results of the research.

Up to the results of our questionnaire we can say, that most of our respondents buy gluten free products for few times in a week (39.23% of respondents), they buy them mostly in stores with healthy food, pharmacy and supermarket (28.46%, 25.38% and 23.85% of respondents), they do not exactly prefer the domestic producers of gluten free products (52.31% of respondents), they prefer mostly the labels of Schär, Novalim and Schnitzer (42.31%, 36.23% and 10.2% of respondents; Figure 5), they use the possibility to buy them abroad only randomly (63.85% of respondents do not use this possibility at all) and they use it only in the case of neighbouring countries because of the better quality and price of these products (81.11% and 79.3% of those respondents who use this possibility).

The above mentioned results correspond to some extant

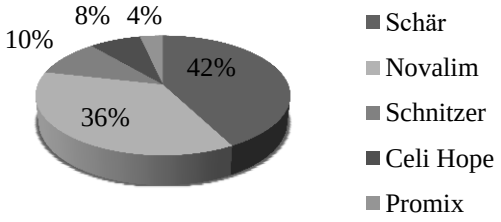

Figure 5 Mostly preferred labels of gluten free products.
Source: Results of the research.

to the results of research conducted by **Hes et al.,(2014)** on the sample of 289 randomly selected Slovak celiacs, where the authors have found out that the most important criterion in the purchase of gluten free products is their freshness and quality (57% of respondents), resp. the price (23% of respondents); that most of Slovak celiacs buy gluten free products for once or few times in a week (44% and 27% of respondents); most of them (45% of respondents) prefer to purchase gluten-free products in specialized stores, supermarkets (18% of respondents), hypermarkets (18% of respondents)and in pharmacies (10% of respondents) and that they prefer mostly the labels of Schär, Novalim and Pečivárne Liptovský Hrádok (96.19%, 28.35% and 23.18% of respondents); resp. with the results of research conducted by **Košičiarová et al., (2015)**on the sample of 506 randomly selected Slovak celiacs, where the authors have found out that the most important criterion in the purchase of gluten free products is their freshness and quality (56.56% of respondents), price level (21.06% of respondents) and their range width (14.04% of respondents), that most of Slovak celiacs buy gluten free products in specialized shops (52.17% of respondents), hypermarkets (18.77% of respondents) and supermarkets (17.98% of respondents) and that most of them are not satisfied with the range width of the gluten free products (69.96% of respondents). Unfortunately, there are no more researches with which we could compare our results, because our research is very specific and in the area of Slovak republic there was not implemented a similar research. This disadvantage brings with it also additional advantage in the form of the possibility of implementing other similar studies, resp. other studies focused on the situation of Slovak celiacs.

Evaluationoftheformulatedhypotheses

Connected with few of above evaluated questions, there have appeared also the questions of the dependence resp. indepence between few variables. This is, why in the part Material and Methodology were formulated ten different hypotheses, which have been tested with the use of Pearson´s chi-square test, Mann-Whitney U-Test and Cramer´s contingency coefficient; and which evaluation is following one:

1. H_{01} – there does not exist the dependence between the frequency of purchase of gluten free products and the respondent´s place of living – accepted.
2. H_{02} – there does not exist the dependence between the place of purchase of gluten free products and the respondent´s age – accepted.
3. H_{03} – there does not exist the dependence between the decisive criteria in the purchase of gluten free products and the respondent´s age – accepted.
4. H_{04} – there does not exist the dependence between the decisive criteria in the purchase of gluten free products and the respondent´s level of education – accepted.
5. H_{15} – there exists the dependence between the respondent´s opinion on the adequacy of available information and the region from which the respondent comes from – accepted.
6. H_{06} – there does not exist the dependence between the respondent´s opinion on the scope of gluten free products on the Slovak market and the region from which the respondent comes from – accepted.
7. H_{07} – there does not exist the dependence between the respondent´s opinion on the scope of gluten free products in his region and the region from which the respondent comes from – accepted.
8. H_{08} – there does not exist the dependence between the respondent´s opinion on the availability of gluten free products on the Slovak market and the region from which the respondent comes from – accepted.
9. H_{09} – there does not exist the dependence between the respondent´s opinion on the availability of gluten free products in his region and the region from which the respondent comes from – accepted.

H_{110} – there exists the dependence between buying gluten free products abroad and the region from which the respondent comes from – accepted.

CONCLUSION

Up to the results of our own research, which was conducted on the sample of 130 randomly selected celiacs from different parts of Slovak republic, we can conclude, that while the situation with the information and availability of gluten free products on the Slovak market is nowadays on a better level as it was before, there are still some drawbacks, which must be reduced – still too high price of gluten free products (only 6.92% of respondents think that the price is adequate), lack of people who use so called possibility of granting a monetary contribution for compensation of increased expenses on a special diet (only 45.39% of respondents use this possibility), insufficient scope of range of gluten free products in the Slovak market (almost 65% of respondents), insufficient scope of range of gluten free products in the respondent´s region (more than 58% of respondents), inadequate availability of gluten free products in the Slovak market (53% of respondents), inadequate availability of gluten free products in the respondent´s region (almost 58% of respondents) and small preference of domestic producers of gluten free products (only 47.69% of respondents prefer them).

Because of the need to execute a deeper analysis of the obtained results, as well as to determine the dependence between variables, four assumptions and ten hypotheses have been formulated and finally tested. Up to their evaluation we can say, that only three assumptions were true – the assumption no. 1, which has said that most of our respondents are women (exactly 92% of our respondents were women, which confirms also the statement that the celiac disease occurs more often in the case of women than of men); the assumption no. 2, which has said that most of our respondents have celiac disease diagnosed from 0 to 5 years (exactly 68.46% of our respondents, which confirms our previous notes about the increasing number of celiacs in the last few years); and the assumption no. 4, which has said that most of our respondents think that the scope of range of gluten free products in the Slovak market is insufficient (exactly 64.62% of our respondents, which is a very interesting result because exactly 89.23% of our respondents stated in an another questions, that the present scope of range of gluten free products is better as it was before); and only two hypotheses have been confirmed. These hypotheses show, that between the respondent´s opinion on the adequacy of available information and the region from which he/she comes from, there exists a small, but statistically significant dependence (the result of Cramer´s contingency coefficient was equal to 0.10085, what can be interpreted as a weak dependence, and the result of Mann-Whitney´s U-Test was − the U-value was 12.5, the critical value of U at $p \leq 0.05$ was 13, which means, that the result is at $p \leq 0.05$significant) and between buying gluten free products abroad and the region from which the respondent comes from there exists also some dependence, but it is statistically not significant (up to the results of Pearson´s chi-square test, the H_0 hypothesis must be on the level of significance 5% rejected and adopted must be its alternative H_1 hypothesis talking about the dependence between tested variables (TC = 18.988 >CV = 14.067), the result of Cramer´s contingency coefficient was equal to 0.04242, what can be interpreted as almost none dependence, and the result of Mann-Whitney´s U-Test was − the U-value was 24, the critical value of U at $p \leq 0.05$ was 13, which means that, the result is at $p \leq 0.05$not significant).

Based on the results of our research, we can propose the following recommendations for not just the Ministry of Health of Slovak Republic, but also for the Office of Labour, Social Affairs and Family, doctors as well as producers, suppliers and sellers of gluten free products:

- to increase the level, but also the promotion of so called possibility of granting a monetary contribution for compensation of increased expenses on a special diet – most of celiacs know about this possibility, but they do not use it, because they think that it is still very small and insufficient, as well as they say, that it is also difficult to obtain it,
- this is why it is needed also to reduce the bureaucracy and difficulty of its obtaining,
- to support the producers of gluten free products so that they could decrease their price,
- to organize free tasting of gluten free products,
- to increase their quality – exactly in the case of their taste,
- to organize free courses how to buy gluten free products, where to buy them, how to cook for celiacs, how to change to gluten free diet,
- to create separate corners with gluten free products, which will be visible for celiacs and where they could find also some added information about them, etc.

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Contactaddress:

Ľudmila Nagyová, Slovak University of Agriculture, Faculty of Economics and Management, Department of Marketing and Trade, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: ludmilanagyova@hotmail.com.

Ingrida Košičiarová, Slovak University of Agriculture, Faculty of Economics and Management, Department of Marketing and Trade, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: ingrida.kosiciarova@gmail.com.

Jana Rybanská, Slovak University of Agriculture, Faculty of Economics and Management, Department of Marketing and Trade, Tr. A. Hlinku 2, 949 76 Nitr,a Slovakia, E-mail: jane.rybanska@gmail.com.

Mária Holienčinová, Slovak University of Agriculture, Faculty of Economics and Management, Department of Marketing and Trade, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: maria.holiencinova@gmail.com.

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INFLUENCE OF THE XANTHAN GUM ADDITION ON THE TECHNOLOGICAL AND SENSORY QUALITY OF BAKING PRODUCTS DURING THE FREEZING STORAGE

Tatiana Bojňanská, Jana Šmitalová, Vladimír Vietoris, Alena Vollmannová

ABSTRACT

The influence of the 0.16% xanthan gum addition in the recipe of the bread production and its influence on the baking and sensory quality of products was monitored during the process of our research. Prepared dough was inserted in the freezing box directly (-18°C) and it was stored for one, two, three, four, five and six months. When the baking process was finished, the products with xanthan gum and the products without it were compared and evaluated by both objective and subjective methods. It was found that freezing, cooling and storage of the products without xanthan gum addition influenced the volume, vaulting and general appearance of the products in a negative way and loaves of bread were evaluated as unacceptable after four months of freezing. The quality of experimental loaves of bread with xanthan gum was, even after six months of freezing storage, comparable with freshly baked products. Despite the freezing, the volume of the products had an increased value. After first month of freezing the volume increased by 28.6% and after two months of freezing it increased by 23.8% both compared to the control. The vaulting in products processed by freezing was in the required optimal level during the whole period of freezing. Sensory evaluation results of loaves of bread with xanthan gum were the best after three, four and five months of storage in a freezer, when 98 points were achieved. During the monitored period of freezing, the addition of 0.16% of xanthan gum markedly contributed to the preservation of sensory and baking quality of the frozen wheat dough.

Keywords: xanthan gum; freezing storage; baking quality; sensory quality

INTRODUCTION

The producer's aim is to preserve the product fresh as long as possible, therefore it is feasible to influence the forms of slowing down the bread staling by regulating the room temperature where the bread is stored and by the modification of production process, or recipes **(Cauvain, 1998; Hampl et al., 1981)**. Hydrocolloids are perspective additives with the ability of effective linking of water molecules which become fixed, thus with this mechanism it is possible to eliminate the presence of unfavourable crystals that influence product texture in a negative way **(Gimeno et al., 2004; Khan et al., 2007)**.

Hydrocolloids are high-molecular hydrophilic biopolymers which has many functions in food industry. The most important function could be the ability to control the rheologic attributes and food texture. In baking industry they are mostly added for the purposes of emulsions, suspensions and foams stabilization, to improve the processing attributes, because of their inhibition ability of starch retrogradation, their efficient humidity retaining, improvement of the whole structure, staling inhibition of the products, but also as the replacement of fat and eggs **(Collar et al., 1999; García-Ochoa et al., 2000; Arozarena et al., 2001;Kohajdová et al., 2008; Kohajdová et al., 2009; Magala et al., 2011)**. The most popular hydrocolloid of the microbial origin is a xanthan gum, which is exocellular polysaccharide produced by aerobical sugar fermentation by the *Xanthomonas campestris* bacterium **(Hojerová et al., 2005; Mikuš et al., 2011; Tao et al., 2012)**; the main bond of xanthan consists of β-D-(1.4) glucosic elements and lateral bonds are formed by the D-glucoronic acid leftover and two leftovers of D-mannose **(Velíšek, 2002)**. According to **Hojerová et al. (2005)**, it was discovered that xanthan gum reaches the fastest hydration, the smallest temperature sensibility and its stability in soft acid to neutral pH only.

The xanthan gum is characterized by the ability of creation the reversible gels in conjunction with galactomannans, e.g. carob gum **(Milani et al., 2012)**. It is possible to use xanthan gum as a partial replacement of egg white in cakes **(Miller and Setser, 1983)**. Moreover it was discovered that the xanthan gum addition during the dough kneading, prevented the shrinking of products and markedly improved the volume and height of a bread in comparison with the specimen **(Miller and Hoseney, 1993)**, provided the highest viscosity of dough **(Ashwini et al., 2009)**, firmed the structure of dough **(Ashwini et al., 2009)** and strengthened the bonds between flour

proteins **(Collar et al., 1999)**. There are various possibilities of xanthan gum usage: applications in dressings, syrups, or diet, frozen and baking products, but also in other branches of industry and agriculture **(García-Ochoa et al., 2000)**. The xanthan gum has a very important function as an additive in gluten-free bread recipes where, due to the absence of gluten, it is improving technological and sensorial quality of the products for celiatics **(Gambuś et al., 2007)**.

MATERIAL AND METHODOLOGY

The first experimental group of loaves of bread were prepared from wheat extra fine flour T 650 (Mlyn Pohronský Ruskov a.s.) in amount of 500 g, sugar (1%), salt (1.6%), yeast (4%) (Trenčianske droždie, OLD HEROLD HEFE, s.r.o.) and water which was added according to farinograph **(ICC - Standard 115/1, 1992, AACC Method 54-21, 1995)** farinograph water absorption of the flour (150 cm^3). The second experimental group of bread loaves was created with the same ingredients in same proportions, but in addition it contained 0.16% of xanthan gum (f. Natural Jihlava) in regard to the flour weight.

The dough was kneaded in a laboratory kneader Diosna SP 12. Then it was designed into loaves of bread, which rose in rise rooms for 20 minutes at 30°C and after that were baked at 240°C for 20 minutes with a steaming in Miwe Condo oven. The control specimen of wheat bread loaves was prepared this way. The rest of the loaves was put without yeasting into the freezer with temperature of -18°C (AFG 070 AP, company: Whirlpool Slovakia spol. s.r.o.,) and stored at this temperature for one, two, three, four, five and six months. The defrosting of the loaves before the yeasting and baking lasted 2 hours at room temperature of 22°C ±2°C.

The flour was quantitatively evaluated and the following attributes were set: *determination of the moisture content, %* **(ICC Standards No. 110/1 (1976))**, *ash on products, %* **(ICC Standard No. 104/1, (1990))**, *determination of the "Falling number", s* **(Falling Number, measurer FN 1800, Perten, according ICC Standard No. 107/1, (1995))**, *the wet gluten rate, %* Glutomatic 22000, Perten, (**ICC Standard No. 155, (1994))**, *determination of the sedimentation value by Zeleny, cm^3* **(ICC Standard No. 116/1, (1994))**, *determination of crude protein* **(ICC Standard No. 105/2 (1994))**.

Baked loaves of bread were evaluated by objective methods for baking quality examination during the storage at -18°C. Within them the following attributes were set by standard procedures and calculations used at the working place: *volume of the products* (cm^3), *specific volume of the products* ($cm^3.100g^{-1}$), *volume recovery* ($cm^3.100g^{-1}$ flour), *yield of products* (%), *baking losses* (%) and *vaulting of the products* (the ratio of the height and width of the loaves). Also, the loaves were evaluated sensorically by using the one hundred points test applied by the retrained evaluators at the Department of Plant Products Storing and Processing. These attributes were evaluated: *general appearance and shape* (the coefficient of the importance 1), *surface and characters of the crust* (the coefficient of the importance 2), *rising and the appearance of the crumb* (the coefficient of the importance 4), *structure and elasticity of the crumb* (the coefficient of the importance 4), *smell and taste* (the coefficient of the importance 9) with the maximum possible reached points of 100. In terms of sensory evaluation, the general profile of experimental controlled loaves (which were stored for six months in a freezer) with xanthan gum, was created and compared. The experiment results are pictured graphically and they were evaluated by the application of non-parametric statistic methods: Wilcoxon test and Kruskal-Wallis test and evaluated in **R Core Team (2014)**.

RESULTS AND DISCUSSION

The year consumption of pastry for one inhabitant of the Slovak Republic is approximately 40 kg of bread and 30 kg of wheat pastry **(ŠÚ SR)**. The quality of bread and pastry as the essential food is a very current topic. It is markedly influenced by the quality of used ingredients, especially flour and additives. In the flour we used (T650) it was detected: moisture content 13.9%, ash on products 0.49%, the Falling Number test 324 s, the wet gluten rate 32.6%, sedimentation value according to Zeleny 40.0 cm^3, crude protein (Nx5.7) 11.5%. Based on the analysis of **Muchová et al. (2001)** the used flour can be characterized as mid-strong with proportional amylases activity and sufficient amount of good quality gluten; so it can be used for risen products.

The prepared dough in the bowl mixed directly after the rising and the qualitatively evaluated specimen was used for the control. After the process of dough freezing, storage (from one to six months) and defrosting, the next specimens were risen again and baked according to the

Table 1 Results of the experiment with wheat bread loaves without the xanthan gum addition

period of deep-freeze storage	volume of products cm^3	specific volume $cm^3.100g$	volume recovery $cm^3.100$ g flour	yield of products %
immediately baked/ control [a]	262.5	297.1	420.0	141.3
one month [a]	212.5	231.9	340.0	146.5
two months [a]	225.0	258.3	360.0	139.3
three months [a]	240.0	264.6	384.0	145.1
four months [a]	235.0	283.7	380.0	133.9
five months [a]	215.0	239.1	344.0	143.8
six months [a]	200.0	230.9	320.0	138.5

methodology described above. The chosen results of the baking experiment without the xanthan gum are pictured in Table 1. From the Table 1, it is seen that the volume of the baked loaves without the addition of xanthan gum has been gradually decreasing till the third month, then the decrease was stopped and the slight increase of volume was observed compared to the first and the second month of freezing. Based on the results of Kruskal – Wallis test it was discovered that there is no statistic difference between the samples (every month of freezing was compared with the control), because the rate of p = 0.45. After five and six months of freezing, the loaves of bread achieved the lowest volume and insufficient vaulting (Figure 1) in comparison to the control and thus these products can be evaluated as unsatisfactory.

Results of the work, which confirm the similar decreasing baking quality during the freezing storage, were also noticed and described after six months of freezing storage by the authors **Berglund et al.** (**1991**).

The disrupted and cracked gluten structure, which was separated from the starch granules, was discovered by them. More authors mention that the most significant changes of frozen dough were related to yeast, because dead ice damaged yeast cells release glutathione, which disables the gluten structure. This consequently leads to the worse retention of gases and extension of the rise time of dough **(Kline et al., 1968; Hsu et al., 1979; Autio et al., 1992; Gelinas et al., 1995; Pepe et al., 2005)**. A decreased ability of the dough to retain emergent gases during the rising process is practically expressed by insufficient and low volume of experimental loaves of bread. So it is possible to state, that freezing storage gradually degrades the baking quality of loaves of bread, which was confirmed also by our experiments.

Table 2 shows experimental loaves of bread staling, which in contrast to products pictured in Table 1 contained

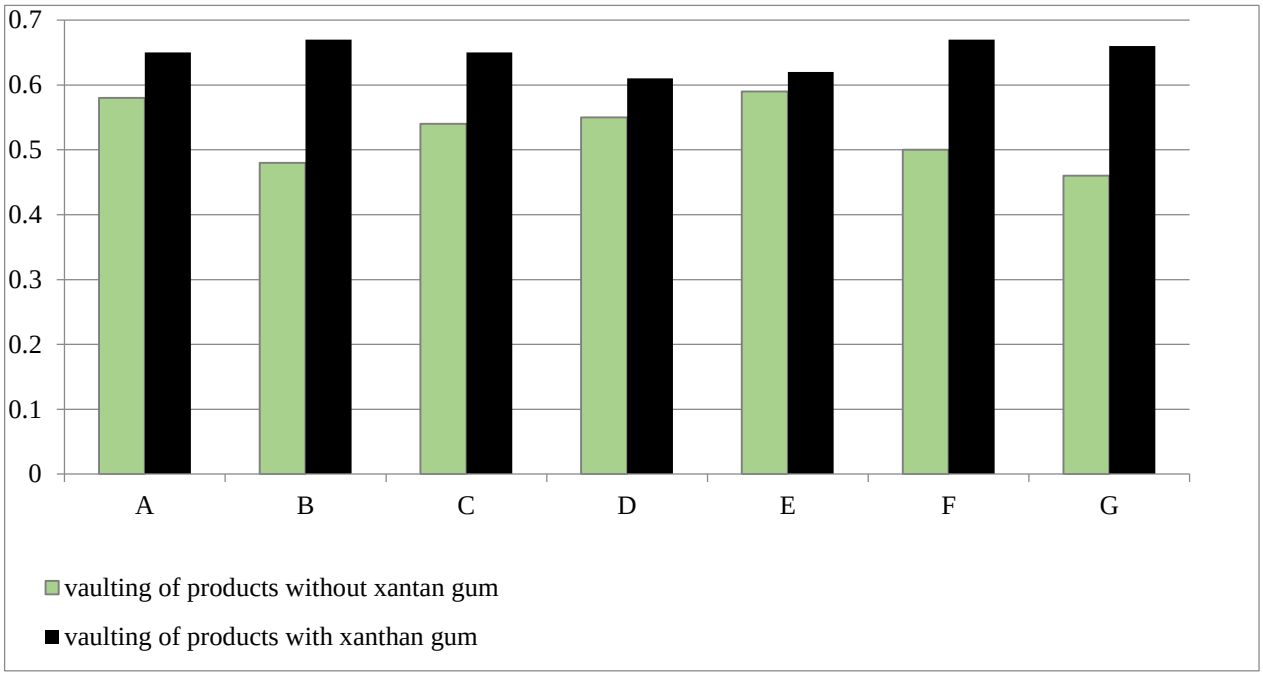

Figure 1 Comparison of the vaulting of the products with and without xanthan gum.

Note: A – products baked at the beginning - the control, B – products stored in freezer for one month, C – two months, D – three months, E – four months, F – five months, G – six months. The 0.65 value is optimal for vaulting of the loaves of bread. The values under 0.6 and over 0.7 are insufficient for vaulting of the products.

Table 2 Baking experiment results with the wheat loaves of bread containing 0.16% of xanthan gum.

period of deep-freeze storage	volume of products cm^3	specific volume $cm^3.100g$	volume recovery $cm^3.100$ g flour	yield of products %
immediately baked/ control [a]	262.5	286.4	420.0	146.6
one month [a]	337.5	362.5	540.0	148.9
two months [a]	325.0	351.5	520.0	147.9
three months [a]	262.5	241.8	420.0	153.4
four months [a]	312.5	338.2	500.0	147.8
five months [a]	275.0	301.2	440.0	146.0
six months [a]	262.5	280.9	420.0	149.5

0.16% of xanthan gum. The dough prepared in kneader was risen and baked. The qualitatively evaluated sample was used as the control. The other samples (experimental loaves of dough) were put in a freezing storage (from one to six months) and then defrosted, risen, baked and evaluated. Consequently, their baking quality was compared to the products without the addition of xanthan gum.

The Table 2, where results of baking experiment with frozen and baked loaves of bread with 0.16% of xanthan gum are introduced, shows that freezing storage does not affect the quality of products negatively. After the first month the volume of later baked loaves of bread increased by 28.6% compared to the control, after two months of freezing storage it increased by 23.8% also compared to the control and the vaulting rates (Figure 1) were on the required optimal level constantly. After three and six months of freezing storage the same volume as in the control was discovered and after four and five months of storage the increase of volume was observed, while the vaulting was constantly good (0.65). Statistic results reached by Kruskal – Wallis test (every month of freezing storage is compared to the control again) indicated the value of $p = 0.7$ which means that no statistical difference was found between compared samples and the control. By the Wilcoxon test, the results of particular months of freezing storage without xanthan gum were statistically compared to the results of products, which were frozen for the same time span, but they contained xanthan gum. It is possible to claim again that there was no statistically significant difference between compared samples.

In the past, many scientific groups were engaged in removing and eliminating negative impacts on baking products by the addition of xanthan gum, e.g. **Collar et al. (1999)**, who published that xanthan gum in recipes improves the retention of gases which is visible by bigger volume, **Rosell et al. (2001)** discovered the positive effect of increased water activity in a crumb, **Dodić et al. (2007)** measured out the higher specific volume in comparison with frozen products without the addition of hydrocolloids and **Mandala (2005)** discovered that the xanthan gum addition higher than 0.16% causes a decrease of specific volume of breads, so it recommended not to exceed this level. The results of works of the authors who were engaged in this issue were also confirmed by our experiments. It is possible to claim that the baking quality of the products with 0.16% xanthan gum addition was perfect even after six months of freezing storage and it was comparable to freshly baked products. After the evaluation of baking experiment, the loaves of bread were subjectively rated by 100 points questionnaires for the comparison of the xanthan gum influence on sensory quality of the products. Figure 2 shows the results of sensory evaluation.

As it results from Figure 2, the sensory quality of experimental loaves of bread without the addition of xanthan gum has been proportionally declining as the time of freezing storage has been prolonging. The most expressive decline of sensory quality was observed after four months of storage (Letter E). The experimental loaves of bread which contained xanthan gum reached almost the same sensory quality during the whole period of freezing. That proves the positive influence of the additive.

For better comparison of xanthan gum influence on a general appearance, an appearance of crust and crumb, elasticity, smell and taste; the sensory profile of bread loaves without xanthan gum (Figure 3) and sensory profile of measured index of bread loaves with xanthan gum (Figure 4) was created. By comparison of the Figure 3 and the Figure 4, it is possible to claim that the addition of 0.16% of xanthan gum had a positive effect on surface of loaves of bread, quality of crust and crumb and elasticity

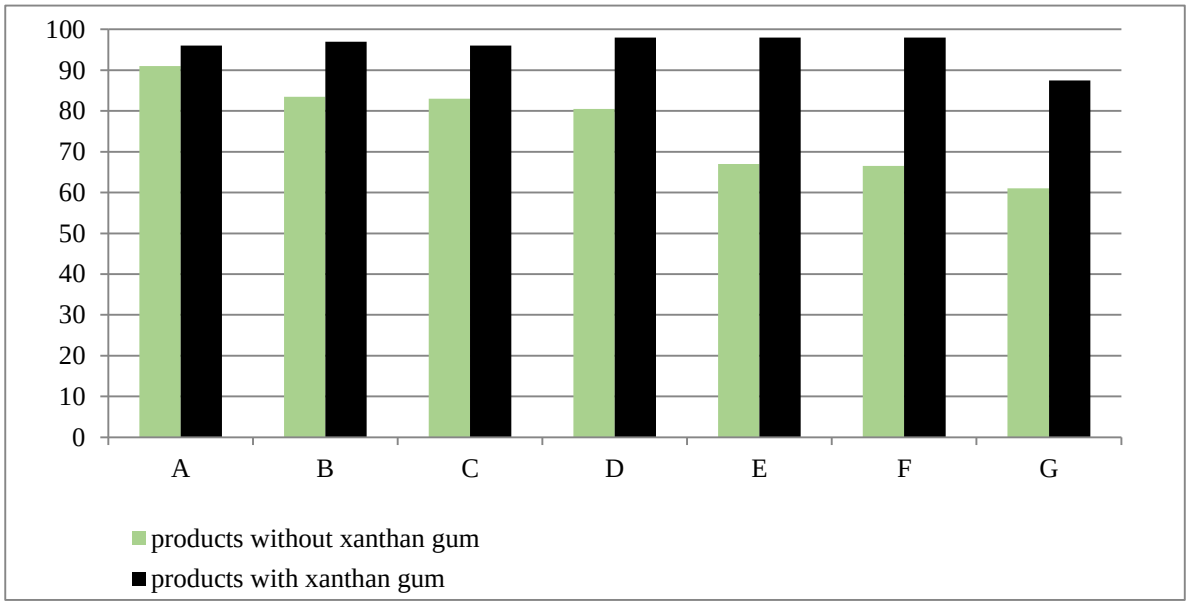

Figure 2 The comparison of sensorial qualities process during the freezing storage.

Note: A – products baked at the beginning - the control, B – products stored in freezer for one month, C – two months, D – three months, E – four months, F – five months, G – six months.

of crumb. The evaluators claimed that even after six months of freezing storage, the crust was softer, crispier and easier to chew in the products with xanthan gum, compared to the products without it.

Moreover, the positive influence of the xanthan gum addition was discovered in the quality of crumb, especially its structure, elasticity and uniform porosity of the products.

The sensory and technological baking quality of baked bread loaves without the addition of xanthan gum were after six months of freezing storage evaluated by **Bojňanská et al.** (**2013**), as decreased in comparison to the control was. Our experiments confirm the results for example of **Gimeno et al.** (**2004**). The sensory quality of experimental loaves of bread with xanthan gum was in balance for almost the whole period of the storage. A mild decline was observed after six months of freezing (85.5 points), but the point score can still be considered as convenient. The addition of xanthan gum had clearly positive effect on the sensory quality of the products during the whole period of freezing storage.

By comparison of the Figure 3 and the Figure 4, it is possible to claim that the addition of 0.16% of xanthan gum had a positive effect on surface of loaves of bread, quality of crust and crumb and elasticity of crumb. The evaluators claimed that even after six months of freezing storage, the crust was softer, crispier and easier to chew in the products with xanthan gum, compared to the products without it. Moreover, the positive influence of the xanthan gum addition was discovered in the quality of crumb, especially its structure, elasticity and uniform porosity of the products.

The sensory and technological baking quality of baked bread loaves without the addition of xanthan gum were after six months of freezing storage evaluated by **Bojňanská et al.** (**2013**), as decreased in comparison to the control was. Our experiments confirm the results for example of **Gimeno et al.** (**2004**). The sensory quality of experimental loaves of bread with xanthan gum was in balance for almost the whole period of the storage. A mild decline was observed after six months of freezing (85.5 points), but the point score can still be considered as convenient. The addition of xanthan gum had clearly positive effect on the sensory quality of the products during the whole period of freezing storage.

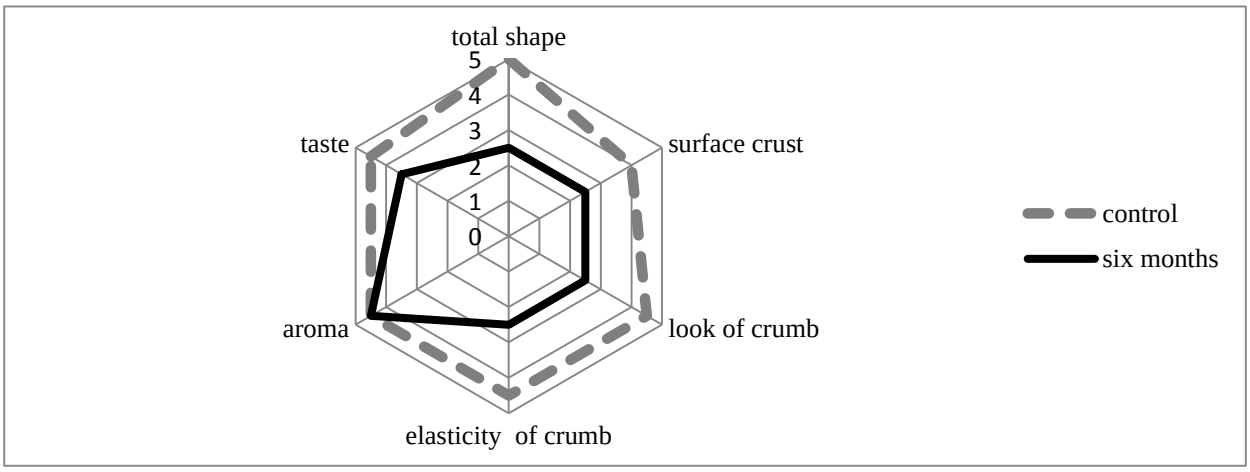

Figure 3 Sensory profile of the products without xanthan gum.

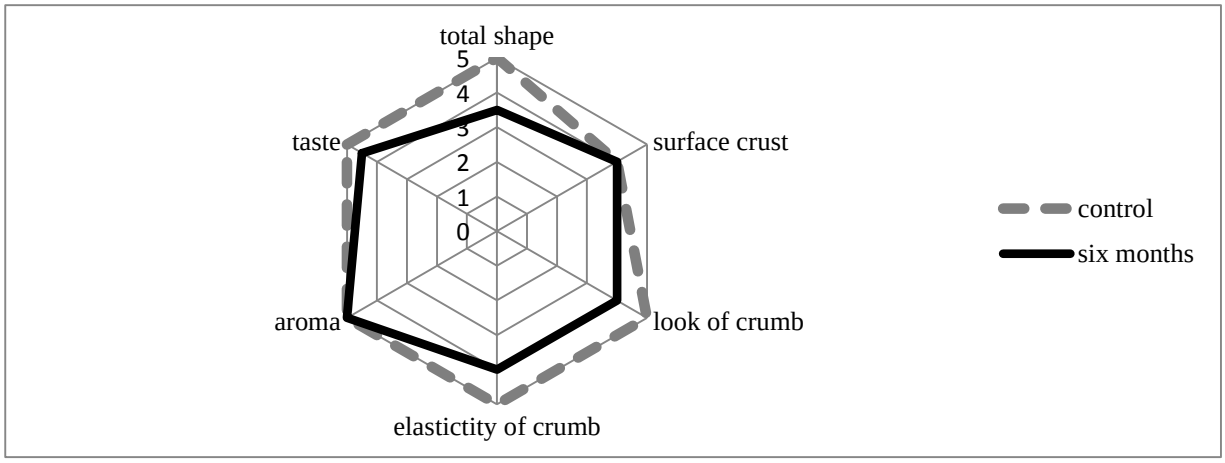

Figure 4 Sensory profile of the products with xanthan gum.

CONCLUSION

Based on the comparison of the baking experiment results and the sensory evaluation of baked wheat bread loaves and bread loaves with xanthan gum addition, it is possible to claim that this additive in recipe has markedly contributed to the elimination of undesirable effects of freezing storage and the products achieved perfect quality also after six months of storage. Technological and sensory quality of the products without xanthan gum can be, after four months of the storage in a freezing box, evaluated as unsatisfactory. This was in contrast to the loaves of bread with 0.16% xanthan gum addition which volume, specific volume, vaulting and sensorial indicators were, even after six months of freezing storage, still excellent and comparable with freshly baked products (the control).

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Contact address:

doc. Ing. Tatiana Bojňanská, CSc. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storage and Processing of Plant Products, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, Tel. 037 6414 703, E-mail: tatiana.bojnanska@uniag.sk.

Ing. Jana Šmitalová, PhD. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storage and Processing of Plant Products, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, Tel. 037 6414 933, E-mail: jana.smitalova@gmail.com.

doc. Ing. Vladimír Vietoris, PhD. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Storage and Processing of Plant Products, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: vladimir.vietoris@unaig.sk

prof. RNDr. Alena Vollmannová, PhD. Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: alena.vollmannova@uniag.sk

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Intersection of mycotoxins from grains to finished baking

Viera Šottníková, Luděk Hřivna, Iva Burešová, Jan Nedělník

ABSTRACT

This work is focused on the evaluation of the content of deoxynivalenol and zearalenone in samples of winter wheat of the following varieties: Sultan, Cubus, Akteur, Seladon, Mulan, Chevalier, Evina, Hewitt, Bohemia, Baletka. The total amount of 10 samples harvested in 2011 and 2012 was evaluated and included variants both treated and untreated against fungal diseases. The samples were adjusted for mycotoxicological determination and subsequently measured by the ELISA method. The content of deoxynivalenol (DON) and zearalenone (ZEA) was measured in grain, flour and breadrolls in all samples. Out of all samples 43% were found to have positive content of DON and 75% of ZEA. In the treated variants, the average DON content was found to be 115 $\mu g.kg^{-1}$ in grain, 77 $\mu g.kg^{-1}$ in flour and 97 $\mu g.kg^{-1}$ in pastries. In the untreated variants, the average DON content was found to be 208 $\mu g.kg^{-1}$ in grain, 103 $\mu g.kg^{-1}$ in flour and 128 $\mu g.kg^{-1}$ in pastries. Moreover, the average ZEA content in the treated variant was 4.95 $\mu g.kg^{-1}$ in grain, 3.38 $\mu g.kg^{-1}$ in flour and 4.51 $\mu g.kg^{-1}$ in pastries, in the non-treated variant average ZEA content in grain was 3.07 $\mu g.kg^{-1}$, 4.97 $\mu g.kg^{-1}$ in flour and 2.81 $\mu g.kg^{-1}$ in pastries. The maximal acceptable limits given by the valid legislation were not exceeded in any analysed sample. It can be concluded wheat grain grown in the Czech Republic, whether it is treated or untreated by fungicides, is not dangerous for consumers. The content of both mycotoxins is not dependent on each other, and the untreated variant has a slightly higher dependency between DON and ZEA.

Keywords: mycotoxins; deoxynivalenol; zearalenone; grain; flour; finished product

INTRODUCTION

Microscopic fibrous micromycetes (moulds) create an important part of all organisms, especially in relation to humans and animals. They can cause skin, mucosal and internal organ diseases, collectively they are called mycoses **(Cempírková et al., 1997)**. Mycotoxicosis occurs after consumption, inhalation or after contact with toxic secondary metabolites of microscopic fungi. In favourable conditions, microscopis fungi may contaminate and destroy huge quantity of stock, food and feed by its adverse actions **(Tančinova et al., 2012)**. Every type of micromycet has different requirements for environment conditions, in which it grows, and this is also valid for types of the same species **(Diekman and Green, 1992)**. We can divide them into external factors (temperature, relative humidity, oxygen, period of stock storage), and internal factors (water activity, pH, texture, composition of stock, antimicrobial substances in stock) **(Tančinová et al., 2001, Mašková et al., 2012)**.

Danger of majority of mycotoxins lies rather in chronical effect of slight amounts than in actual toxicity **(Patočka et al., 2004)**. Deoxynivalenol (DON) is probably the most known and the most common mycotoxin contaminating feed and food made of grain crops. DON was for the first time isolated from corn infested by mould *Fusarium graminearum* in 1973. Further it is produced by toxicogenic species of *Fusarium culmorum, F. graminearum, F. sporotrichioides*a*F. poae*. It occurs anywhere in the world where grains are grown. It has been found in a set of other food, e.g. in baby food made of grains, in corn, rice, millet, bran, ginger, garlic, beer, muesli, spaghetti and soybeans **(Malíř and Ostrý, 2003)**.

Zearalenone (ZEA, also F-2 toxin) was for the first time isolated in 1964 from the culture *Gibberellazeae* (anamorph of *F. graminearum*) of corn. It is produced by the genus *Fusarium*, especially *Fusarium graminearum, F. culmorum, F. equiseti, F. moniliformea* and *F. semitectum* belong to the most significant producers. The ideal temperatures for production of zearalenone are in the range of 12 – 14 °C, but also lower than 10 °C. *F. graminearum* is able to produce zearalenone even in the concentration 1900 µg/kg **(Magan and Olsen, 2004)**. First indirect evidence about masked mycotoxins appeared already in the first half of 80s in the 20th century. In animals, there were largely observed symptoms typical for mycotoxicosis in spite of the quantity of mycotoxins set in feed did not correspond with that. High toxicity of feed was apparently caused by conjugated forms of mycotoxins, which leaked from analytical determination **(Berthiller et al., 2013)**. While observing dynamics of mycotoxins in intentionally infected wheat, the increase of deoxynivalenol and subsequently its decrease was proved, which was probably caused by transformation of initial deoxynivalenol into its metabolites **(Hajšlová et al., 2009)**. Studies dealing with transformations of mycotoxins proved that conjugated forms of mycotoxins apparently occur during detoxification processes of grain crops. Until today, metabolites of deoxynivalenol, zearalenone, ochratoxin A and T-2 toxin have been identified **(Berthiller et al., 2013)**. Currently, only deoxynivalenol and zearalenone are

observed as markers of grain contamination caused by fusarium mycotoxins. However, many studies proved that in a majority of cases, trichotecenenivalenol, T-2 toxin or HT-2 toxin are dominant. However, if deoxynivalenol is the only one analytically observed representative, the severity of contamination can be underestimated **(Nedělník et al., 2005)**. Commission Regulation (ES) No. 1126/2007 from 28th September 2007 determines maximum limits for deoxynivalenol, zearalenone (cereals - 100, flour - 75 and breadrolls - 50 µg per 1 kg) and tolerable daily intakes of these substances to 1 kg of body weight.

The objective of work was to compare and evaluate changes in the content of mycotoxins DON and ZEA in wheat grain, flour and breadrolls.

MATERIAL AND METHODOLOGY

Samples of winter wheat Sultan, Cubus, Akteur, Seladon, Mulan, Chevalier, Evina, Hewitt, Bohemia, Baletka were used for measuring, while they were obtained from experiments of the Central Controlling and Testing Institute for Agriculture, from its research station Hradec nad Svitavou. Grain was harvested from standardly treated crops (seed disinfected against bunt, dwarf bunt, morph regulator was used during vegetation, fungicide was applied against illnesses of haulm heels and against leaf and spikelet illnesses: T1 – treatment end of bracketing, T2 – beginning of heading until flowering). The test stations were located in the altitude 450 m, where long-term average temperature is 7.4 °C, long-term average rainfall is 616 mm. The soil type was typical brown soil and the soil class clay-loam (heavy soil). Content of DON and ZEA was observed in grain samples (especially whole grain groats, flour and breadrolls made of it were analysed).

Milled flour was let to ripen for a month. Breadrolls were made from the flour by the RMT method adjusted to laboratory conditions of MENDELU Brno. For quantitative evaluation of content of both mycotoxins, ELISA kits Veratox from the company Neogen were used. Kits contain plates with microtiter wells with bound antibody.

Results were evaluated through Statistika 8, the variable projection method into a factorial plane and pair test.

RESULTS AND DISCUSSION

All tested samples of wheat were positive for content of DON in case of the treated as well as non-treated variant. Measured values of DON were quite low in spite of the year 2013 was characteristic by considerable rainfall in the period of fungicide application and its efficiency did not have to be absolute. None of measured values exceeded the legislative limit. Concerning cereal supplementary food for infants and little children, the limit (200 µg.kg^{-1}) was exceeded in 5 samples of the non-treated variant. In the treated variant, DON in flour decreased on average by 33.04% in comparison to grain, in pastries it increased by 25.97% in comparison to flour, and in pastries it decreased by 15.65% in comparison to grain. In case of the non-treated variant, content of DON in flour in comparison to grain on average decreased by 50.48%, in pastries in comparison to grain it decreased by 38.46%. Decrease of values in both variants of flour in comparison to grain can be explained by low penetration of mycotoxins into endosperm, thus higher content was concentrated in outer covering layers, which were removed during milling. Increase of DON values in breadrolls in both variants can be explained by release of masked forms of mycotoxins (derivatives and conjugates of DON) and thus increase of their free forms, what was confirmed also by **Malachová et al., (2010)**. In addition to this, DON is relatively thermally stable, and therefore there are minimal losses during baking. Thus, passing of DON into finished products was confirmed. Moreover, yeast is added into recipe, enzymes of which are able to transform some of precursors of mycotoxins contained in flour into mycotoxins and thus to increase their quantity in pastries, what was also proved by **Young et al., (1984)**, that during fermentation processing of flour, the level of DON increased almost by 100%.

According to **Horáková (2013)** Cubus, Hewitt and Seladon belong to varieties very prone to content of DON, which can be also confirmed by measured results. The lowest content of DON was in the variety Akteur. Resistant varieties have not been bred yet.

In the Figure 1 and 2 we can see excessive quantity of DON and ZEA in both observed years.

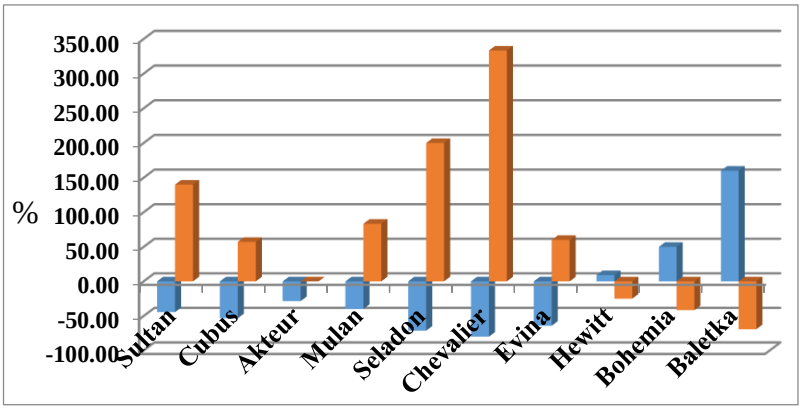

Figure 1 Change of DONcontent after milling and baking in the treated variant in %.

Note: blue-changing content DON grain / flour, red- changing content DON flour/pastries.

By comparison of the treated and non-treated variant, measured values of DON were clearly higher in the non-treated variant, on average by 80.87 % for grain, 33.77 % for flour and 31.96 % for pastries. Thus, we can say that positive effect of fungicide treatment was proved.

Statistical evaluation of measured values of DON is stated in the Table 1, where the two sided T-test for files of the same size was used. Decrease of DON in flour in comparison to grain seems to be statistically significant in case of the treated as well as the non-treated variant and further difference of DON quantity in grain in the treated in comparison to the non-treated variant. Many studies state that DON is thermally stable even during baking. **Scott et al., (1983)** and also **Lancová et al., (2007)** state that baking did not have any influence on DON content.

Zhang and Wang (2015) found**,** that concentrations of DON were verifiably higher in baked bread than in flour. On the contrary, according to **Young et al (1984)** baking has verifiable influence on decrease of DON concentration in bread, on average by 17 to 33% in comparison to the dough. **Boyacioglu et al., (1993)** state that content of DON decreased by 7% after baking bread, however the content of DON in bread with L-cystein as an additive in flour before baking decreased by 38 to 46 %. **Lešnik et al., (2008)** in his study baked 6 types of bread from 3 types of differently milled flour and in two different ovens (industrially used and classic ceramic). Average decrease of DON concentration in bread was 47. 2% in industrial ovens and 48.7% in ceramic ovens. The flour used was strongly contaminated by DON (average concentrations were 850–950 $\mu g.kg^{-1}$), however, breads after baking reached under 500 $\mu g.kg^{-1}$, and thus they met legislative limits. We can summarize that during technological processing, there occurs reduction of DON, but it is not completely removed from a finished product. **Hajšlová et al., (2008)** confirm that the most of waste fractions and bran tend to be the most contaminated and the lowest levels are in scouring flour. Further they stated that with increased time of dough ripening, there occurs increase of free DON and at the same time decrease of the conjugated form of D3G and that with increasing baking time, DON content decreases on average by 32% in comparison to shorter baking time.

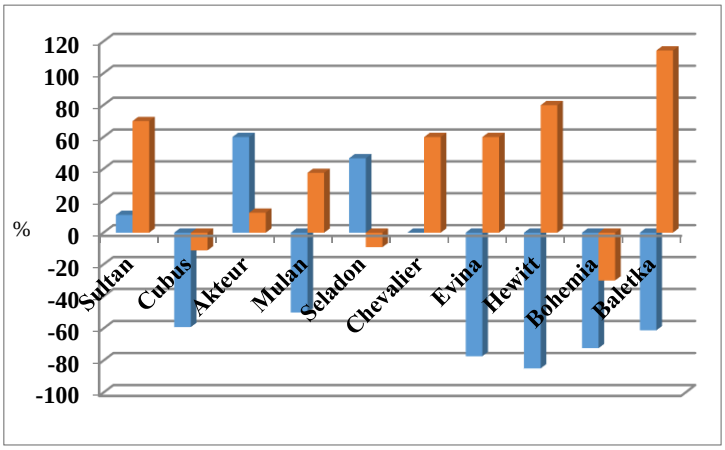

Figure 2 Change of DON content after milling and baking in the non-treated variant in %.

Note: blue-changing content DON grain / flour, red- changing content DON flour/pastries.

Table 1 Statistical significance (P <0.05) decrease/increase of DON values and comparison of the treated and the non-treated variant.

		p	Statistically significant difference
DON treated	grain / flour	0,028	+
	flour / pastries	0,188	-
	grain/ pastries	0,213	-
DON untreated	grain / flour	0,029	+
	flour / pastries	0,282	-
	grain/ pastries	0,079	-
DON treated/ untreated	grain T/ flour U	0,04	+
	flourT/ pastries U	0,232	-
	grain T/ pastries U	0,08	-

Note: *T-treated, U-untreated.

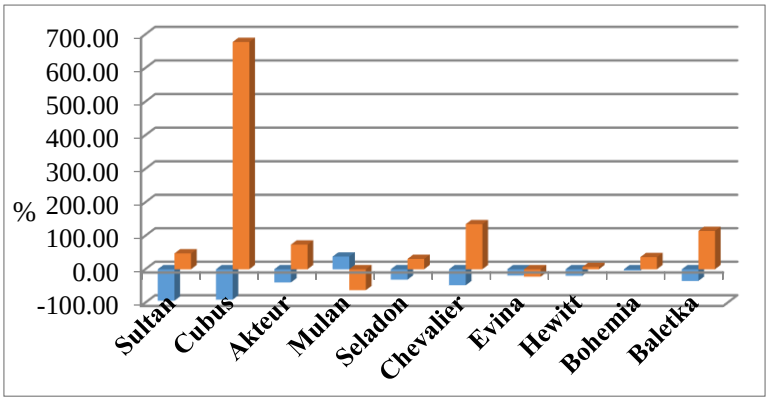

Figure 3 Change of ZEA content after milling and baking in the treated variant in %.

Note: blue-changing content ZEA grain / flour, red- changing content ZEA flour/pastries.

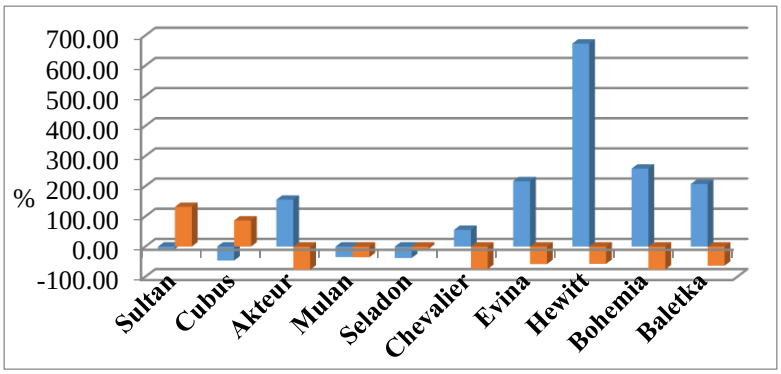

Figure 4 Change of ZEA content after milling and baking in the non-treated variant.

Note: blue-changing content ZEA grain / flour, red-changing content ZEA flour/pastries.

In the Figure 3 and 4 there are illustrated differences in the treated and non-treated variants and changes in ZEA content after milling and baking and in a finished product in comparison to grain.

All measured samples of wheat were positive for content of ZEA. None of the measured values of the treated and non-treated variant exceeded legislative limit. In total, values were very low and at so low level of contamination it is not possible to make clear conclusions.

When comparing the treated and the non-treated variant, higher ZEA values were measured in the treated variant, on average by 62.00% for grain and 62.23% for pastries. In flour, higher values were in the non-treated variant, on average by 46.85 %. Thus, the ZEA fungicide treatment did not have positive effect. This fact can also be caused by high rainfall at the time of fungicide application and its

Table 2 Evaluation of statistical significance (p <0.05) decrease/increase of ZEA values and comparison of the treated and non-treated variant

		p-value	Statistically significant difference
ZEA treated	grain / flour	0,07	-
	flour / pastries	0,273	-
	grain/ pastries	0,64	-
ZEA untreated	grain / flour	0,032	+
	flour / pastries	0,012	+
	grain/ pastries	0,713	-
ZEA treated/untreated	grain T/ flour U	0,019	+
	flour T/ pastries U	0,09	-

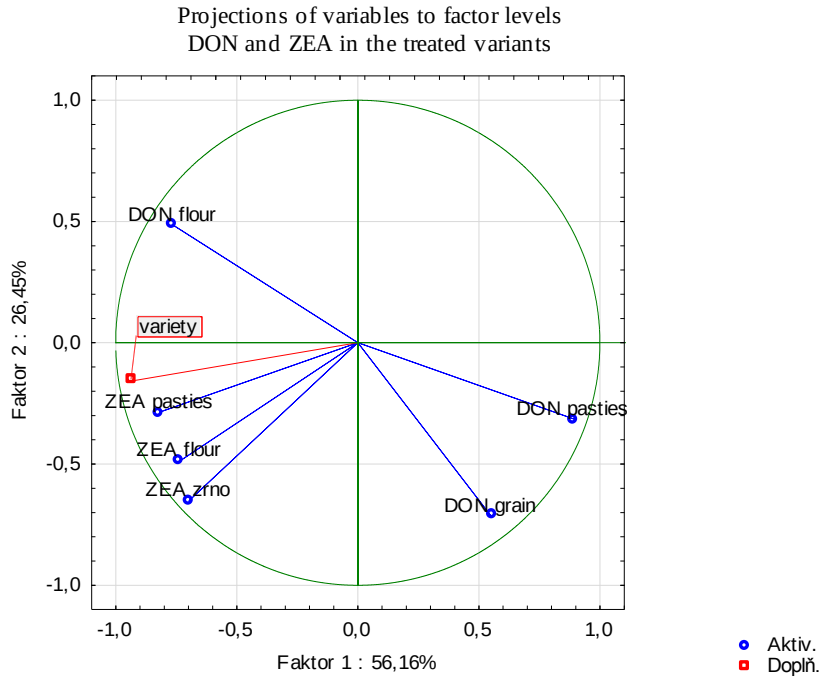

Figure 5 Projection of variables into factor plane – DON and ZEA in the treated variant.

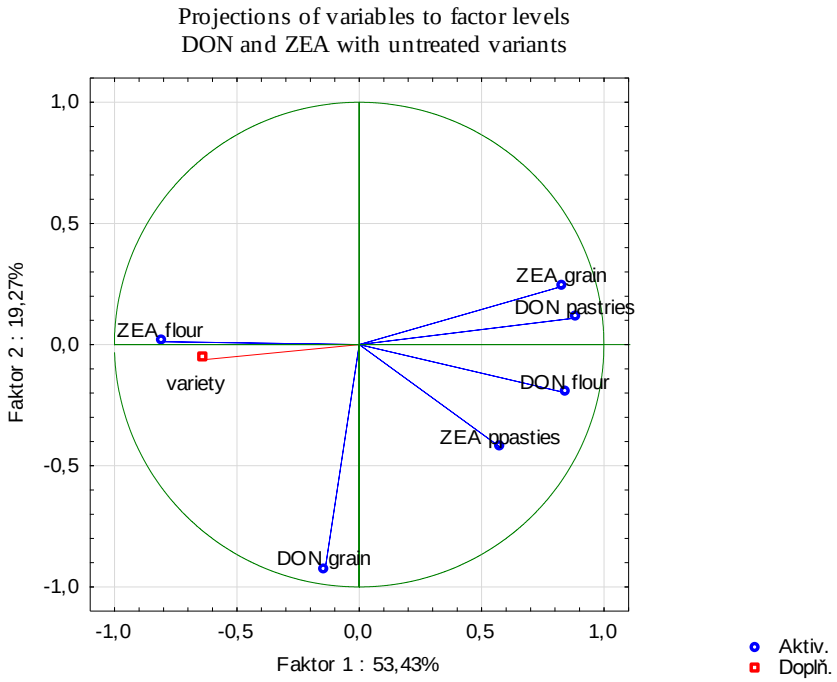

Figure 6 Projection of variables into factor plane – DON and ZEA in the non-treated variant.

efficiency thus did not have to be absolute. According to **Nedělník et al., (2005)**, it commonly happens that after fungicide treatment (especially of strobilurins) the increase of mycotoxins occurs because the stress factor affects fungus and it tries to save itself before extinction and thus it produces even higher quantity of mycotoxins, which is also confirmed by **Šafránková et al., (2010)** and **Malachová et al., (2010)**.

In the treated variant, ZEA in flour in comparison to grain decreased on average by 31.63%, in pastries in comparison to flour increased by 33.34% and in pastries in comparison to grain it decreased by 8.83%. In the non-treated variant ZEA content in flour in comparison to grain increased on average by 61.93% and in pastries in comparison to flour it decreased by 43.50% and in pastries in comparison to grain it decreased by 8.51%.

Decrease of values in flour in comparison to grain in the treated version can be explained by low penetration of mycotoxin into endosperm, and thus higher content was concentrated in outer covering layers, which were removed during milling. On contrary, in the non-treated variant, there is clear increase in flour in comparison to grain,

which can be explained by opposite situation that penetration was high thus mycotoxins were concentrated in grain endosperm. Increase of ZEA values in pastries in the treated variant can be, similarly as for DON, explained by release of masked forms of mycotoxins. ZEA is also relatively thermally stable and losses in baking are small.

Statistical evaluation of measured ZEA values is stated in the Table 2. The two sided T-test was used for files of the same size. Increase of ZEA in flour in comparison to grain and decrease of ZEA in pastries in comparison to flour in the non-treated variant appears to be statistically significant. Furthermore, difference between ZEA quantity in grain of the treated version and non-treated version is statistically significant

Figure 5 and 6 are outcomes of processing of measured data in the program Statistica 8 and they illustrate projection of variables into the factor plane. There is the level of dependence of individual variables evaluated according to the angle size that they enclose. The smaller the angle, the stronger the dependence. At the same time, the angle cosine determines approximate value of correlation coefficient. If arrows are horizontally in the same direction, it means that variables are positively dependent on each other. If arrows are horizontal but in opposite direction, it means that variables are negatively dependent on each other. If arrows are perpendicular on each other, variables can be considered as independent. Length of an arrow indicates variability of measured data.

At the Figure 5 we can see that in the treated variant, species influence ZEA content in all products and at the same time, there is strong correlation dependency. DON content in individual products is independent on each other. DON and ZEA content are not dependent on each other. The highest variability of measured values was recorded in ZEA content in grain (the longest arrow).

In the Figure 6 in the non-treated variant, we can see strong dependency of species on ZEA content in flour. DON content (breadrolls, flour) and ZEA (grain, breadrolls) area closely related, especially ZEA content in grain and in breadrolls and DON in breadrolls and flour. The highest variability of measured values was also recorded in DON content in grain.

CONCLUSION

Average DON content in the treated variant was 115 $\mu g.kg^{-1}$ in grain, 77 $\mu g.kg^{-1}$ in flour and 97 $\mu g.kg^{-1}$ in pastries, in the non-treated variant average DON content in grain was 208 $\mu g.kg^{-1}$, 103 $\mu g.kg^{-1}$ in flour and 128 $\mu g.kg^{-1}$ in pastries. Average ZEA content in the treated version was 4.95 $\mu g.kg^{-1}$ in grain, 3.38 $\mu g.kg^{-1}$ in flour and 4.51 $\mu g.kg^{-1}$ in pastries, in the non-treated version average ZEA content in grain was 3.07 $\mu g.kg^{-1}$, 4.97 $\mu g.kg^{-1}$ in flour and 2.81 $\mu g.kg^{-1}$ in pastries. Measured ZEA contents are on a very low level of contamination. The maximal acceptable limits given by the valid legislation were not exceeded in any analysed sample. It implies that grain grown in the Czech Republic, whether it is treated or non-treated by fungicides, is not dangerous for consumers.

Content of both mycotoxins is not dependent on each other, in the non-treated variant there is slightly higher dependency of DON and ZEA. Species that have the highest influence on ZEA content, did not have any influence on DON content. In the treated variant ZEA contents in grain, flour and breadrolls contents closely related.

In the treated variant, DON content was decreased by milling on average by 33.04% in comparison to grain and on the other hand, its content in breadrolls was increased by 25.97%. In the non-treated variant, DON content decreased by 50.48% in comparison to grain and by baking, it again increased in comparison to flour by 24.27%. ZEA content in the treated version in flour decreased on average by 31.63% in comparison to grain, in pastries in comparison to flour, it increased by 33.34%. On the other hand, in the non-treated version, ZEA content was increased through milling by 61.93% in comparison to grain and during baking, its content decreased by 43.50% in comparison to flour. Decrease of mycotoxin content after milling means that mycotoxins were concentrated more in outer covering layers and less in endosperm. Covering layers are removed during milling and thus mycotoxin levels are decreased in flour. On the contrary, in the non-treated variant ZEA was more concentrated in endosperm and thus there was its increase in flour. Subsequently, increase of mycotoxins after baking in pastries can be explained by release of masked forms of mycotoxins or by activity of yeast during fermentation, what was confirmed by number of studies stated above. Both mycotoxins are quite thermally stable and thus they are not degraded during baking too much.

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Contact address:

Ing. Viera Šottníková, Ph.D., Mendel University in Brno, Department of Food Technology, Zemědělská 1, 61300 Brno, E-mail: viera.sottnikova@mendelu.cz.

Prof. Dr. Ing. Luděk Hřivna, Mendel University in Brno, Department of Food Technology, Zemědělská 1, 61300 Brno, E-mail:hrivna@mendelu.cz.

doc. RNDr. Iva Burešová, Ph.D., Tomas Bata University in Zlín, Department of Food Technology, nám. T. G. Masaryka 5555, 760 00 Zlín, E-mail:buresova@ft.utb.cz.

RNDr. Jan Nedělník, Ph.D., Zemědělský výzkum, spol. s. r. o, Zahradní 1, 664 41 Troubsko, E-mail: nedělník@vupt.cz.

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OXIDATIVE STABILITY OF CHICKEN'S BREAST AFTER VACUUM PACKAGING, EDTA, SAGE AND ROSEMARY ESSENTIAL OILS TREATMENT

Adriana Pavelková, Marek Bobko, Peter Haščík, Miroslava Kačániová, Jana Tkáčová

ABSTRACT

In the present work, the effect of the sage and rosemary essential oils on oxidative stability of chicken breast muscles during chilled storage was investigated. In the experiment were chickens of hybrid combination Cobb 500 after 42 days of the fattening period slaughtered. All the broiler chickens were fed with the same feed mixtures and were kept under the same conditions. The feed mixtures were produced without any antibiotic preparations and coccidiostats. After slaughtering was dissection obtained fresh chicken breast with skin from left half-carcass, which were divided into five groups (n = 5): C - control air-packaged group; A1 - vacuum-packaged experimental group; A2 - vacuum-packaged experimental group with EDTA solution 1.50% w/w; A3 - vacuum-packaged experimental group with *Salvia officinalis* L. oil 2.0% v/w and A4 - vacuum-packaged experimental group with *Rosmarinus officinalis* L. essential oil 2.0% v/w. The sage and rosemary essential oils were applicate on surface chicken breasts and immediately after dipping, each sample was packaged using a vacuum packaging machine and storage in refrigerate at 4 ±0.5 °C. The value of thiobarbituric acid (TBA) expressed as amount of malondialdehyde (MDA) in 1 kg sample was measured during storage in 1st, 4th, 8th, 12th and 16th day. The treatments of chicken breasts with sage and rosemary essential oils show statistically significant differences between all testing groups and control group, where higher average value of MDA measured in breast muscle of broiler chickens was in samples of control group (0.396 mg.kg^{-1}) compared to experimental groups A1 (0.060 mg.kg^{-1}), A2 (0.052 mg.kg^{-1}), A3 (0.042 mg.kg^{-1}) and A4 (0.041 mg.kg^{-1}) after 16-day of chilled storage. The results of experiment showed that the treatment of chicken breast with sage and rosemary essential oils had positive effect on the decrease of oxidative processes in breast muscles during chilling storage and use of plant essential oils is one of the possibilities increase shelf life of fresh chicken meat.

Keywords: oxidative stability; chicken breast; essential oil; sage, rosemary

INTRODUCTION

Meat and meat products are essential components in the human diets and their consumption is affected by various factors, e.g. product characteristics, consumer and environment related **(Jiménez-Colmenero et al., 2001)**. Chicken meat has many desirable nutritional characteristics such as a low lipid content and relatively high concentration of polyunsaturated fatty acids (PUFAs) which can be further increased by specific dietary strategies **(Bourre, 2005)**. However, a high degree of polyunsaturation accelerates oxidative processes leading to deterioration in meat flavour, colour, texture and nutritional value **(Mielnick et al., 2006)**.

Lipid oxidation causes degradation of polyunsaturated fatty acids (PUFA) and generation of residual products, such as malondialdehyde (MDA) and lipid-derived volatiles leading to sensory and nutritional deterioration of meat **(Kanner et al., 1991)**. Oxidative reactions in foodstuffs are enhanced after cooking and refrigerated storage through the increase of their oxidative instability due to the degradation of natural antioxidants and the release of free fatty acids and iron from the haem molecule **(Estévez and Cava, 2004; Kingston et al., 1998; Kristensen and Purslow, 2001)**.

The higher level of PUFAs in muscle membranes increases the susceptibility of oxidative deterioration of lipid **(Engberg et al., 1996)**, which impairs the organoleptic characteristics and shortens the shelf-life of meat and meat products.

The major strategies for preventing lipid oxidation are the use of antioxidants and restricting the access to oxygen during storage vacuum-packaging **(Tang et al., 2001)**. The antioxidant additives are added to fresh and further processed meats to prevent oxidative rancidity, retard development of off-flavours, and improve colour stability **(Nam and Ahn, 2003)**.

For chicken meat products, freshness, as one of the most important quality attributes, has attracted attention from producers and consumers and has a strong relationship with product sales and consumption **(Rzepka et al., 2013)**. One option for reducing lipid oxidation is the use of various natural plant antioxidants presented in essential oils.

The use of natural preservatives to increase the shelf life of meat products is a promising technology since many

vegetal substances have antioxidant and antimicrobial properties. Functional ingredients in meat products may improve the nutritional and health qualities and prolonging their self-life **(Fernández-Ginés et al., 2005)**. Plants' extracts rich in polyphenols are good candidates, since they are easily obtained from natural sources and they efficiently prevent lipid oxidation in food products.

Studies have shown wide effective in spices to retard lipid oxidation in meat products **(Juntachote et al., 2006, 2007; Chouliara et al., 2007; Mariutti et al., 2008; Sasse et al., 2009; Lee et al., 2010; Marcinčák et al., 2010; Viuda-Martos et al., 2011; Tkáčová et al., 2015)**.

Essential oils (EOs) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). They can be obtained by expression, fermentation, enfleurage or extraction but the method of steam distillation is most commonly used for commercial production of EOs **(Van de Braak and Leijten, 1999)**. EOs obtained from various herbs are widely used in cosmetics and food manufacturing and can be used for prolonging the shelf-life of food for their antimicrobial **(Skandamis et al., 2002; Mihajilov-Krstev et al., 2009)**, and antioxidant activities **(Burt, 2004; Bobko et al., 2015a, b)**.

In the last years, many researchers have evaluated the antioxidant properties of extracts from different plants and vegetables **(Chen et al., 2002; Ibanez et al., 2003; Ichikawa et al., 2003)**.

Essential oils represent a small fraction of the plant composition; the main compounds are terpenes and sesquiterpenes, and several oxygenated derivatives compounds (alcohols, aldehydes, ketones, acids, phenols, ethers, esters, etc.) all of them responsible for the characteristic plant odour and flavour **(Yanishlieva et al., 2006)**. These compounds include natural flavourings such as sage, oregano, rosemary and others **(Mariutti et al., 2008)**.

Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officianalis*) are popular *Labiatae* herbs with a verified potent antioxidant activity **(Dorman et al., 2003)**. The antioxidant activity of sage and rosemary essential oils is mainly related to two phenolic diterpenes: carnosic acid and carnosol which are considered two effective free-radical scavengers **(Dorman et al., 2003; Ibanez et al., 2003)**.

Sage (*Salvia officinalis*) is a variety of aromatic herb which has been planted widely throughout much of the world. It is not only used as raw material in the pharmaceutical and cosmetic industries but also used to improve flavours of foods **(Tepe et al., 2006)**. Sage has been reported to have excellent activities in scavenging radicals, reducing metal ions and inhibiting lipid peroxidation **(Dorman et al., 2003; Grzegorczyk et al., 2007)**. The phenolic compounds, such as carnosol, carnosic acid and rosmarinic acid, in the plant may account for the antioxidant activity of sage. Some researchers have reported that sage, or sage extracts, can effectively retard lipid oxidation of muscle foods **(Fasseas et al., 2007; McCarthy et al. 2001a; Tanabe et al., 2002)**.

Among natural antioxidant sources, rosemary (*Rosmarinus officinalis* L.), a woody aromatic herb that is native to the Mediterranean countries, has recently been authorized by the European Union under Directive 95/2/EC and assigned E-392 as its E number (European Union Directives 2010/67/EU and 2010/69/EU) for use in meat product preservation. The addition of rosemary extract to poultry products has been shown to be effective in retarding lipid oxidation, and previous studies in chicken sausages **(Liu et al., 2009)** and patties **(Naveena et al., 2013)** have pointed to the protective effect of rosemary extract (500–1500 ppm) and leaves (22.5–130 ppm) in inhibiting lipid oxidation.

Rosemary antioxidant activity is related to components such as phenolic diterpenes, carnosol (CAS No. 5957-80-2) and carnosic acid (CAS No. 3650-09-7) **(Rodriguez-Rojo et al., 2012)**. The antioxidant capacity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms and chelate metal cations **(Shan et al., 2005)**. Previous studies **(Azmir et al., 2013; Wang et al., 2013)** have reported that the yield of bioactive compounds can be changed or modified by using different extraction procedures, solvents, temperatures, pressures and times.

In this study we aimed to investigate the combined effect of ethylenediaminetetraacetate (EDTA) and plant essential oils (*Salvia officinalis* L. and *Rosmarinus officinalis* L.) on the oxidative stability of fresh chicken breasts stored under vacuum packaging (VP), at 4 ±0.5 °C for a period of 16 days.

MATERIAL AND METHODOLOGY

The experiment was implemented in the local poultry station (Hydinaren a.s., Zamostie). The tested were broiler chickens of hybrid combination Cobb 500 both sexes. All the broiler chickens were fed with the same feed mixtures and were kept under the same conditions. The feed mixtures were produced without any antibiotic preparations and coccidiostats. At the end of the fattening period (42. day) were chickens slaughtered for analysis in laboratory of Slovak University of Agriculture in Nitra. After slaughtering was dissection obtained fresh chicken breast with skin from left half-carcass, which were divided into five groups (n = 5):

- Air-packaged (C, control group): chicken breast fresh meat was packaging to polyethylene backs and stored aerobically in refrigerator;
- Vacuum-packaged (A1, experimental group): chicken breast fresh meat was packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator;
- VP with EDTA solution 1.50% w/w (A2, experimental group): chicken breast fresh meat was treated with EDTA for 1 min and packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator;
- VP with *Salvia officinalis* L. 2.0% v/w (A3, experimental group): chicken breast fresh meat was treated with *Salvia officinalis* L. oil for 1 min and packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator;
- VP with *Rosmarinus officinalis* L. 2.0% v/w, (A4, experimental group): chicken breast fresh meat was treated with *Rosmarinus officinalis* L. oil for 1 min and packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator.

Immediately after dipping, each sample was packaged using a vacuum packaging machine type VB-6 (RM Gastro, Czech Republic).

Ethylenediaminetetraacetic acid (EDTA) (C10H14N2O8.Na2.2H2O) was 99.5% purity, analytical grade, (Invitrogen, USA). A stock solution of 500 mM concentration was prepared by diluting 186.15 $g.L^{-1}$ distilled water. A final concentration of 50 mM EDTA solution was prepared from the stock solution. The pH of the solution was adjusted to 8.0 with the addition of the appropriate quantity of NaOH solution. The amount of EDTA added to the treat chicken breasts was 0.28 $g.kg^{-1}$. Essential oil (Calendula, Nova Lubovna, Slovakia) were added to the coated chicken beast surface (both sides) of each sample using a micropipette so as to achieve a 0.2% v/w final concentration of essential oils.

TBA value expressed in number of malondialdehyde (MDA) was measured in the process of first storage day of 1^{st}, 4^{th}, 8^{th}, 12^{th} and 16^{th} day. TBA number was determined by **Marcinčák et al. (2004)**. Absorbance of samples was measured on UV-VIS spectrophotometer T80 (PG Limeted Instruments, UK) at a wavelength of 532 nm, the translation results on the amount of MDA in 1 kg samples.

Results of the experiment were evaluated by statistical program SAS 9.3 with using application Enterprise Guide 4.2. The variation-statistical values (mean, standard deviation) were calculated and to determine the significant difference between groups was used variance analyse.

RESULTS AND DISCUSSION

Jo et al. (2006) stated that oxidation of lipids can have significant impact to meat industry. Meat containing unsaturated fatty acids is very sensitive to lipid oxidation especially during storage, because polyunsaturated fatty acid esters are easily oxidized by molecular oxygen. This kind of oxidation is called autoxidation and proceeds by a free radical chain mechanism **(Brewer, 2011)**.

The results of the oxidation stability of fresh chicken breast muscles of chicken Cobb 500 after application EDTA and plant essential oils (*Salvia officinalis* L. and *Rosmarinus officinalis* L.) during 16 days storage at 4 °C are shown in Table 1 and Figure 1.

The higher average value of MDA measured in breast muscle in 0 day of experiment was in samples of vacuum-packaged chicken breasts group with *Rosmarinus officinalis* L. oil 2.0% v/w group A4 (0.026 $mg.kg^{-1}$) compared to experimental groups A1 (0.022 $mg.kg^{-1}$), A2 (0.023 $mg.kg^{-1}$), A3 (0.024 $mg.kg^{-1}$) and air-packaged control group (0.024 $mg.kg^{-1}$). We have not found statistically significant differences between testing groups chicken breasts. During chilled storage of the breast muscles were noticed increased content of malondialdehyde in comparison to the first day of storage.

On the fourth day of storage were measured below the values of malondialdehyde in all experimental groups (0.028 $mg.kg^{-1}$ in group A2, 0.030 $mg.kg^{-1}$ in group A3, 0.034 $mg.kg^{-1}$ in group A4, and 0.036 $mg.kg^{-1}$ – group A1) opposite control group C (0.182 $mg.kg^{-1}$). We have found statistically significant differences ($p \leq 0.05$) between control group C and all tested groups.

A similar tendency of improving the oxidation stability after eight days of refrigerate storage in the breast muscle of hybrid combination Cobb 500 we found in the experimental groups (0.031 $mg.kg^{-1}$ – A3, A4 to 0.048 $mg.kg^{-1}$ - A1) compared with control group C (0.191 $mg.kg^{-1}$).

After 12 days of breast muscle storage was statistic significantly ($p \leq 0.05$) improved the oxidative stability of all test groups chicken breasts (0.033 $mg.kg^{-1}$ – A4 to 0.055 $mg.kg^{-1}$ – A1) compared to the control group C (0.229 $mg.kg^{-1}$). We have found statistically significant differences ($p \leq 0.05$) between control group C and tested groups, between group A1 and A2, A4 and between tested group A2 and groups A3, A4.

During testing period of chilled storage were higher values of malondialdehyde measured in control group C

Table 1 Effect of sage and rosemary essential oils on the concentration of MDA ($mg.kg^{-1}$) in breast muscle (mean ±*SD*) (n = 5).

Day	C	A1	A2	A3	A4
0	0.024 ±0.006	0.022 ±0.007	0.023 ±0.006	0.024 ±0.005	0.026 ±0.007
4	0.182 ±0.007^{a}	0.036 ±0.004^{b}	0.028 ±0.007^{b}	0.030 ±0.004^{b}	0.034 ±0.004^{b}
8	0.191 ±0.006^{a}	0.048 ±0.005^{b}	0.044 ±0.007^{b}	0.031 ±0.008^{c}	0.031 ±0.007^{c}
12	0.229 ±0.019^{a}	0.055±0.006^{b}	0.043 ±0.005^{c}	0.037 ±0.009^{cd}	0.033 ±0.005^{d}
16	0.396 ±0.027^{a}	0.060 ±0.005^{b}	0.052 ±0.004^{c}	0.042 ±0.004^{d}	0.041 ±0.005^{d}

Note: C - air-packaged control group; A1 - vacuum-packaged control group; A2 - vacuum-packaged control samples with EDTA solution 1.50% w/w; A3 - vacuum-packaged experimental group with *Salvia officinalis* L. oil 2.0% v/w; A4 - vacuum-packaged experimental group with *Rosmarinus officinalis* L. oil 2.0% v/w. Mean values in the same lines with different superscripts (a, b, c) are significantly different at $p \leq 0.05$ level.

compare to experimental groups. The higher average value of MDA measured in breast muscle of broiler chickens Cobb 500 was in samples of control group C (0.396 mg.kg^{-1}) compared to experimental groups A1 (0.060 mg.kg^{-1}), A2 (0.052 mg.kg^{-1}), A3 (0.042 mg.kg^{-1}) and A4 (0.041 mg.kg^{-1}) after 16-day of chilled storage. At the end of the test period we have found statistically significant differences ($p \leq 0.05$) between all testing groups and control group of chicken breasts.

Botsoglou et al. (2007) reported that a higher concentration of antioxidants in poultry meat has the effect of reducing lipid oxidation, i.e. there is a reduction in malondialdehyde values during chilling storage. **Gong et al. (2010)** used TBARs values as an indicator of secondary lipid oxidation products, which were determined in minced breast and thigh muscles from chicken, turkey and duck during -4 °C storage. TBARs formation was slowest in minced chicken thigh, intermediate in duck thigh and fastest in turkey thigh ($p < 0.01$).

The plant essential oils such as oregano, thyme, sage etc. **(Economou et al., 1991; Yanishlieva and Marinova, 1995; Man and Jaswir, 2000)**, show positive effect on oxidation stability of lipids in meat.

In contrast to synthetic antioxidants, the use of natural antioxidants from spices is increasing since their application is less stringently regulated in most countries around the world. Active essential oil compounds in rosemary, oregano, borage and sage are for example phenolic diterpenes, derivates of hydroxycinnamic acid, flavonoides and triterpenes **(Oberdieck, 2004; Ryan et al., 2009; Sanchez-Escalante et al., 2003).** For rosemary, sage and oregano, the most active substances with a high antioxidant potential are carnosic acid, carnosol, and rosmarinic acid **(Oberdieck, 2004).**

Estévez et al. (2007) evaluated the antioxidant effect of two plant essential oils (sage and rosemary essential oils) and one synthetic antioxidant (BHT) on refrigerated stored liver pâté (4 °C/90 days). The addition of antioxidants significantly ($p \leq 0.05$) reduced the total amount of lipid-derived volatiles isolated from liver pâtés HS. Plant essential oils inhibited oxidative deterioration of liver pâtés to a higher extent than BHT did.

Fasseas et al. (2007) showed that porcine and bovine ground meat treated with the essential oils of oregano and sage (3%w/w) had increased oxidative stability and the antioxidant capacity of the raw and cooked meat (85 °C for 30 min) was high during storage at 4 °C for 12 days. They also suggested that addition of antioxidants is much more important for cooked meat products than the raw products.

Mohamed et al. (2011) reported that addition of herbal extracts of marjoram, rosemary and sage at concentration of 0.04% (v/w) to ground beef prior to irradiation (2 and 4.5 kGy) significantly lowered the TBARS values, off odour scores and increased colour and acceptability scores.

Sampaio et al. (2012) examined the effect of combinations of sage, oregano and honey on lipid oxidation in cooked chicken meat (thigh and breast) during refrigeration at 4 ±0.5 °C for 96 h as measured by TBARs numbers. The analysis of variance on the TBARs data indicated that the TBARs values were significantly affected by natural antioxidants throughout refrigeration ($p < 0.05$). Analysis their data showed that all of the three combinations of natural antioxidants tested would be beneficial for reducing the velocity of lipid oxidation in

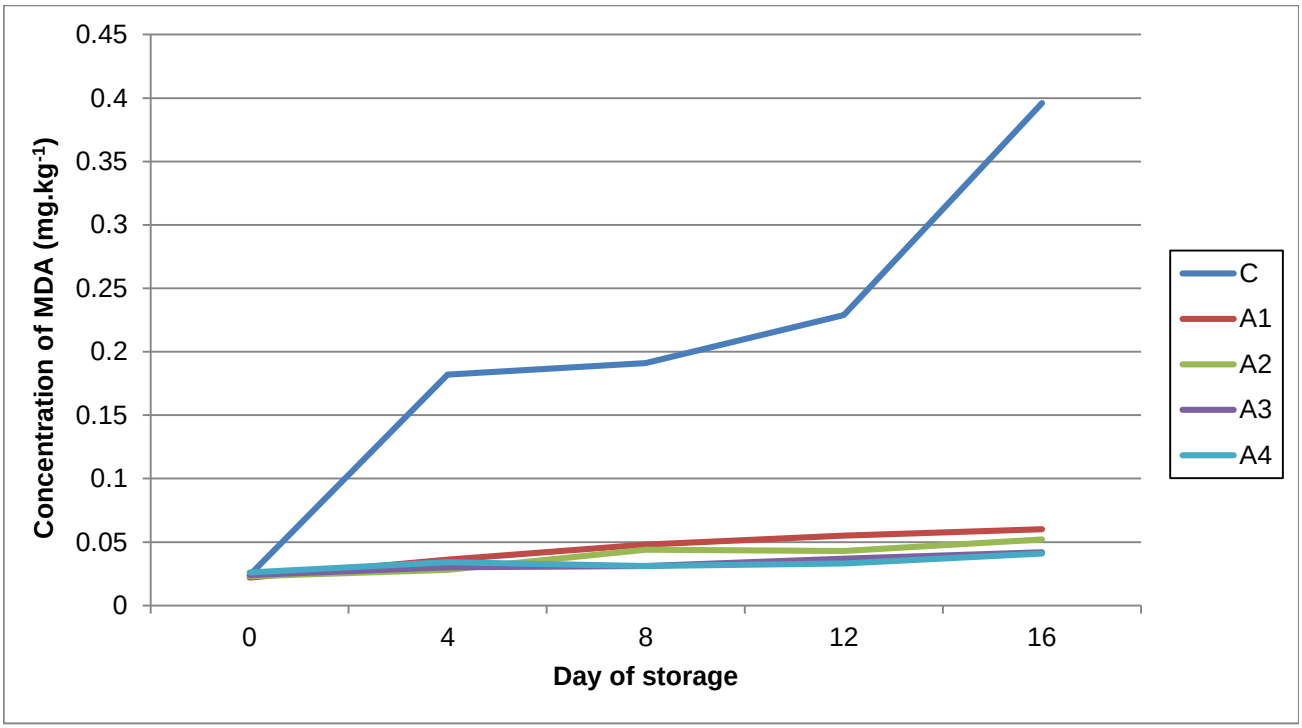

Figure 1 Concentration of MDA (mg.kg^{-1}) in breast muscle.

Note: C - air-packaged control group; A1 - vacuum-packaged control group; A2 - vacuum-packaged control samples with EDTA solution 1.50% w/w; A3 - vacuum-packaged experimental group with *Salvia officinalis* L. oil 2.0% v/w; A4 - vacuum-packaged experimental group with *Rosmarinus officinalis* L. oil 2.0% v/w.

both chicken meats during storage, what are corroborated by other authors who have added honey and herbs and thereby inhibited the development of lipid oxidation in cooked meats during refrigeration time **(McKibben and Engeseth, 2002; Juntachote et al., 2007)**.

Mohamed et al. (2011) reported that addition of herbal extracts of marjoram, rosemary and sage at concentration of 0.04% (v/w) to ground beef prior to irradiation (2 and 4.5 kGy) significantly lowered the TBARS values, off odour scores and increased colour and acceptability scores.

The effectiveness of rosemary essential oil as an inhibitor of lipid oxidation in meat products has been documented **(Esteévez and Cava, 2006; McCarthy et al., 2001; Sebranek et al., 2005)**.

Plant essential oils have been successfully introduced to inhibit oxidative deterioration of meat and fat products, this deterioration being generally referred to the accumulation of lipid-oxidation-derived products and to the generation of lipid-derived volatiles in meat products **(Ahn et al., 2002; Yu et al., 2002)**. **Formanek et al. (2001)** and **McCarthy et al. (2001)** reported the high effectiveness of antioxidants from natural resources against oxidative reactions that showed similar activity to those from synthetic origin such as BHT. **Sebranek et al. (2005)** reported similar antioxidant activities of rosemary essential oils and synthetic ones (BHT/BHA) regarding MDA generation in refrigerated sausages.

Ramos Avila et al. (2013) stated that the degradation pathways of fatty substances play one of the main causes of foods deterioration and unpleasant odours. This factor is also responsible for the loss of sensory properties.

Rhee et al. (1996) observed that raw poultry meat is less prone to lipid oxidation than beef or pork meat because of its lower iron content.

CONCLUSION

The essential oil as well essential oils from *Labiatae* herbs can be used as substitutes to chemical food additives which could prolong of shelf life of the meat and meat products. Results achieved in the experiment show that the treatment of chicken breast muscles with *Salvia officinalis* L. and *Rosmarinus officinalis* L. essential oils in concentration 0.20% v/w with combination vacuum packaging had positive effect on the decrease of oxidative processes in chicken breast muscles during chilling storage at 4 ±0.5 °C in comparative with tested groups - control air-packaged group, vacuum-packaged experimental group and vacuum-packaged experimental group with EDTA solution 1.50% w/w.

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Contact address:

Adriana Pavelková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: adriana.pavelkova@uniag.sk

Marek Bobko, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marek.bobko@uniag.sk.

Peter Haščík, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.hascik@uniag.sk.

Miroslava Kačániová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@uniag.sk.

Jana Tkáčová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.tkacova@uniag.sk.

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THE ROLE OF COLOR SORTING MACHINE IN REDUCING FOOD SAFETY RISKS

Eleonóra Kecskésné Nagy, Péter Korzenszky, Péter Sembery

ABSTRACT

It is the very difficult problem how we can decrease food safety risks in the product, which was polluted in process of cropping. According to professional literature almost the prevention is considered as an exclusive method to keep below safe level the content of DON toxin. The source of food safety in food chain is that the primary products suit the food safety requirements. It is a very difficult or sometimes it is not possible to correct food safety risk factors - which got into the products during cultivation - in the course of processing. Such factor is fusariotoxin in fodder and bread wheat. DON toxin is the most frequent toxin in cereals. The objective of the searching was to investigate, if it is possible to decrease DON toxin content of durum wheat and to minimize the food safety risk by application milling technology with good production practice and technological conditions. The samples were taken in the first phase of milling technology just before and after color sorting. According to measuring results Sortex Z+ optical sorting decreased DON toxin content of wheat. This mean that the food safety risks can be reduced by Sortex Z+ optical sorting machine. Our experiments proved if there is color sorting in the cleaning process preceding the milling of wheat then a part of the grain of wheat infected by Fusarium sp. can be selected. This improves the food safety parameters of given lot of wheat and decrease the toxin content. The flour made from contaminated grains of wheat can be a serious food safety risk. We would like to support scientifically the technical development of milling technology with our experimental data.

Keywords: food safety; DON toxin; fusarium; wheat; *Triticum durum*

INTRODUCTION

During the course of wheat process, we have to make efforts to minimize toxin content of wheat before milling, but at the least to keep below allowable limits.

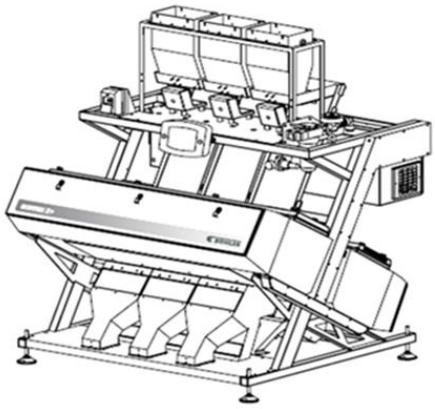

Figure 1 Sketch of Sortex Z+ optical sorter.
(Source: Sortex Z operator's manual).

Basic requirement is that these products don't contain microbiological, chemical and other contaminations, or at least not more than the maximum allowable limits **(Commission regulation (EC) No 1881, 2006)**. Products made of cereals, such as flour, bread and bakery products are classifiable as basic foods, but we consume lot of pasta and cake also. Large-scale consumption of basic foods is a feature of adult and children population equally. At these products, the fundamental importance to use primary materials which are free from biological and chemical pollutants is unambiguous.

The mycotoxins in food are secondary metabolic products of moulds, which have strong toxic effect. These can cause heavy complications in human and animal organizations, can result illness shorter or longer time, and can cause lasting damage. The species belong to Fusarium family produce significant quantity of toxin, which contain several fuzariotoxin. The Fusarium species are parasites on several cultivated plants. In most cases they infect the cereals. So they might cause significant damage both in plant cultivation and animal husbandry as well as there might be considerable human-health consequences. **(Mesterházy, 2015; Summerell at al., 2010; Stanic at al., 2015; Leslie and Logrieco, 2014; Remža at al., 2011; Adam at al., 2002)**. Deoxinivalenol, otherwise vomitoxin became known as DON toxin, is frequent representative of fusariotoxins. This toxin can be found in wheat very often according to **Mesterházy (2007)**. This toxin might be present both in cereals and in processed cereal products, so it is important from the point of food safety **(Hrubošová, 2015)**.

Contrary to data of literature we started from the hypothesis, that nowadays toxin content can be decreased

after harvesting and storing also by application of modern milling industry equipments and technical conditions.

Our first investigations were focused on toxin decreasing effect of Sortex Z+ color sorting machine (Figure 1). This is a new, more precise, quicker technology with less loss in milling processing. It's possible to select the components which size are similar to unbroken, health grain, but they are optically different. The sorting machines applied previously were not satisfactorily efficient to select components which brought down the quality. The manufacturer offers the application of color sorting machine Sortex Z+ as the alternative of the mechanical cleaning equipments. But it possible to gain more efficient sorting than at the mechanical cleaners, and the result is cleaner, contaminants-free product. Color sorting is not yet generally applied in the milling technology. As it was mentioned the application of Sortex Z+ makes possible not just to remove the physical dirt but the grains with different color can be selected also. It is known the color of infected grains can change depending on characteristic and time of infection.

Meteorological factors have significant influence on development of fusarium infection and on degree of toxin content also. The weather conditions are risk factors which can't be influenced during the wheat growing **(Commission recommendation, 2006)**. The prevention against these factors maybe the usage of resistant species. But it is known the species-structure has not changed in the last years in this regard. This means we have to calculate with fusarium infection in the following periods also, especially afterward rainy early summer weather. The degree and the characteristic of fusarium infection depends in which phenology phases was the wheat contaminated. This determines whether just the seed-coat is infected, or the endosperm also. If the weather is favourable for fusarium infection after the fertilization and at the beginning of development of cereal grains, then the rate of cereal grains with fungi in the endosperm is higher. This case the color of grains changes. It becomes bright, primarily greyish-white, but lilac or pinky color might occur also. The color change is very important in the respect of our investigation. In case of early infection a part of wheat grains are smaller and their texture are softer, in parallel with the above mentioned characteristics. As far as the weather becomes rainy in more advanced status of wheat that is at the beginning of the full ripening, then the infection of fungi represents lower rate in the inner part of wheat grain, and the center of infection develops in the layers of seed-coat. In cases of that kind the change of grain color is less typical and the size of grain is not considerably smaller. But the texture of infected grains are softer than the healthy ones. At the end of full ripening the infected seeds barely differ from healthy ones and the inner part of grains remains intact. Accidently mycelium on surface or slight discoloration indicates the infection.

The experimental results of **Hrubošová (2015)** clearly confirm the above mentioned process. The analysis of infected lots of wheat proved there is no correlation between internal and external infection that is they develop independently from each other. Based on the above mentioned results it is difficult to remove the infected seeds from wheat lots after harvest period. It is not possible to decrease the toxin content reliably and efficiently by simple cleaning, selection process. Therefore, scientific literature assumes that the opportunity of decreasing mycotoxin content and thereby the food safety risk is very restricted and uncertain during processing.

Font et al., (2013) implemented model research to decrease DON toxin content, when they created and built machineries in laboratory which were operated similarly to surface cleaning treatment applied in the milling process. The starting hypothesis was that majority of toxin concentrates in the coat and the germ of the wheat. They evaluated the test of a small number of samples which was taken from a given lot of wheat. Their results are important because they proved it is possible to decrease the toxin content of wheat by application certain surface cleaning methods. However, we shouldn't ignore that the degree and characteristic of fusarium infection depends in which phenology phases was the wheat contaminated. This determines whether just the coat and the germ infected or the endosperm affected also. But it shouldn't be ignored that the cleaning treatment and its efficiency is different in mill industrial and in laboratory circumstances due to blending of raw material lots and different processes. Conversely it is very important from food safety aspect to get information not only about theoretical possibilities, but about efficiency of process which actually takes place.

Presumable beyond removing physical dirt the quantity of mycotoxin being the cause of chemical danger can be decreased also. We try to prove the rightness of our hypothesis by our experiments and data.

Toxin-test of wheat harvested in 2013 indicated that the color sorting of grain resulted decrease of toxin content. But the other hand the investigation of the degree of decrease didn't resulted correlation between toxin content before and after color sorting **(Kecskés-Nagy et al., 2015)**. The reason for this is that toxin concentration can be different in the internal layers of the grain depending on the characteristic of the Fusarium infection **(Veha et al., 2015)**. This is why we continued our experiment. We tested wheat samples harvested in 2014 to prove it is possible to decrease toxin level in the mill technology under different ecological circumstances by operation of modern equipment like Sortex Z+ color sorter.

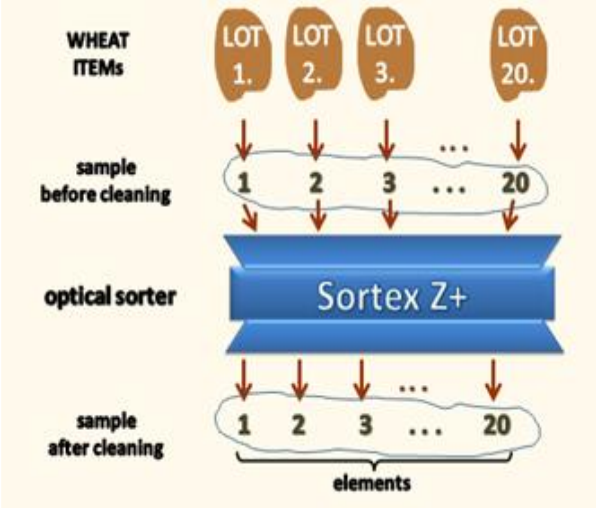

Figure 2 The method of sampling.

MATERIAL AND METHODOLOGY

We carried out the experiment at Júlia Malom Ltd. and investigated the DON toxin content of durum wheat (*Triticum durum*) before and after the cleaning process in the course of milling.

Durum wheat which was the subject of our investigation is cultivated among the wheat species on the second largest territory in the world. It is particularly popular in the mediterranean region. The flour made from hard, glassy wheat grains is used primarily by pasta industry but Sicilians bake bread from that also. Its nutritional value is better than that of *T. aestivum*, which is widely grown in our area. Its consumption is advantageous because of its beta-carotene content, amino acid and protein composition, slowly resorbable carbohydrate content. Owing to its beta-carotene and protein content, pasta can be produced from durum wheat without eggs. But growing experiences indicate that durum wheat is more sensitive to Fusarium infection than other wheat species.

The samples were taken in the first phase of milling technology just before and after color sorting. The time of sampling was settled in accordance with performance of Sortex Z+ machine. This method ensured the test same samples before and after sorting.

We investigated DON toxin content of 20 samples during the experiment. The samples taken before sorting by Sortex Z indicate the initial toxin content of investigated wheat (Figure 2). After sorting the mycotoxin decreasing efficiency of the process can be evaluated by means of analytical results of relevant samples.

Toxin analysis was carried out in own laboratory of Júlia Malom Ltd with AgraQuant Deoxinyvalenol test kit.

The evaluation was made by hypothesis analysis. The elements of two samples came in pairs (before and after sorting element in a pair) from the same lot of wheat, and DON toxin content was tested in each element. Thus the lot and the elements of samples are not independent from each other from mathematical respect. We applied „one-sample T-test" to the statistical analysis, in which the difference of two values that is difference (d_i - t) was ordered to the element.

We used „null-hypothesis" at 5% significance level to answer whether the difference between DON values before and after color sorting under same condition is negligible.

From mathematical aspects we can investigate in four logical steps whether the hypothesis is correct or it should be rejected. Values of t-probe function were calculated with MS Excel software, thus we investigated the rightness of null-hypothesis in two steps. (The last three steps were drawn together.)

First step:

We analyzed fulfillment of precondition to carrying out pared t-probe According to our assumption the distribution of population is normal. The results of measuring was completed with equipment in practice and employed chemical and physical rules. Thus they fulfilled the precondition of normality.

Mathematically we should verify normality with so-called $\chi 2$-probe, but owing to number of data (n = 20 <50) this wouldn't be exact. Presumably if we would make quite a number measurement, we would experience that DON values and their differences have normal distribution.

Second (drawn together) step:

We defined the value of „t-probe function" with Excel program. The mathematical basis of calculation can be described by the following equations:

$$t = \frac{\bar{d}}{s_{\bar{d}}}, \text{ where:}$$

$$\bar{d} = \frac{\sum_{i=1}^{n} d_i}{n} \quad \text{and} \quad s_{\bar{d}} = \sqrt{\frac{\sum_{i=1}^{n}(d_i - \bar{d})^2}{n(n-1)}}$$

Thereafter we compared the values of t-probe belonging to the relevant degree of freedom (which can be seen in the Excel table) with t values of probe function. That was the basis for acception or rejection the null-hypothesis.

RESULTS AND DISCUSSION

Data of initial DON toxin content of wheat can be seen in the Figure 3, where the elements of sample arranged by size. The figure indicates well that in the case of tested elements Sortex Z color sorting decreased DON toxin content of wheat. But there is big difference between degree of decrease if we examine the individual elements.

The hypothesis analysis is necessary because the effectiveness of sorting must be proved undoubtedly. We have to clearly express, the decrease is not owing to chance.

Drawn up the starting point: there are tandem samples with „n" elements and it is supposed those come from population with normal distribution. The arithmetic mean and standard deviation isn't known. Toxin data of samples before cleaning is indicated by „x", and data of cleaned wheat samples by „y". Namely:

Elements of wheat samples before cleaning (X): x_i

Elements of cleaned wheat samples (Y): y_i

where i= 1,…, n

As it was above-mentioned according to arrangement of research samples data that belonging together were analyzed by paired t-probe. The average of data before cleaning indicated μ_1, and standard deviation σ_1. Accordingly with this logic the average of cleaned wheat samples is μ_2, and standard deviation is σ_2.

The hypothesis are following:

H0 = null-hypothesis when there is no significant difference between theoretical mean of two samples. That is

$$\mu_1 = \mu_2$$

H1= according to alternative hypothesis theoretical average of samples before mean is significantly higher than the average of sample after cleaning

$$\mu_1 > \mu_2$$

In our case the unilateral alternative hypothesis has sense. Figure 3 clearly presents the color sorting have an influence on decreasing of DON toxin content of wheat.

The Table 1 demonstrates the critical value of Student's

t- distribution at 5% significance level less than calculated value. That is null-hypothesis should be rejected, because theoretical means of two samples present significant difference. That is

$$\mu_1 \neq \mu_2$$

So it can be stated the selection by color proved to be effective in certain circumstances, at 95% probability level. Results were not induced by chance.

In the matter of food safety questions it is worth to examine null-hypothesis at lower significant level also. Data of the table displays, that

$$\alpha=0.0005 \rightarrow t=3.883$$

That is the effect of treatment is justifiable at higher probability level also, and the null-hypothesis can be rejected.

Similarly to examination of wheat samples harvested in 2013, we proved the efficiency of color sorting on decreasing of DON toxin content of wheat in samples cultivated in 2014. The plan of the experiment was started from the results of **Veres and Borbély (2007)** and **Kótai et al., (2012)**. They didn't find correlation between external and internal infection of grains, respectively toxin content. Depending on the characteristic of the infection the toxin concentration might be different in the grain **(Mesterházy, 1995)**. Thus we got to the color sorting method. Using this process principally those grains can be selected, which were infected in early stage of grain development. We come to the conclusion from the examination of results, that color sorting of wheat has statistically verifiable effect on the reduction of toxin content. That is the effect of cleaning is provable on 95% probability level. In this way our experiments proved that the toxin content of wheat can be efficiently decreased by application of modern machinery during the processing. Since this is a food safety question, it is important to clarify the results to what extent can be considered stable and repeatable depending on the different weather conditions of the years. We didn't get unambiguous answer to this. Further investigations are required to determine the extent of correlation between different initial mycotoxin content of wheat before sorting and efficiency of cleaning. Although the efficiency of a Sortex Z+ color sorting machine was justifiable in two very different years with different weather and infection circumstances, but the degree of decreasing of risk is can not be predetermined, because it depends on characteristic of fusarium infection. The grains infected in the full ripening period can not be selected by color sorting, because they are not discolored. In this case DON toxin accumulates in the wheat coat, which becomes bran during the processing. **Sándor et al., (2010)** and **Frank (2010)** investigated the efficiency of traditional surface cleaning in model experiments. Although they experienced different results, these methods had influence on decreasing of DON toxin content. For that very reason we plan to examine a modern surface cleaning method further on. The surface of grain can be cleaned by intense scrubbing machine with good

Table 1 Two sample paired t-probe for probable value.

	DON value before cleaning	DON value after cleaning
Experted value	0.6005	0.5320
Variance	0.0824366	0.084154
Observations	20	20
df	19	
t value	8.2097227	
P(T <=t) unpaired	5.694E-08	
t critical unpaired	1.7291328	
P(T <=t) paired	1.139E-07	
t critical paired	2.093024	

Note: Table style: Top and bottom border lines 1.5 point, other lines 1 point.

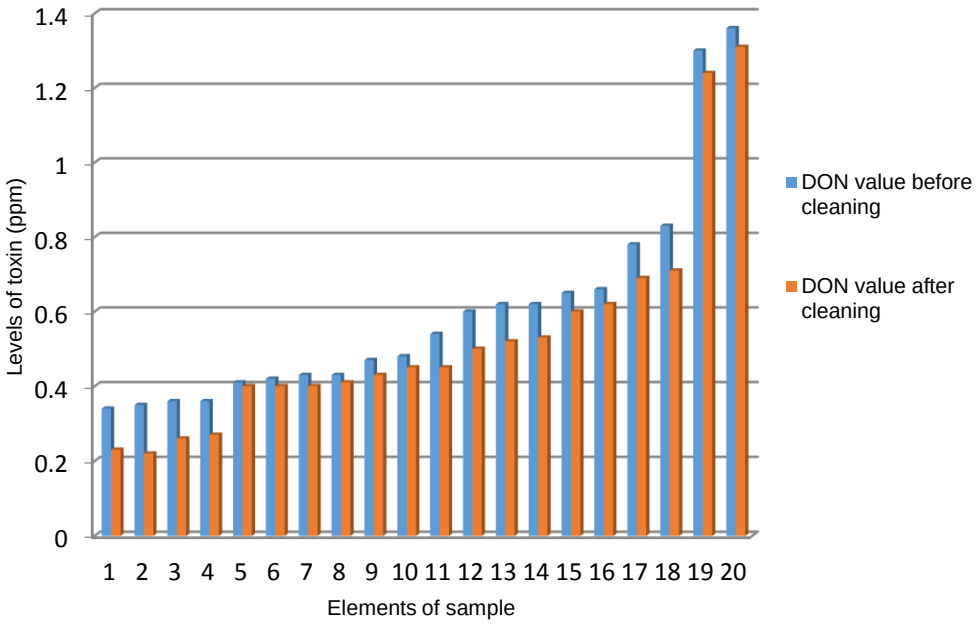

Figure 3 DON-toxin content of wheat before and after cleaning.

efficiency. Júlia Malom Ltd. applies Schule Verticone VPC 480 intense surface cleaner, which is a modern machine. In the next phase of experiment we will examine the efficiency of DON toxin content by application this machine.

CONCLUSION

Requirements of good manufacturing practice (GMP) and good hygiene practice (GHP) must be followed with attention and must be kept in mill industrial production also. On the one hand this means just as instruments and machinery can be applied in production which comply with these requirements. On the other hand it must be kept in mind that by the application of proper machinery in technological process enable to keep the regulation and to decrease risks. For the latter it is good example the opportunity of decreasing of DON toxin during processing. During our research work we prove by experiments, that by applying adequate instruments and machinery the degree of food safety risk can be decreased and the requirements of good manufacturing practice can be fulfilled. It can be summarized, that application of adequate technical equipment contributes to fulfil food safety requirements on a higher level in the course of food processing. According to the results of the experiment the application of Sortex Z+ color sorting can be suggested in the milling industry.

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Contact address:

Eleonora Kecskés Nagy, Kecskemét College Faculty of Horticulture, 6000 Kecskemét Erdei Ferenc tér 1. Hungary, E-mail: nagy.nori@kfk.kefo.hu.

Péter Korzenszky, Szent István University, Faculty of Mechanical Engineering, Institute of Mechanics and Machinery, 2100 Gödöllő Páter K. út 1. Hungary, E-mail: Korzenszky.Peter@gek.szie.hu.

Péter Semberyy, Szent István University, Faculty of Mechanical Engineering, Institut of Process Engineering, Department of Measurement Technic, 2100 Gödöllő Páter K. út 1. Hungary, E-mail: Sembery.Peter@gek.szie.hu.

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INDUSTRIALLY PROCESSED OILSEED RAPE IN THE PRODUCTION OF TABLE EGGS

Mária Angelovičová, Michal Angelovič, Zdenko Tkáč, Juraj Jablonický, Marek Angelovič

ABSTRACT

The purpose of this study was to investigate the effectiveness of feed mixtures with varying proportions of rape cakes to the weight of table eggs, its components, thickness and strength of egg shell. The eggs were from the final laying hybrid ISA Brown reared in the enriched cage system under experimental conditions. An age of laying hens was from 48 to 54 weeks. Egg weight and its components were measured on scales type KERN 440-35N, with an accuracy of 0.01 g and a maximum weight of 400 g. Egg white weight was calculated. The thickness and strength of the egg shell were measured from the dried samples at 55 °C. From each egg shell were cut 3 pcs of samples in the equatorial plane, one sample from the blunt end and one sample from the sharp end. Egg shell thickness was measured by test instrument SOME, type 60/0.01mm with a range of 0 – 10 mm. Egg shell strength was measured according to test instrument Instron with the small body, having a diameter 4.48 mm to exert pressure on the egg shell. The obtained data were assessed in the program system SAS, version 8.2. Based on the results observed in egg weight of our experiment we can conclude that in the group with share 5% of rape cakes was non-statistically significant (p >0.05) decreased egg weight compared to the control group. Egg weight was reduced in the group with share 10% of rape cakes, which confirmed a statistically significant difference compared to egg weight of control group (p <0.05). The differences among experimental groups with share 5% and 10% of rape cakes in feed mixture and as well as to control group were not statistically significant (p >0.05) in weight of egg yolk, egg white, egg shell and egg shell strength. **E**gg shell thickness was no statistically significant (p >0.05) increased in experimental group with share 5% of rape cakes and decreased in experimental group with share 10% of rape cakes versus control group. Increase of egg shell thickness in experimental group with share 5% of rape cakes versus decrease in experimental group with share 10% of rape cakes was statistically significant (p <0.05).

Keywords: table egg; white; yolk; shell; quality; oilseed rape

INTRODUCTION

Poultry breeding recorded a boom in technology of nutrition but also breeding in the twentieth century. The average body weight and the number of laid the eggs were increased. It changes the methods used in poultry breeding, the programs of hybridization and breeding, as well as the principles of nutrition and feeding. The production of hybrid chickens a laid type of hens is in the developed world by breeding provided through specialized reproductive breeding **(Capcarová et al., 2009)**.

Modern chickens were domesticated from the Red Jungle Fowl (*Gallus gallus*) over the last four or five thousand years for eggs and meat, for game and for exhibition **(Klasing, 2005)** and they are scientifically classified as the same species **(Wong et al., 2004)**.

Consumers of agricultural commodities are increasingly sensitive to animal welfare, and new systems aiming at improving this have recently been introduced to EU livestock production. For example, the conventional battery cage system used for chicken egg production was banned in the European Union in 2012 **(Council Directive 1999/74/EC)**, and fully housed production was mainly replaced by new enriched colony cages (or free range production) **(Leinonen et al., 2014)**.

According to a study conducted in the U.S.A., the quality of eggs from different production systems does not substantially differ. United States department of agriculture-agricultural research service developed a study in which examined various quality criteria for eggs. One of the many findings was that the organically produced eggs and eggs of usual production, there is no significant difference in quality **(Kvasničková, 2010)**.

Nutrition of laying hens is an important factor for the quality and safety of the table egg production. A feeding of laying hens with share 20% of secondary industrial products of oilseed rape has no negative effects on egg weight. These results indicate that the utilization of nutrients from the feed mixture containing secondary industrial products were similar as in the control group without secondary rapeseed products. Based on these results it can be assumed that laying hens good use the nutrients from rapeseed meal **(Gheisari et al., 2011)**.

The selection of appropriate feedstuffs for nutrition of the laying hens is limited to concentrated kinds with a high concentration of nutrients and energy. Corn together with

wheat and soybean meal presents the basis of feed mixture. Currently, the corn and wheat constitute a proportion 70% and soybean meal 16 – 20% of feed mixture **(Angelovičová, 1999)**.

The oil crops are considered a strategic material. The seeds contain economically significant quantity of oil. The fat is irreplaceable in human nutrition, in animal feed rations but also for an increasing proportion of oil in biodiesel. **Angelovič et al., (2013)** state that rapeseed oil constitutes a main raw material in the EU and presents two thirds of total input in biodiesel production.

Rapeseeds are a rich source of oil and their industrial remnants also of proteins. Oilseed rape is one of the most important and most fertile oilseeds in Slovakia **(Božík, 2007)**. Oilseed rape ranks the second among oilseeds **(USDA, 2011)**.

The chemical composition of oilseed rape was significantly changed by breeding. The term "rape" is currently used in Canada. It is incorporated in Great Britain, Australia and the United States to the characteristics of the species *Brassica napus* L. Oilseed rape provides edible oil with an erucic acid content of less than 2% and less than 30 mmol per gram of aliphatic glucosinolates. The use of rapeseed meal or cakes is restricted to full replacement of soybean meal due to the low level of available energy and the occurrence of anti-nutritional factors **(Khajali and Slominski, 2012)**.

Anti-nutrients of secondary rapeseed products include glucosinolates, sinapines, tannins and phytates, indigestible oligosaccharides and non-starchpolysacharides **(Kocher et al., 2000)**.

So far it has been identified more than 120 different species of glucosinolates **(Chen and Andreasson, 2001)**.

It is generally assumed that the glucosinolates by themselves are non-toxic. However, they are always accompanied by the enzyme myrosinase (thioglucoside glucohydrolase) in seed. Glucosinolates are subject to hydrolysis in moist and cracked seed. The results of glucosinolate hydrolysis are degradation products, such as isothiocyanates, goitrin, nitriles and isothiocyanates, which interfere with the function of the thyroid gland **(Tripathi and Mishra, 2007)**.

As a result of these effects on thyroid function, it is affected the metabolism of almost all tissues, including the reproductive system. In addition, various hydrolysis products of glucosinolates are irritating to the mucous membranes of the gastrointestinal tract and consequently are result local necrosis and hepatotoxicity **(Mawson et al., 1994b; Mithen et al., 2000; Burel et al., 2001; Conaway et al., 2002)**.

The negative consequences are described in farm animals as growth retardation, decreased production, impaired reproductive activity, and hepatic and renal function **(Mawson et al., 1994a)**.

The earlier published work indicating that the effect of glucosinolates is associated with reduced production of eggs, and the mortality syndrome related to bleeding of the liver **(Ibrahim and Hill, 1980)**.

In another study warn **Smith and Campbell (1976)** on the harmful effects of high levels of glucosinolates in rapeseed meal and rapeseed cake for laying hens. According to them, these adverse effects may be caused by the degradation products of glucosinolates, such as nitrile. They found that these decomposition products of glucosinolates are in the digestive tract of laying hens.

In the conclusions of the published work by **Mawson et al., (1994a)** is indicated the recommended share of low glucosinolate oilseed rape in feed mixture for laying hens.

The recommended proportion of low glucosinolate oilseed rape ranges up to 10%, in which there were no adverse effects on egg production **Khajali and Slominski (2012)**. They recommend share 20% of low oilseed rapes. Over the past few years, glucosinolate content of was decreased by breeding, particularly as regards double zero oilseed rapes compared to indigenous species.

According to the current annual report of the Canadian International Grains Institute, current oilseed rapes contain 10 μmol per gram of seed (in non-fat dry matter) **(Newkirk, 2009)**. Earlier report indicates reduced glucosinolate content, an average 11 μmol per gram of rapeseed meal **(Mailer and Cornish, 1987)** or 18 μmol per gram (in non-fat dry matter) **(Brand et al., 2007)**.

Oilseed rape cultivated in conditions of Europe, concretely in France, reached glucosinolate content 10 μmol per gram **(Labalette et al., 2011)**.

The level of glucosinolates 4.3 μmol per gram of grown oilseed rapes was also achieved in conditions of Poland **(Mikulski et al., 2012)**.

Glucosinolate content 1.5 μmol per gram could correspond to a share of rape cakes or rapeseed meal from 15 to 20% of current types '00' oilseed rape **(Khajali and Slominski, 2012)**.

The genetic potential of laying hens can be fully utilized. The condition is that laying hens must efficiently convert feed nutrients to eggs as food for human consumption. Laying hens must be healthy and their breeding must be well managed. Laying hens must consume highly digestible, concentrated and well balanced a feed mixture **(Jeroch et al., 2013b)**.

Industrial secondary products of oilseed rape can constitute a substantial proportion of laying hens feed mixture. The valuable components of feed mixture are modern genotypes (F1, F2 and F3 generation, i.e. 0-, 00- and 000-oilseed rape) **(Jahreis, 2003; Jahreisa Schöne, 2006; Jeroch et al., 2013a)**.

Rapeseed secondary products can replace 35 – 40% of soybean meal from the aspect of physiologically acceptable combination of amino acids **(Roth-Maier et al., 2004)**.

A representation of different share of soybean meal by rapeseed industrial secondary products were recommended based on the results of experiments **(Janjecic et al., 2002; Mushtaq et al., 2007; Tripathi a Mishra, 2007)**.

The purpose of this study was to investigate the effectiveness of feed mixtures with varying proportions of rape cakes to the weight of table eggs, its components, thickness and strength of egg shell.

MATERIAL AND METHODOLOGY

Characteristics of the object for research

The objects of the research were the table eggs and quality their components. The eggs were from the final laying hybrid ISA Brown reared in the enriched cage system under experimental conditions. An age of laying hens was from 48 to 54 weeks.

Experimental conditions of laying hens ISA Brown

Hens of laying type ISA Brown were included in the experiment. They lay eggs with brown shell. ISA Brown hens are a hybrid combination of color sexing type of lower body weight. Body weight to end of young hen rearing is 1450 g and to end of egg laying 2100 g. They reach sexual maturity at 145 days of age, when they begin laying. They reached high laying eggs 295 pcs to 500 days of age. Average egg weight is 63.3 grams. It is currently one of the most common hen hybrid combinations in the European Union, about 60% of large-scale breeding. Feed consumption, is around 115.0 g to 118.0 g per day, and 2.2 kg of feed per one kg of egg mass.

The experiment was conducted at the experimental facilities of Slovak University of Agriculture, Faculty of Biotechnology and Food, Department of Food Hygiene and Safety no. SK P 10011. Hens were housed individually in two-storey enriched cages. The cage space is in accordance with the recommendation for the implementation of the natural activity of laying hens, i.e. 750 cm^2. Laying hens had unrestricted access to feed in the feeder and water in the watering place. Feeder and watering place were completed daily. Laying hens was fed by feed mixture of soybean-cereal type, which is usually used in practical conditions. A share of corn and wheat constitute about 66% (33% corn and 33% wheat) and soybean meal 20% of feed mixture.

This feed mixture was used in control group. In the first experimental group was fed a feed mixture with a 5% rape cakes at the expense of soybean meal and other experimental group with share 10% of rape cakes at the expense of soybean meal. Rape cakes were obtained as remnant after pressing of seeds of oilseed rape. Double-zero rape seeds generally contain less than 30 μmol of glucosinolates per gram **(Widharna, 2012)**.

Replenishment of feed and water, as well as egg collection was carried out by hand, and each day at 9:00 am.

Sampling and investigated indicators

Sampling of eggs was carried out three times for 10 eggs in each group of laying hens. An age of laying hens was 50, 52 and 54 weeks when sampling.

Investigated indicators were: egg weight, yolk weight, white weight, shell weight, shell thickness and shell strength.

The methods of investigation of indicators

An egg weight was measured on scales type KERN 440-35N, with an accuracy of 0.01 g and a maximum weight of 400 g.

Sample preparation: The egg was broken, separated the yolk and the white. The yolk placed in pre-weighed watch glass and the egg shell with membranes were washed with tap water and dried in a drying cabinet preheated to 55 °C.

Yolk and shell were weighed on scales of type KERN 440-35N, with an accuracy of 0.01 g and a maximum weight of 400 g.

White weight was calculated using the formula:

$$x = \text{egg weight, g} - (\text{weight of egg yolk, g} + \text{weight of egg shell, g})$$

The thickness and strength of the egg shell were measured from the dried samples at 55 °C. From each egg shell were cut 3 pcs of samples in the equatorial plane, one sample from the blunt end and one sample from the sharp end.

Egg shell thickness was measured in the laboratory of the *Department of Machines* and Production Systems, Slovak University of Agriculture in Nitra; by test instrument SOME, type 60/0.01mm with a range of 0 – 10 mm.

Egg shell strength was measured in the laboratory of the Department of Machines and Production Systems, Slovak University of Agriculture in Nitra; according to test instrument Instron with the small body, having a diameter 4.48 mm to exert pressure on the egg shell by the method of **Angelovičová et al. (1994)** and **Rataj (1994)**.

Statistical methods

The obtained data were assessed according to basic statistical characteristics ($\overline{x}$ = mean, *SD* = standard deviation and c_v = coefficient of variation). Scheffe´s test at the significance level of $\alpha = 0.05$ was used to compare a difference between indicator values in the program system SAS, version 8.2.

RESULTS AND DISCUSSION

Table eggs are among the valuable foodstuffs **(Sparks, 2006)**. Type of laying hens is used to produce table eggs. In our experiment, it was used type of ISA Brown hens. Composition of eggs is influenced by genetic factors, age and diet. Nutrition is a very important factor for the production of quality and safe table eggs. The feed mixture of soybean-type cereal was used in our experiment.

According to **Angelovičová (1999)**, excluding maize and wheat is the basis of feed mixture the soybean meal too. We do not know soybeans to grow in our country, so we are forced to import it. We are focused on solving partial substitution of soybean meal with our domestic feed-rape cake in our experiment. It is a product that has a relatively high nutritional value and can be a useful component in feed mixture for laying hens in the production of the table eggs.

Mawson et al., (1994a) recommend incorporating into the feed mixture for laying hens with share of low glucosinolated oilseed rape. This amount is up to 10%. These authors conclude that in such proportion were no adverse effects on the production of table eggs. Some authors state that the appropriate proportion may be up to 20% **(Khajali and Slomiński, 2012)**.

The study by **Ibrahim and Hill (1980)** stated that the feed mixture of 20% rapeseed high in glucosinolates suppressed egg production in laying hens. However, the feed mixture with 20% of rapeseed meal produced from low glucosinolated rape did not cause a reduction in egg production.

Weight of eggs, yolk and white

Egg weight

Najib and Al-Kateeb (2004) noted that the results of their experiment confirmed that 10% of rapeseed in the feed mixture of laying hens not adversely affects the egg mass and egg weight. These authors further stated that the

daily egg production, egg mass and egg weight was lower if the in the feed mixture of laying hens was an addition of 30% rapeseed. They also stressed that the highest egg production was found if the laying hens fed feed mixture with a share of 5% and 10% of rape seed.

Based on the results observed in egg weight of our experiment we can conclude that in the group with share 5% of rape cakes was non-statistically significant (p >0.05) decreased egg weight compared to the control group. Egg weight was reduced in the group with share 10% of rape cakes, which confirmed a statistically significant difference compared to egg weight of control group (p <0.05). In the group with share 10% of rape cakes were observed the greatest variation in egg weight values expressed by standard deviation and coefficient of variation.

Table 1 Average egg weight.

Group	n	$\bar{x}$, g	*SD*	c_v, %
Control	30	61.8[a]	1.72	2.78
Experimental 5	30	60.3[ab]	2.10	3.48
Experimental 10	30	59.7[b]	2.36	3.95

n – number of samples, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient of variation,
a, b – value within a column compared between groups with different superscript letter is significantly different (p <0.05).

In contrast to our results, **Gheisar et al., (2011)** reported that feeding of the laying hens with share 20% of rapeseed products in feed mixture did not cause a reduction in egg weight. However **Ciurescu (2009)** takes the opposite view. The author notes based on the results his experiments, if the proportion of the rapeseed meal is greater than 15%, egg weight decreases.

Egg yolk weight

With this statement we can agree with the difference that in our experiment, there was a decrease in egg weight at share 10% of rape cakes. Average egg weight was in our experiment, by the proportion of rape cakes in the feed mixture, from 59.70 g in experimental group 10 and 60.30 g in experimental group 5. Higher egg weight (65.00, 71.25 g, respectively) in laying hens of the same age reported **Angelovičová and Polačková (2015)**. The laying hens were different type, Moravia SSL.

Based on our results of yolk weight we can conclude that differences among experimental groups with share 5% and 10% of rape cakes in feed mixture and as well as to control group were not statistically significant (p >0.05). Our results are in agreement with the results of yolk weight recorded by **Ciurescu (2009)**. However, our results indicate that the greatest variation of yolk weight values were in the group with share 10% of rape cakes.

Table 2 Average yolk weight.

Group	n	$\bar{x}$, g	*SD*	c_v, %
Control	30	15.7	0.83	5.29
Experimental 5	30	15.9	1.14	7.17
Experimental 10	30	15.2	1.19	7.83

n – number of samples, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient of variation,
No significant differences between groups *in* yolk weight between groups (p >0.05).

Egg white weight

Table 3 Average white weight.

Group	n	$\bar{x}$, g	*SD*	c_v, %
Control	30	40.2	2.17	5.4
Experimental 5	30	38.5	2.09	5.43
Experimental 10	30	38.8	3.14	8.09

n – number of samples, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient of variation,
No significant differences between groups in white weight between groups (p >0.05).

We did not met s literary knowledge about the impact of rape products on an egg white quality. The results of our experiment showed that between groups (control, with share 5% of rape cakes and 10% of rape cakes) no statistically significant difference (p >0.05). Even at egg white weight was observed the biggest variation of values in the experimental group with share 10% of rape cakes.

Egg shell quality

Egg shell weight

The egg shell constitutes the skeletal or external support of the egg **(Ar et al., 1979)** and, as such, egg shell quality is very important to the poultry industry **(Takahashi et al., 2009)**.

Table 4 Average egg shell weight.

Group	n	$\bar{x}$, g	*SD*	c_v, %
Control	30	5.9	0.12	2.03
Experimental 5	30	5.9	0.16	2.71
Experimental 10	30	5.7	0.19	3.33

n – number of samples, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient of variation,
No significant differences between groups *in* shell weight between groups (p >0.05).

We did not met s literary knowledge about the impact of rape cakes on an egg white quality. Literary knowledge is known about the impact of rapeseed meal on this indicator. **Riyazi et al., (2009)** indicate that the proportion 10% of rapeseed meal in feed mixture caused an increase of the egg shell weight. Our results disagree with this conclusion. We have found that, among the groups no statistically significant difference (p <0.05) in weight of the egg shell being compared according to the share 5 and 10% of rape cakes in feed mixture or compared to control group.

Egg shell thickness

Likewise at a thickness of the egg shell **Riyaz et al. (2009)** state that was observed no significant difference in the thickness, as well as in strength of egg shells, if laying hens fed rapeseed meal. Authors further state that the values of these indicators were higher in group of laying hens that were fed share 10% of rapeseed meal in feed mixture compared with the control group. Our results are consistent with ones of these authors.

Table 5 Average egg shell thickness.

Group	n	$\bar{x}$, mm	*SD*	c_v, %
Control	30	0.38	0.012	3.16
Experimental 5	30	0.4[a]	0.021	5.25
Experimental 10	30	0.361[b]	0.019	5.26

n – number of samples, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient of variation,
a, b – value within a column compared between groups with different superscript letter is significantly different (*p <0.05).*

Egg shell strength

Our results on the strength of egg shell are different compared to ones by **Khajali and Słomiński (2012)**. They argue that the quality of the egg shell was statistically significant in the control group compared with groups of laying hens, which fed feed with a share of industrial rapeseed products.

Table 6 Average egg shell strength.

Group	n	$\bar{x}$, N	*SD*	c_v, %
Control	30	33.1	1.36	4.11
Experimental 5	30	33.5	1.59	4.75
Experimental 10	30	33.8	1.72	5.09

n – number of samples, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient of variation,
No significant differences between groups in shell strength between groups (*p >0.05).*

Khajali and Słomiński (2012) reported on the base of experimental results that the values of quality indicators of egg shell were significantly higher (p <0.05) in the control group compared with the indicators of quality of the egg shell hens that fed feed mixture with a share of industrial by-products of oilseed rape. It is believed that the presence of phytic acid in the by industrial oilseed rape forms an insoluble complex with the protein and some minerals, such as e.g. calcium, iron, zinc, manganese and magnesium in a biologically unavailable for laying hens. This complex in turn leads to difficulties in the use of these minerals, protein and other nutrients for the organism of laying hens **(Šašytė et al., 2006)**. In addition, a high level of sulfur in rapeseed meal causes indigestion and absorption of calcium **(Summers et al., 1992)**. Increasing the pH of the small intestine chyme is another reason that might have impact on the quality characteristics the egg shell, if rapeseed meal is fed, in comparison with the pH of the small intestine chime, if soybean meal is fed **(Zdunczyk et al., 2013)**.

Oilseed rape is a farm significant crop suitable for cultivation in our country. Its products after industrial processing of seeds for oil are an important feed material for animals intended for food production. However, definite conclusions about their use as feed material require further investigation. Conclusions of existing experiments are not unanimous as to the extent of their inclusion in feed mixture in relation to the quality production of table eggs, or their safety.

CONCLUSION

Seeds of oilseed rape are an important raw material for obtaining oil. By-product after industrial processing has a relatively high nutritional value. By-product may be classified as feed material for laying hens. More research is needed for single-valued recommendation of by-product share of oilseed rape in the feed mixture for laying hens. Oilseed rape and industrial by-products contain anti-nutrients according to literary knowledge.

In our work we focus on the verification of rape cake, for their use as feed for laying hens. Rape cakes were obtained as a by-product after pressing of seeds. By pressing of seeds was obtained oil to produce of biodiesel.

On the basis of evaluated results, we can state the following:

- share 5% of rapeseed cakes in the feed mixture had no statistically significant effect on egg weight, its component parts, egg shell thickness and strength,
- share 10% of rapeseed cakes in feed mixture statistically significant negative influenced on egg weight and measured values showed the greatest fluctuations in investigated indicators expressed as the standard deviation and coefficient of variation,
- the issue of the use of rapeseed cakes as feed is highly topical in terms of their production in obtaining of oil for human consumption and biodiesel,
- the conditions for inclusion of rapeseed cakes in the feed mixture for laying hens remain open for further research.

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Contact address:

Mária Angelovičová, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra Slovakia, E-mail: maria.angelovicova@gmail.com.

Michal Angelovič, Slovak University of Agriculture in Nitra, Faculty of Engineering, Department of Transport and Handling, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: michal.angelovic@gmail.com.

Juraj Jablonický, Slovak University of Agriculture in Nitra, Faculty of Engineering, Department of Transport and Handling, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: juraj.jablonicky@uniag.sk.

Zdenko Tkáč, Slovak University of Agriculture in Nitra, Faculty of Engineering, Department of Transport and Handling, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zdenko.tkac@uniag.sk.

Marek Angelovič, Slovak University of Agriculture in Nitra, Faculty of Engineering, Department of Machines and Production Systems, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: marek.angelovic@uniag.sk.

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A COMPARISON OF THE DETERMINATION OF THE RENNET COAGULATION PROPERTIES OF BOVINE MILK

Roman Pytel, Květoslava Šustová, Vojtěch Kumbár, Šárka Nedomová

ABSTRACT

The aim of the work was compared of two different methods (the visual method and the nephelo turbidimetry method) for determination of rennet coagulation time. It was observed the effect of heat treatment of milk; types of rennet and addition of different amount of $CaCl_2$ into the pasteurized milk. It was used two different chymosin rennet. For the visual method was milk sample (100 mL) equilibrated at 35 °C, 1 mL of rennet was added into milk and was measured the time required for the first visible flakes (visual method). For the determination rennet coagulation time by nephelo-turbidimetry was removed part of milk with rennet and placed into nephelo-turbidimetry. Milk had a titratable acidity in the range from 6.5 to 7.0 °SH, average pH of milk was 6.68. Dry matter content was in range from 12.351 to 13.142%. The average content of protein by Kjeldahl was 3.14%, fat by Gerber 4.34%, lactose by polarimetry 4.68% and calcium content 1.1%. The pasteurized milk had the worst rennet coagulation time about 32 s compared to the raw milk. The difference coagulation time between milk with addition of 20 µL $CaCl_2$ and 40 µL $CaCl_2$ was in range 21 s to 26 s by visual method. The difference coagulation time between milk with addition of 20 µL $CaCl_2$ and 40 µL $CaCl_2$ was 15 s by nephelo-turbidimetry method. There occurred statistically non-significant differences in most of the measurements, comparing the visual and the nephelo-turbidimetric method. The heat treatment, addition of $CaCl_2$ and using of different rennet had an influence on the curd category. It was obtained, that using nephelo-turbidimetry shown objective results for measuring the rennet coagulation time contrary the subjective visual method. Further, the results obtained by nephelo-turbidimetry are accurate and determined with the lower variation.

Keywords: rennet coagulation time; nephelo-turbidimetry; bovine milk; calcium chloride

INTRODUCTION

Milk coagulation properties are one of the most important technological properties of milk which have influence on the cheese production. The addition of rennet into the sample of milk will be cause of physical and biochemical changes in milk, which include modification of casein micelles. The result of renneting is the change of viscosity and elasticity. Milk coagulation properties is collection of property which are traditionally expressed as the rennet coagulation time, time to curd firmness of 20 mm and curd firmness 30 min after enzyme addition **(Bittante, 2011)**.

The principle of cheese production is the conversion of a viscous liquid (milk) into solid material (curd) which retain casein protein and fat (which was included in milk). If needs a solid material, the whey from curd have to be removed. In the whey is left a major part of water, whey protein and majority of lactose. For this step is important to precipitate the casein from the milk and release the whey from the curds **(Law and Tamime, 2010)**. After the addition of rennet para casein micelles starts to aggregate, thereby increasing the viscosity and elasticity. This transition may also change other physical properties of milk e.g. a light reflectance or a thermal conductivity. Several methods are principally based on the detection of these changes. These methods were developed for measuring and determination of rennet coagulation properties **(Fuquay et al., 2011)**.

The milk coagulation property is influenced by many factors, which have an effect on primary or secondary phase of precipitate of milk or both. The milk composition has direct effect on precipitate of milk and total yield of cheese. Especially important is the content of calcium, particularly the ionic form, which has influence on formation of curd gels consistency **(Roginski et al., 2003)**. Further factors which had an effect on rennet coagulation time are coagulation temperature, pH and concentration of $CaCl_2$. The coagulation temperature had highly significant effect on rennet coagulation time, coagulum firmness, gel firming rate and curd firmness. The effect of pH was highly significant for all parameters too, especially on curd firmness. Concentration of $CaCl_2$ was significant only for rennet coagulation time and coagulum firmness **(Nájera et al., 2003)**. The milk salts have an influence on rennet coagulation of milk and structure of cheese **(Lucey and Fox, 1993)**. The addition of $CaCl_2$ had an effect on the yield of cheese **(Wolfschoon-Pombo, 1997)**. The main salts are Ca and PO_4. Addition of Ca decrease the rennet coagulation time of milk that is due to neutralization of negatively charged residues of casein, which increased the aggregation
of renneted micelles **(Lucey and Fox, 1993)**. **Yuksel**

(2013) confirmed that rennet flocculation and clotting times are decreased with increasing concentration $CaCl_2$.

In this study was showed that $CaCl_2$ has effects on both – the primary and secondary phase of renneting. $CaCl_2$ reduced the time of coagulation. On the other hand addition of disodium phosphate into homogenized milk increases the time of coagulation **(Maxcy et al., 1955**).

High heat treatment or a low calcium concentration had an effect on prolonged flocculation (slow aggregation rate) with slow firming rate in the samples of reconstituted milk. The coagulation time was negatively correlated with the firming parameters **(Klandar et al., 2007)**.

Several methods were used for measuring and determining the rennet coagulation property of milk. The visual determination of rennet coagulation time is very often used. A nephelo-turbidimetry and the measurement on the formagraph are also used.

The visual method – the coagulation of proteins is often assessed using a visual method that consists of the observation of precipitating milk with naked eye. After the addition of rennet, it was measured the time until the first visible flakes of aggregation casein could be spotted with naked eye. The sample of coagulation milk is observed under continuous gentle mixing, mostly against the light **(Sbodio and Revelli, 2012)**. The result of visual method affects subjective assessment and the experience of the person monitoring the rennetability. For these reasons, it is not regarded as an objective **(Čejna and Přibyla, 2006)**.

The characteristic of rennet was based on the determination of rennet strength. To determine the strength of the rennet, there can be often used these methods: The rennet strength by Soxhlet is defined as the quantity of raw milk, which can be precipitated by 1 mL rennet at 35 °C for 40 min **(Law and Tamime, 2010)**. The method by Berridge – this method is used for determination of the defined strength of rennet to precipitate 10 mL of reconstituted standardized milk (standardized sample of powdered milk reconstituted 0.01 mol/L $CaCl_2$ at pH 6.3 at 30 °C for 100 s **(Fuquay et al., 2011)**. The method of Rolling bottle which is based on giving milk samples in special tubes or bottles. These bottles are heated up to 30 °C, adding 1 mL of diluted chymosin and placed in water bath at an angle of approximately 20 °. Bottles are spinned in this bath at speed of 8 rpm. This test is finished when the film of small casein flocks is formed on the inner wall of the bottle. The coagulation time is determined from number of revolutions from start to the end of this test **(Sommer and Matsen, 1935)**. This method is among nondynamic method. Nondynamic methods are mostly empirical and based on the measurement of rheological or optical properties **(Castillo, 2006)**.

The nephelo-turbidimetry works in the principle of the nephelometry and the turbidimetry. The nephelometry is an optical method, engaged in measuring the intensity of diffusely scattered light to the dispersed particles **(Sojková et al., 2011)**. The optical detector device converts light intensity to an electrical signal. The strain at the output is a function of the intensity of light incident on the optical detector. The milk coagulate reduces the optical signal (turbidimetry) and thereby also a reduction of the measured strain. The resulting recording signal is immediately derived. This result corresponds to the precipitate para-casein and the maximum value of derivate curve **(Chládek and Čejna, 2005)**.

The detection of rennet coagulation properties by formagraph is based on the principle of the movement of small pendulum which is linearly moved in the milk coagulation sample. This movement is recorded on a photographic paper and this curve gives us the dependence of strength to the time. When milk is in liquid form and has a low viscosity, this milk doesn´t have a sufficient strength to move the pendulum, whereas the coagulation milk has a higher viscosity and causes synchronous movement of the pendulum **(McMahon and Brown, 1982)**. Other methods for determination rennet coagulation time are: refractometry, where was measured change in the refractive index during milk coagulation **(Korolczuk et al., 1988)**, Near Infrared spectroscopy (NIR) used a light transmission or reflectance measured in the NIR range for coagulation milk **(O´Callaghan et al., 2000)**, vibration viscometry **(Marshall et al., 1982; O´Callaghan et al., 2000)**, fluorescence spectroscopy where is monitored emission fluorescence spectra of tryptophan residues during coagulation **(Herbert et al., 1999)**, electroacoustic **(Wade and Beattie, 1998)**, ultrasound low frequency used a frequencies in range 50 to 100 kHz **(Nassar et al., 2001)** and reflection photometry where are monitoring coordinates L* and b* which rise during coagulation of milk **(Hardy and Fanni, 1981)**.

MATERIAL AND METHODOLOGY

The bovine milk used for this work was collected in summer 2015. The samples of milk were heated up prior to analyses to 40 °C and then cooled down to 20 °C for better dispersion of the fat globules. After heating and cooling, the samples were immediately analysed. Before measuring the milk samples, there were done some basic laboratory analyses that have an effect on rennet coagulation time. The titratable acidity, pH, the calcium content and the lactose by polarimetry were determined according to Czech state standard No 57 0530. The content of fat was determined by Gerber's method **(ISO 2446:2008)**, the protein content was determined by Kjeldahl's method **(EN ISO 8968-1:2002)**, dry matter content was determined by gravimetry **(ISO 6731:2010)**.

After these analyses, the part of milk for rennet coagulation time was removed. This part of milk was analysed as raw milk. The remaining part of milk was pasteurized at 72 °C for 20 s. This combination is very often used at cheese production. It was premised that heat treatment has an influence on the rennet coagulation time (that the pasteurized milk needs more time for coagulation then raw milk).

Subsequently, the pasteurized milk has been divided into four groups. The first group was only pasteurized. The solution of 36% $CaCl_2$ was added into the next three groups at the quantities of 20 μL, 30 μL and 40 μL 100 $mL^{-1}$of pasteurized milk.

In our work was used a proteolytic enzyme which causes precipitation of proteins. It was used two different chymosin RENNET A (CHY-MAX M200, CHR. HANSEN, Denmark, 190 IMCU/1 mL) and RENNET B (Laktochym CZ 1068 ES, MILCOM a.s.,

Czech Republic, 59.5 IMCU/1 mL). These rennet were diluted so, that the precipitation was in range from 120 s to 240 s.

Each group (raw milk, pasteurized milk, pasteurized milk with 20 μL 100 mL^{-1}, pasteurized milk with 30 μL 100 mL^{-1}, pasteurized milk with 40 μL 100 mL^{-1}) had 6 samples for RENNET A and 6 samples for RENNET B. The measurements were repeated three times.

The measurement of rennet coagulation time of milk was carried out by visual methods and method using the nephelo-turbidimetric sensor for milk coagulation property.

The milk sample (100 mL) was equilibrated at 35 °C, 1 mL of rennet was added into milk and was measured the time required for the first visible flakes (visual method). For the determination rennet coagulation time of coagulation, which was measured by nephelo-turbidimetry.

The milk with adding rennet was placed in a thermostat at 35 °C. Samples of coagulation milk were evaluated according five grade scale (in the Table 1) after one hour in a thermostat.

Statistically, the difference between the two methods using the t-test was evaluated. The results were statistically processed by program STATISTICA 12.

The aim of the work was compare of two difference methods (the visual method and nephelo-turbidimetry method) for determination of rennet coagulation time. It was observed the effect of heat treatment of milk; types of rennet and addition of difference concentration of $CaCl_2$ into the pasteurized milk.

Table 1 Evaluation of rennet curd quality (Kuchtík et al., 2008).

Category	Appearance and firmness of curd and appearance of whey
1	very good and hard curd, keeping its shape after its removal from the container; whey is clear, of yellowgreenish colour
2	good but a little softer curd, not keeping its shape quite perfectly; excretion of whey not perfect; whey is greenish
3	not good, soft curd, partly not keeping its shape; milky white whey
4	very bad curd, not keeping its shape; milky white whey
5	very weak or invisible flocculation of casein

Table 2 The composition of milk and milk properties.

	Average	Standard deviation	Minimum	Maximum	Coefficient of variation (%)
Titratable acidity (°SH)	6.7	0.3	6.5	7.0	3.7
pH	6.68	0.04	6.63	6.71	0.62
Dry matter content (%)	12.740	0.396	12.351	13.142	3.106
Protein content (%)	3.14	0.11	3.04	3.26	3.58
Fat content (%)	4.34	0.31	4.01	4.62	7.11
Lactose content (%)	4.68	0.08	4.61	4.77	1.75

Table 3 The comparison of rennet coagulation time of raw and pasteurized milk by the visual and the nephelo-turbidimetry method (the average value with the standard deviation).

Groups of milk	Rennet	Visual method (s)	Nephelo-turbidimetry (s)	Significance
Raw	A	165 ±4	148 ±6	*
	B	135 ±15	130 ±16	NS
Pasteurized	A	184 ±23	162 ±23	*
	B	159 ±27	142 ±15	NS

Note: * – significant difference at $p < 0.05$; NS – nonsignificant difference at $p > 0.05$.

by nephelo-turbidimetry was removed part of milk with rennet and placed into nephelo-turbidimetry. The result of nephelo-turbidimetry measurement of coagulation of milk is a curve with an inflection point representing the milk coagulation time. From each group, there was calculated an average compared to the time

RESULTS AND DISCUSSION

Milk, which has been used for comparing of rennet coagulation time, had a titratable acidity in the range from 6.5 to 7.0 °SH, average pH of milk was 6.68. Dry matter content was in range from 12.351 to 13.142%. The average content of protein by Kjeldahl was 3.14%, fat by Gerber

Groups of milk	Rennet	Visual method (s)	Nephelo-turbidimetry (s)	Significance
Raw	A	165 ±4	148 ±6	*
	B	135 ±15	130 ±16	NS
Pasteurized	A	184 ±23	162 ±23	*
	B	159 ±27	142 ±15	NS

4.34%, lactose by polarimetry 4.68% and calcium content 1.1%. The detail results of milk composition and properties are shown in Table 2.

It was compared visual and nephelo-turbidimetry method for determination of rennet coagulation time in raw and pasteurized milk. The rennet coagulation time determined by two different methods is shown in the Table 3.

It was used two different rennet for determination of coagulation time in this work. The RENNET A was added in the raw milk. The coagulation time determinated by visual method was 165 s and the coagulation time determined by nephelo-turbidimetry was 148 s. According **Bujko et al., (2011)** this rennet coagulation time is evaluated as "less good" because coagulation time is in range 140 – 200 s. The difference between the visual and the nephelo-turbidimetry method was 17 s. For raw milk, there is statistically significant difference between these methods.

The same samples of milk were obscured by RENNET B. The coagulation time determinated by visual method was 135 s and the coagulation time determined by nephelo-turbidimetry was 130 s. According **Bujko et al., (2011)** this rennet coagulation time is evaluated as "good" because coagulation time is in range 110 – 140 s. The difference between the visual and the nephelo-turbidimetry method was 5 s. This difference is not statistically significant.

The pasteurized milk had the worst rennet coagulation properties because heat treatment caused changes in solubility of calcium ions. The rennet coagulation time was increased by about 32 s compared to the raw milk. The pasteurized milk with RENNET A gave a statistically significant difference between both methods. However comparing these methods with RENNET B, it was found that there is no significant difference.

It was observed an influence of different amount $CaCl_2$ to rennet coagulation time. The results of rennet coagulation time are shown in the Table 4.

The rennet clotting time for pasteurized milk with 20 µL by visual method (RENNET A) was 150 s. The rennet clotting time by nephelo-turbidimetry was 134 s. Difference between these methods was 16 s with a statistically significant difference.

The rennet clotting time for pasteurized milk with 20 µL by visual method (RENNET B) was 129 s. The rennet clotting time by nephelo-turbidimetry was 124 s. Difference between these methods was 5 s. This difference between these methods is a statistically not significant.

This statistically not significant difference is due to the fact that RENNET B with $CaCl_2$ forms larger flakes which are better visibly detected by naked eye.

The higher addition than 20 µL of calcium chloride into the milk caused that the difference between the visual and the nephelo-turbidimetry methods is not statistically significant. With the increasing levels of calcium chloride begin to form the larger flakes without influence to type of rennet. The rennet coagulation time are decreasing with increasing the addition of $CaCl_2$. The difference

Table 4 The comparison of rennet coagulation time of pasteurized milk with the addition of different amount of $CaCl_2$ by the visual and the nephelo-turbidimetry methods (average value with standard deviation).

Groups of milk	Rennet	Visual method (s)	Nephelo-turbidimetry (s)	Significance
Pasteurized with 20 µL	A	150 ±20	134 ±11	*
	B	129 ±22	124 ±18	NS
Pasteurized with 30 µL	A	136 ±20	124 ±13	NS
	B	121 ±17	111 ± 9	NS
Pasteurized with 40 µL	A	124 ±19	119 ±15	NS
	B	108 ±17	109 ±15	NS

Note: * – significant difference at p <0.05; NS – nonsignificant difference at p >0.05.

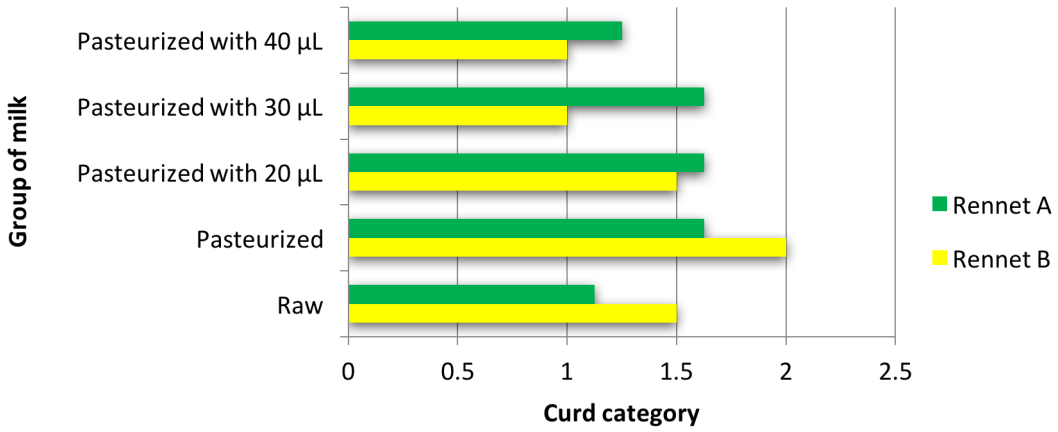

Figure 1 Curd category evaluation of milk group.

coagulation time between milk with addition of 20 µL $CaCl_2$ and 40 µL $CaCl_2$ was in range 21 s to 26 s by visual method. The identical results present **Erdem (1997)**, which studied the effect of $CaCl_2$ concentration on the clotting time. **Fox et al. (2004)** report, that the best rennet coagulation time had pasteurized milk with added calcium chloride. This finding was confirmed by our results – rennet coagulation time for milk with addition of 40 µL was 124 s while the rennet coagulation time for pasteurized milk was 184 s (RENNET A).

According to the study by **Čejna and Přibyla (2006)** the good milk coagulation property means that milk needs a short time to precipitate the milk by adding rennet. The results of this study confirmed possibility to reduce rennet coagulation time by addition of $CaCl_2$.

When was compared the curd quality, it was confirmed the premise that the heat treatment had an effect to curd quality. These curd were soft but the curd harder released a whey. The released whey was a slightly turbid. The curd quality was also affect by rennet which was used (Figure 1). At the higher amount $CaCl_2$ is better used a RENNET B. This curd was solid, keep the shape and whey was yellowgreen clear colour. At amount of 30 or 40 µL $CaCl_2$ was formed a first category of curd. At amount of 20 µL $CaCl_2$ was formed a worse curd category. The curd was a less solid and releasing of whey was more difficult. RENNET A formed a better curd category without addition of $CaCl_2$ or with an addition of 20 µL $CaCl_2$.

CONCLUSION

The visual method for determining rennet coagulation time is a subjective method, which is influenced by the experience of person monitoring rennet coagulation time, by the type of rennet and its concentration. It was obtained, that using nephelo-turbidimetry shown objective results for measuring the rennet coagulation time contrary the subjective visual method. There were found statistically non-significant differences in most of the measurements, comparing these methods. Using nephelo-turbidimetry was obtained the objective results for measuring the rennet coagulation time. Further, the results obtained by nephelo-turbidimetry are accurate and there are determined with the lower deviation. The determination rennet coagulation time by nephelo-turbidimetry is more expensive, but is unaffected by the experience of person monitoring these properties. Thus, this method can replace the subjective visual method.

The heat treatment, addition of $CaCl_2$ and using of different rennet had an influence on the curd category. For milk where is used a less amount of $CaCl_2$ or without $CaCl_2$ is better used RENNET A, which formed a better curd category. The combination of higher amount $CaCl_2$ with RENNET A is not suitable for formed good curd category. RENNET B is better used with higher amount of $CaCl_2$ for formed good curd category.

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Contact address:

Ing. Roman Pytel, Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: roman.pytel@mendelu.cz.

prof. Ing. Květoslava Šustová, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: sustova@mendelu.cz.

Ing. Vojtěch Kumbár, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Technology and Automobile Transport, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: vojtech.kumbar@mendelu.cz.

doc. Ing. Šárka Nedomová, Ph.D., Mendel University in Brno, Faculty of AgriSciences, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: snedomov@mendelu.cz.

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STEREOLOGICAL ANALYSIS OF PEA PROTEIN IN MODEL SAMPLES

Zdeňka Javůrková, Matej Pospiech, Michaela Petrášová, Bohuslava Tremlová, Ludmila Luňáková

ABSTRACT

With the growing popularity of various plant proteins used as raw materials for meat production, interest of manufacturers to extend the range of such raw materials is increasing as well. Manufacturers are trying to minimize the cost of manufacturing their products with simultaneous preserving the nutritional value of their products to the maximum extent possible. Such cheaper raw materials, which are also nutritionally rich, include pea protein. Another advantage for manufacturers is the fact that legislation does not order them to indicate pea protein presence in case of its addition, as it does for other allergenic ingredients, although this legume contains storage proteins which can cause a variety of allergic reactions, just like other legumes. Currently no method used for its qualitative determination has been described in literature, let alone its quantitative determination. Our work describes a possible method that can be applied for its quantification. It is a stereological method applied to microscopic sections stained by immunohistochemical staining based on the avidin-biotin complex using monoclonal legumin (1H9) as the primary antibody. The stereological method is based on geometry, it applies knowledge of geometry to analyze a sample of diverse origin, size and internal structure. Despite potential shortcomings in staining microscopic preparations, stereology allows us to perform quantification based on knowledge of morphology of the observed structures. This work describes a procedure of a known pea protein addition quantification in model meat products by means of Ellipse software. Pea protein quantification was performed in two ways. In the first case ten microimages of all sections prepared were examined, while in the second case one scan of the entire section was analyzed. Based on the results, Spearman's correlation coefficient was calculated, which confirmed our assumption of correlation between the protein added into the product and the measured area in microimages. In both ways Spearman's correlation coefficient was rSp = 1000. We obtained regression equations in MS Excel, which can be used for calculation of pea protein addition based on measured area of this protein in microscopic section.

Keywords: Vegetable proteins; microscopy; immunohistochemistry; allergens; meat products

INTRODUCTION

In the meat industry, raw materials in the form of vegetable proteins, which are used as a meat substitute, are very common **(Modi et al., 2004)**. During meat production is frequently used various vegetable and animal proteins. The most commonly employed plant - origin proteins are wheat and soy proteins. Meat product also contain from animal - origin proteins as plasma, collagen or milk protein **(Petrášová, 2015)**. Some of these vegetable proteins are classified as allergens in the legislation **(Regulation (EU) No. 1169/2011)**. Besides other reasons, this motivates the producers' efforts to replace them with other vegetable proteins that are not ranked among the allergens that must be indicated in harmony with the aforementioned legislation. Pea protein belongs among the most common ones **(Baticz, 2001)**. Like other legumes, however, pea proteins also include storage proteins which can cause allergic reactions. The literature identifies analysis of polyphenols characteristic of certain legumes and HPLC method which can detect up to 0.1% addition of soy protein in a meat product, as potential detection methods. Detection of lupine can be performed similarly, nevertheless, reliable detection of pea has not been achieved yet **(Mession et al., 2012; Mellenthin and Galensa, 1999)**. Other possible methods that can be used to detect vegetable protein are microscopic methods.

Microscopic methods belong among the oldest analytical methods and can be applied to demonstrate food components. These methods are simple, able to differentiate and identify individual basic components in the foodstuffs. The most commonly used methods in practice are histochemical methods, but now there is a wide range of options for processing and preparation of samples and also investigative techniques from classic to those that apply the most innovative technical equipment. Imaging techniques belong among the most suitable techniques to examine the structure of food **(Kaláb et al., 1995)**. As argued by **Tremlová et al. (2013)**, addition of vegetable protein can be detected using microscopic methods if they are present in the product in a sufficient size for light microscopy.

Javůrková et al. (2015) mentioned the use of modern microscopy methods for a qualitative as well as quantitative examination of the products. These methods provide information about location all components of the sample examined. One of the methods is image analysis.

Image analysis is often using as qualitative methods for meat products. The image obtained by microscopic methods can undergo quantitative analysis while preserving all the advantages of microscopy. In such a case, the input is image data and the output is a description of the image. Quantitative microscopic examination may be indicative or accurate **(Pospiech, 2008)**. Quantitative image analysis allows us to describe and specify all information obtained by microscopic (as well as macroscopic) scanning. It allows a detailed comparison of samples, accurate processing of information obtained and different ways of expressing the results achieved. The procedure for image analysis consists of creating photographs and their subsequent analysis using a program. To scan microscopic slides, a set composed of a light microscope and a digital camera or camcorder can be utilized. The very analysis involves creating a template (colour and brightness are usually selected from among image parameters) to identify the selected components and subsequently to measure their surface area and the entire section area. This results in numerical data obtained from the image, thereby permitting a detailed comparison of different samples, accurate processing of information obtained and different ways of expressing the results. Recorded data can be evaluated using different statistical methods. Another great advantage is the ability to compare objects scanned currently with objects stored previously. Integration of image analysis into the manufacturing process allows on-line measuring which is very useful, even necessary, in the inspection process in food production. The main advantage is the possibility to obtain a result without direct contact with the sample. This completely minimizes the risk of e.g. cross-contamination **(Javůrková, 2014)**. Image analysis based on computer technology is developing rapidly and allows to obtain objective results, because it uses a large number of images in statistical processing. This means that one of the biggest pitfalls of microscopy can be avoided, namely selecting and publishing only the best images as sample „representatives“ for demonstration of results and publishing **(Tremlová et al., 2013)**. Therefore, the literature considers the results obtained by image analysis in the examination of meat and meat products objective, accurate and comparable with data produced by chemical methods.

Development of image analysis in the field of microscopy largely coincides with the development of stereology. Stereology is based on geometry, it applies its knowledge to analyze samples of diverse origin, size and internal structure. It deals with statistical derivation of geometric properties of the examined structures and object from test probes applied to oriented sample sections **(Glaser and Glaser, 2000)**. Stereology is used by **Flintová and Meech (1978)** in their work. They used a method based on counting the points in a grid in quantifying textured soy protein, where estimated surface area of the object being measured was based on counting the area belonging to one point and the number of grid intersections with the object being measured. The advantage of this measurement includes its ease and affordability and the possibility to examine the image not only based on colour contrast, but also on the basis of morphological criteria. The disadvantage of stereology is manual processing that is time consuming and not always more accurate than automatic examination. Image analysis used as quantification method requires optimum contrast between the monitored component and other components in the product **(Aguilera and Stanley, 1990)**, while stereology does not have this requirement **(Lukášková Řezáčová, 2011)**.

An integral part of quantitative studies is statistical evaluation of results. Correctness of the analysis may be affected by so-called deflections of the measuring system itself, processing (various thickness of sections, uneven stainability, creation of artifacts, change of protocols etc.), examiner (whether in manual measuring or error rate in mathematical processing of results) or improper calibration of the digital recording collection equipment. In current practice, variability of sample processing can be reduced by standardizing and automating the examination workflow **(Tonar, 2008)**.

Currently, there is no commercially available method for demonstrating the addition of pea protein, let alone its quantification in a meat product. Therefore, the aim of our work was to create a method and protocols for its quantitative determination in meat products.

MATERIAL AND METHODOLOGY

Model meat products (MMP) containing pea protein additions in concentrations of 0.1, 1.0, 2.0, 3.0, 4.0 and 5.0% were produced for the examination. The products were made of ground chicken breast meat with the addition of pea protein. These model products were cooked at 70 °C for 10 minutes. Four blocks (A, B, C, and D) of 1 mL were collected from each product and frozen. These blocks were then sliced into sections 10 µm thick using cryostat HM 550 (Germany, Microm).

Subsequently, these cryosections were stained with immunohistochemical (IHC) staining method of ABC complex. The primary antibody used was monoclonal legumin (1H9). With respect to previous testing of immunohistochemical staining and consideration of costs, antibody concentration of 1:1000 was selected.

Quantification of the immunohistochemical examination results was performed in two different ways. First, quantification was performed in digital images of MMP, which had been taken in Eclipse E200 microscope (Nikon, JPN) using EOS 1100D camera (Canon, JPN) and processed by DSLR REMOTE Ver. 2.2.2.1 (UK) at a magnification of 100x (Ellipse 1). The entire sections were scanned in this way and a random selection of 10 images from all blocks of the sample was performed. As reported by **Řezáčová Lukášková (2011)**, who used stereology to quantify the addition of wheat protein in her work, in order to achieve the coefficient of error (CE) <0.2, at least 8 images of the sample with added proteins should be investigated.

Also, these samples were scanned using Eclipse Ci-L microscope (Nikon, JPN), DFK 23U274 camera (Imaging Source, GER) and motorized stage of Prosca III (Prior, USA) in NIS Elements Basic Research 04.13.04 software (Laboratory Imaging, Czech Republic) at magnification of 40x (Ellipse 1). Thanks to the motorized stage and NIS software, the entire sections could be scanned and

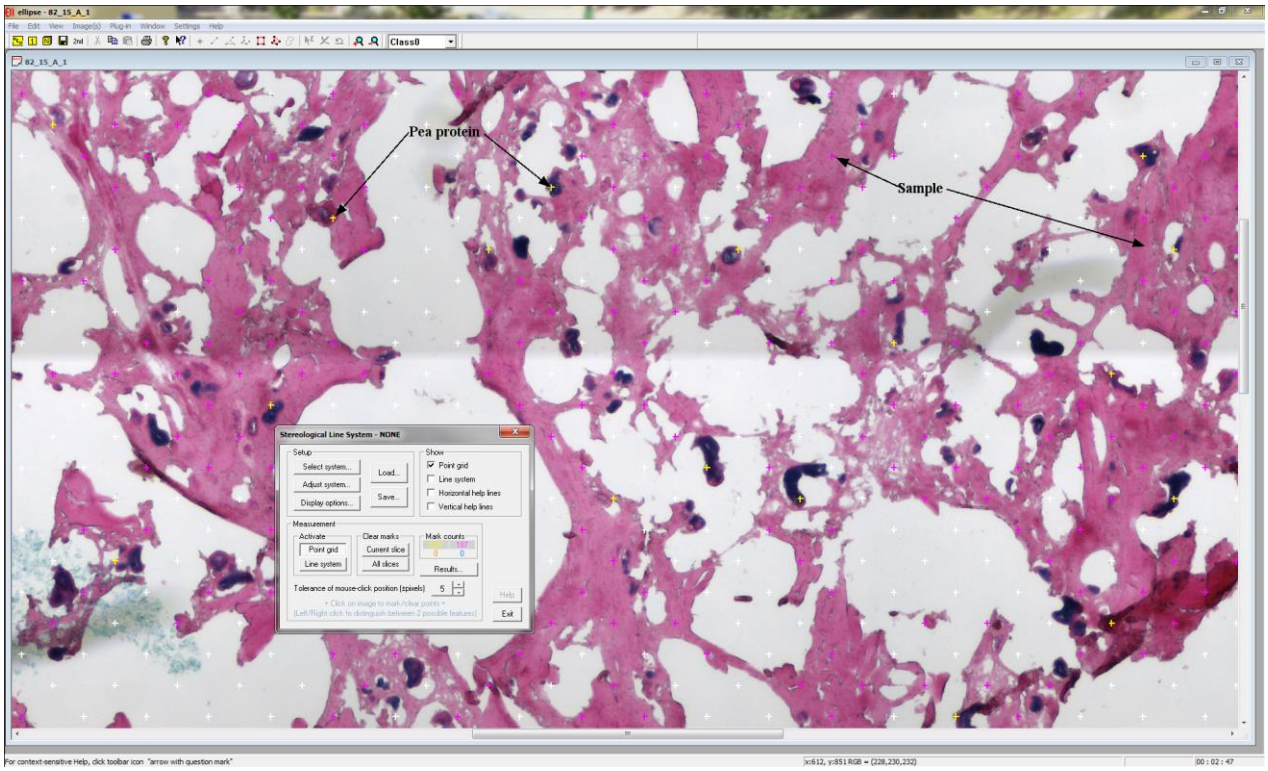

Figure 1 Example of pea protein quantification in the Ellipse software, sample no. 82_15 with 4% pea protein addition, IHC staining method, 40x magnification.

subsequently merged into a single image by the program and thus the Ellipse 1 software was able to examine 1 image (the entire section) from each sample.

Subsequently, the actual quantification of the pea protein addition was performed using Stereological Line System program by Ellipse version 2.0.7.1. (ViDiTo, Slovakia) (Figure 1) with adjusting the size of the grid point for the quantification of individual images and for the entire sections to 20745.5 μm^2 (a total of 157 points in the image) and 20764.8 μm^2 (a total of 7616 points in the image), respectively.

Results obtained by the stereological method of microimages of model meat product sections were contrasted to the contained values in the prepared concentrations of protein additions by means of the Spearman's correlation coefficient rSp (a nonparametric method that uses the order of values of the monitored variables in the calculation, and which can be used to describe any relation (linear and nonlinear). Relation of variables may have a generally upward or downward character **(Bedáňová and Večerek, 2007)**. The coefficients were calculated in the UNISTAT ver. 6.0 software. Moreover, a regression analysis (studying what relationship exists between the variables – linear, quadratic, logarithmic, etc. – and how a dependent variable Y changes depending on changing its predictor (independent variable) X. It is thus a one-sided dependence, unlike the correlation analysis studying bilateral reciprocal relation between two random variables was performed in MS Excel **(Bedáňová and Večerek, 2007)**.

RESULTS AND DISCUSSION

The measured areas of pea protein added for each concentration for both methods of scanning are listed in Table 1 and Figure 2. Table 1 and Figure 2 compare addition of proteins in the weight percentage and section areas in area percentages measured. The results indicate that with increasing addition of the proteins increases the measured area in section by lowest concentration (0.1 percentage).

Figure 3 illustrates a comparison of the examined areas of microscopic slides of model meat product samples when the examined area was 2.9x to 4.7x greater in the event of Ellipse 2 than the examined area Ellipse 1.

Using the first method of capturing images (Ellipse 1) by means of the Ellipse SW, a total of 60 images (10 images of a sample for each concentration) were quantified. Protein surface areas of 1.71%, 3.40%, 6.18%, 8.47%, 9.42%, and 11.26% were detected for the meat product samples with pea protein additions of 0.1%, 1.0%, 2.0%, 3.0%, 4.0%, and 5.0%, respectively. Based on the calculated Spearman's correlation coefficient, statistical dependence (rSp = 1000) was demonstrated for each concentration of pea proteins addition in model meat products.

In the latter method of capturing images (Ellipse 2), where sections were scanned whole, six images (one image of the entire section for each concentration) were examined. Protein surface areas of 0.66%, 2.82%, 4.46%, 6.09%, 7.71%, and 9.52% were measured for pea protein additions of 0.1%, 1.0%, 2.0%, 3.0%, 4.0%, and 5.0%, respectively. Statistical relation was also confirmed by the calculated Spearman's correlation coefficient (rSp = 1000), which confirm high dependence between pea protein addition and measured area of this protein in microscopic sections.

Table 1: Measured areas of pea protein for each MMP concentration.

Prepared MMP concentrations	Measured area of proteins [%] by Ellipse SW	
	Ellipse 1	Ellipse 2
0.1	1.71	0.66
1.0	3.40	2.82
2.0	6.18	4.46
3.0	8.47	6.09
4.0	9.42	7.71
5.0	11.26	9.52

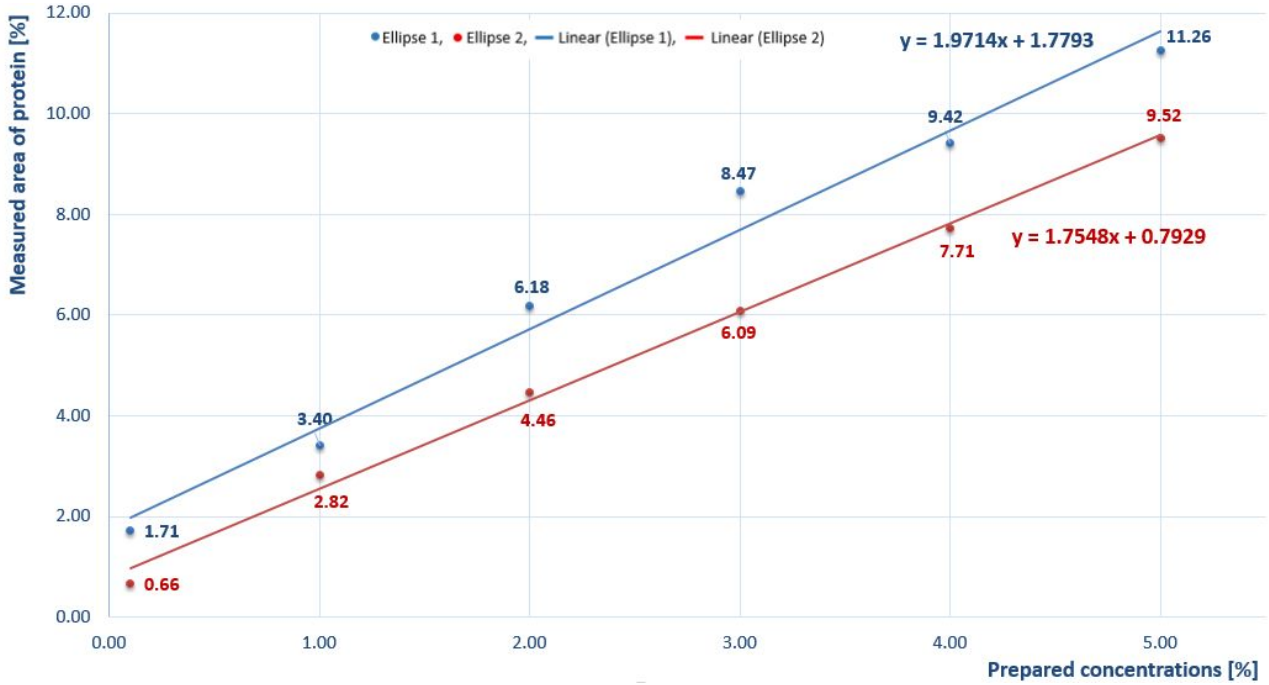

Figure 2 Dependence of protein area measured by Ellipse on the prepared concentrations.

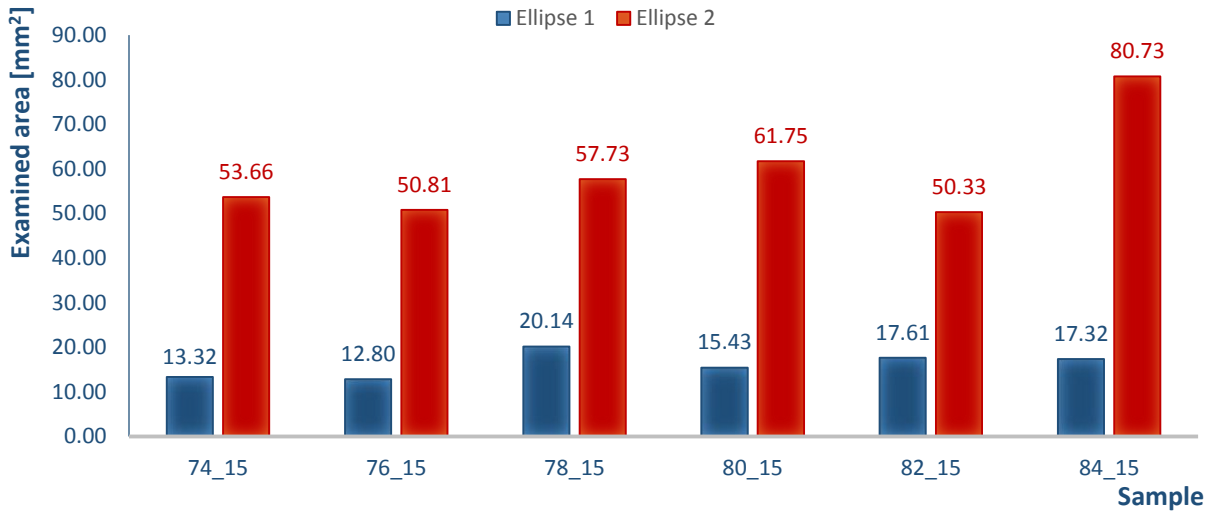

Figure 3 Comparison of areas of the examined sections.

Note: Sample number 74_15 contains 0.1% of pea protein, sample number 76_15 contains 1.0% of pea protein, sample number 78_15 contains 2.0% of pea protein, sample number 80_15 contains 3.0% of pea protein, sample number 82_15 contains 4.0% of pea protein and sample number 84_15 contains 5.0% of pea protein.

In addition to evaluating both procedures of capturing images and their results, regression analysis of the results obtained, which evaluates the dependence of quantitative statistical features, was also conducted. Obtained regression equations are shown in Figure 2. Regression equations can be used for calculation of pea protein addition based on measured area of this protein in microscopic section.

CONCLUSION

Based on the results obtained and calculated Spearman's correlation coefficients, hypothesis regarding the suitability of stereology for the quantitative determination of pea protein additions in model meat products was confirmed. As reported by **Aguilera and Stanley (1990)**, stereological quantification is more time consuming than image analysis. However, in view of incompletely 100% results of immunohistochemical staining, where the image analysis software would fail to mark the protein automatically leading to an incorrect result, this method appears to be appropriate. Also, reduction of stereological points in the grid and thus shortening the time for the quantification itself is worth considering. In case of using scans of entire section, one section would be enough for the quantification, which would also shortened the examination.

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Contact address:

Mgr. Zdeňka Javůrková, Ph.D., University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Plant Origin Foodstuffs Hygiene and Technology, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic, E-mail: javurkovaz@vfu.cz.

MVDr. Matej Pospiech, Ph.D., University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Plant Origin Foodstuffs Hygiene and Technology, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic, E-mail: mpospiech@vfu.cz.

Mgr. Michaela Petrášová, University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Plant Origin Foodstuffs Hygiene and Technology, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic, E-mail: petrasovam@vfu.cz.

doc. MVDr. Bohuslava Tremlová, Ph.D., University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Plant Origin Foodstuffs Hygiene and Technology, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic, E-mail: tremlovab@vfu.cz.

Mgr. Ludmila Luňáková, University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Plant Origin Foodstuffs Hygiene and Technology, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic, E-mail: lida.anezka.l@gmail.com.

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PHTHALATES IN MEAT PRODUCTS IN DEPENDENCE ON THE FAT CONTENT

Alžbeta Jarošová, Soňa Bogdanovičová

ABSTRACT

The content of dibutylphthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) in samples of packages intended for thermally processed meat products and release of phthalates from packages into meat products in dependence on the fat content were observed. 80 samples of packages were analyzed, 5 of them wereselected due to exceeding the specific migration limit. The raw meat was prepared, one type with the fat content of 10% and second one with the fat content of 50%. The both types of raw meat were analyzed for the content of DBP and DEHP and packed into chosen packages.The samples of meat products were thermally processed (70 ℃, 10 min in the core), stored until the expiration date at 4 °C and gradually analyzed after 1st, 7th, 14th, 21st and 28th day of storage. Determination of phthalates was carried out by high performance liquid chromatography (HPLC) in the Zorbax Eclipse C8column and by UV detection at a wavelength of 224 mm. The phthalate content in the raw meat was under the limit of detection. According to the EU Commission Regulation no. 10/2011 the specific migration limit of products intended for the contact with food for DEHP (max. 1.5 $mg.kg^{-1}$of food stimulant and DBP max. 0.3 $mg.kg^{-1}$ of food stimulant), wasexceeded already after first day of storage, in case of DBP in two samples with 10% of fat and after 7^{th} day of storage in one sample. In the samples with 50% of fat, SML was exceeded after first day of storage in four samples and in one sample after 14^{th} day of storage. Regarding DEHP in the samples with 10% of fat SML was exceeded after 1^{st} day of storage in one sample and after 7^{th} day of storage also in one sample and after 21^{st} day of storage similarly in one sample. Four samples with 50% of fat had SML exceeded in case of DEHP already after 1^{st} day of storage. By comparison of PAE migration depending on the fat content we concluded that leaching of PAE from a package into food was 2 – 21 times higher in samples with 50% of fat than in samples with 10% of fat.

Keywords: phthalates; DBP; DEHP; package; migration; fat content

INTRODUCTION

Phthalates are synthetic substances used mainly as plasticizers of polyvinyl chloride (PVC). As additives, they provide plastics with softness and flexibility. Their wide spectrum of use results in the contamination of the environment since pthalates are not firmLy bound by a covalent bond in the plastic, and can leach out, migrate or evaporate into the surrounding air, atmosphere, food or other materials. Phthalates enter the human body via ingestion, inhalation or dermal transfer throughout life, and even during intrauterine development. Due to the potential risks posed to human health and the environment, some phthalates have been added to the list of priority pollutants of the European Union. Although phthalates are not persistent substances, due to the predominance of ingestion when compared to metabolic conversion, the parent compounds and metabolites cumulate in the bodies of both animals and humans. These substances do not remain in the body for long. However, throughout their stay, they are responsible for serious health issues **(Heudorf et al. 2007)**.

Regarding the fact that phthalates (PAE) are not tightly bound in polymer matrices, they can easily migrate from products to the hydrosphere, atmosphere and biosphere during production, usage and liquidation. Regarding the wide use and resistance to microorganism they became omnipresent in the environment and they were found almost in all elements of the environment in the whole world such as soil, precipitation, surface water, sea water, underground water, sediment, biota, air, waste water and sewage sludge **(Schiedek et al., 1995, Zhang et al., 2014, Selvaraj et al., 2015, Net et al., 2015, Wang et al., 2014,Wang et al., 2002)**.

Humans are exposed to phthalates through several ways of exposition including breathing, nutrition and dermal absorption **(Das et al., 2014, Meng et al., 2014)**. The main source of exposition for a human is food, therefore various types of food were researched in various countries of the world from the perspective of phthalate contamination **(Das et al., 2014)**. Phthalates may enter into the food chain also through migration from contact materials during growing, production, storage or even during food preparation in a household.

Evaluation of the dietary intake of phthalates in the Belgian adult population revealed that there is the highest intake of DEHP, followed by di-n-butyl phthalate (DNBP), butyl benzyl phthalate (BBP) and diethyl phthalate (DEP). The groups of food, which contribute to the dietary

exposition to the stated group of phthalates include grain and grain products, milk and dairy products, meat and meat products. According to the executed research the intake of DEHP through food was found ($mg.kg^{-1}.d^{-1}$) in Great Britain, Denmark, Germany, France in the values of 3.40 - 4.00; 2.70 - 4.30; 14.0 and 1.46 respectively **(Yang et al., 2015, Fierens et al., 2014a, Ji et al., 2014, Fierens et al., 2014b)**.

Another studies for example dealt with the phthalate content in caps of beer bottles, in olive oil, in infant feeding bottles, yogurt packages and packages intended for microwave ovens. It was also found that with an increased temperature during preparation there is increasing danger of migration of phthalates from packages into food **(Gonzales-Castro et al., 2011, Li et al., 2012, Nanni et al., 2011)**.

The objective of the work was to carry out the analysis of packages used for production of thermally processed meat products. Another objective was to create products with various fat content, fill them into packages and observe if there occurs release of phthalates into a meat product after thermal processing.

MATERIAL AND METHODOLOGY

Packages (n = 80) used for meat products were supplied by a German company. They included plastic and cellulose packages with printing intended for production of thermally processed meat products. 5 packages were used for filling of the products, in which the specific migration limit was exceeded. The samples were analyzed twice. In total, 80 packages were analyzed and 160 analyses were carried out.

Meat products intended for thermal processing were produced in pilot production conditions at the Department of Food Technology at Mendel University in Brno. Two types of raw meat were prepared (the fat content 10% and 50%), which were filled into the packages (n = 5). For every fat content there were 30 samples produced. The samples were stored at 4 °C and they were taken for analysis after 1st, 7th, 14th, 21st and 28th day of storage. The samples were analyzed twice.

In total, there were 12 samples of raw meat analyzed, 300 samples of meat product and there were 624 analyses carried out.

The package samples were leached in the mixture of dissolvent *n*-hexan:dichlormethan (1:1) for 72 hours and subsequently three times extracted (60, 30, 30 minutes). The combined extracts were filtered, evaporated on a rotary vacuum evaporator and dried by nitrogen. Afterwards, the extract was transferred into vials and centrifuged by hexane (5 mL). The top part of the extract (1.5 mL) was isolated into a vial for determination by HPLC (high-performance liquid chromatography) and dried by nitrogen. Vials were again centrifuged, the top part of the extract was isolated (1.5 mL), dried by nitrogen and subsequently the vials were filled up to the volume of 1 mL. If the extracts were colored or bleary, they were cleared by sulfuric acid.

The verified methods for determination of DBP and DEHP in food were used for the analysis of PAE in the samples of raw meat and meat products **(Jarošová et al., 1998, 1999).**

The samples of raw meat and meat products were homogenized, weighed on metal bowls and frozen. Gradually, the frozen samples were lyophilized and subsequently residues of PAE were extracted by n-hexane. PAE was separated from the co-extracts by gel permeation chromatography on the gel Bio beads S-X3. The cleaning procedure with concentrated sulfuric acid was used for completion of cleaning of eluates. Phthalates were determined by the HPLC method with column of Zorbax Eclipse C8 and UV detection at a wavelength of 224 mm. The injection of samples on the column was 10 µl. The final concentrations were calculated based on the calibration curve by the software Agilent Chemstationfor LC and LC/MS systems. The scope of the calibration curves for DBP was from 1.06 $\mu g.mL^{-1}$to106.00 $\mu g.mL^{-1}$ and for DEHP from 1.01 $\mu g.mL^{-1}$ to 100.50 $\mu g.mL^{-1}$. The correlation coefficient for DBP was 0.9999 and for DEHP also 0.9999. The detection limit for DBP was 0.05 $\mu g.mL^{-1}$ and for DEHP 0.11 $\mu g.mL^{-1}$. The results were statistically processed by the program Statistica 12.

The majority of lab glass was flushed by hexane during preparation of samples. At the same time the dry matter and fat content were determined. Concentrations of DEHP and DBP are related to an initial sample.

RESULTS AND DISCUSSION

The concentration of phthalates in the analyzed packages are expressed in$\mu g.dm^2$ and they are stated in Table 1. Every value represents an average from two parallel determinations.

According to the EU Commission Regulation No. 10/2011 for products intended for the contact with food and dishes, a package cannot release its own components into food in the quantity exceeding10 $mg.dm^2$ or 60 $mg.kg^{-1}$ of food or food stimulant. The stated regulation includes also the specific migration limit which is max. 1.5 $mg.kg^{-1}$ of food stimulant for DEHP and max. 0.3 $mg.kg^{-1}$ of food stimulant for DBP.

Tab. 1 Average concentration of DBP and DEHP ($\mu g.dm^{-2}$) in samples of packages used of packing of meat products

Sample	DBP	DEHP
	$\mu g.dm^{-2}$	
1	21.55	95.45
2	14.12	64.75
3	18.35	88.12
4	39.13	134.97
5	27.43	108.61

From all the analyzed packages (n = 80) there were five packages selected, in which the highest concentration (Table 2) of the both phthalates occurred and there was the assumption that they may contaminate final products after thermal processing and storage. The values of DBP in packages moved from 14.12 to 39.13 $\mu g.dm^{-2}$ and in case of DEHP from 64.75 to 134.97 $\mu g.dm^{-2}$.

The phthalate concentrations (DBP and DEHP) in the raw meat and meat products are expressed in $mg.kg^{-1}$ of the initial sample and they are stated in Table 2. Every value represents an average of 12 values (6 parallel samples and every sample analyzed twice).

In the samples separated immediately after mixingthe raw meat, the concentration of phthalates moved under the limit of detection (≤ 0.2 $mg.kg^{-1}$).

In the first sample of product with 10% of fat, DBP gradually increased from the value 0.21 (after first day of storage) up to 0.68 $mg.kg^{-1}$of the initial sample. In the sample with 50% of fat it reached the values from 3.64 up to 4.7 $mg.kg^{-1}$ of the initial sample. Based on the measured values we can state that at the end of expiration period (28th day) the DBP content was approximately 7x higher in products with 50% of fat than in products with 10% of fat. DEHP reached in the sample with 10% of fat values from 2.19 to 3.57 $mg.kg^{-1}$ of the initial sample and in the sample with 50% of fat from 4.96 to 6.48 $mg.kg^{-1}$ of the initial sample. At the end of the expiration period, there was 2x higher content of DEHP in the product with 50% of fat than in the product with 10% of fat.

In the second sample of product with 10% of fat there was DBP detected only at the end of the expiration period (0.22 $mg.kg^{-1}$ of the initial sample), but it did not exceed SML (0.3 $mg.kg^{-1}$). In the sample with 50% of fat, DBP reached values from 1.18 to 3.61 $mg.kg^{-1}$ of the initial sample. At the end of the expiration period there was 16x higher content of DBP in the product with 50% of fat than in the product with 10% of fat. DEHP in samples with 10% of fat was, similarly as in case of DBP, detected only at the end of the expiration period (0.40 $mg.kg^{-1}$ of the initial sample) but it did not exceed SML (1.5 $mg.kg^{-1}$). In the sample with 50% of fat its content ranged from 4.81 to 8.34 $mg.kg^{-1}$ of the initial sample. At the end of the expiration period, there was 21x higher content of DEHP in the product with 50% of fat than in the product with 10% of fat.

In the third sample of product, DBP was not detected in any sample of both fat contents (10% and 50%) during the whole time of expiration period. DEHP was detected in the sample with 10% of fat only at the end of the expiration period (0.20 $mg.kg^{-1}$ of the initial sample) but it did not exceed SML (1.5 $mg.kg^{-1}$). In the sample with 50% of fat its content ranged from 1.26 to 2.5 $mg.kg^{-1}$ of the initial sample, but it exceeded SML only after 7th day of storage. At the end of expiration period, there was 13x higher content of DEHP in the product with 50% of fat than in the product with 10% of fat.

In the fourth sample of product with 10% of fat DBP gradually increased from the value 0.72 (after first day of storage) up to 1.30 $mg.kg^{-1}$ of the initial sample. In the sample with 50% of fat it reached values from 0.85 up to 1.63 $mg.kg^{-1}$ of the initial sample. At the end of the expiration period (28th day), the content of DBP in both products (10% and 50%) did not differ too much. DEHP in the sample with 10% of fat reached values from 0.77 to 2.01 $mg.kg^{-1}$ of the initial sample and in the sample with 50% of fat from 3.87 to 11.67 $mg.kg^{-1}$ of the initial sample. At the end of the expiration period (28th day) there was almost 6x higher content of DEHP in the product with 50% of fat than in the product with 10% of fat.

In the fifth sample of product with 10% of fat, DBP gradually increased from the value of 0.68 (after first day of storage) up to 1.19 $mg.kg^{-1}$of the initial sample. In the sample with 50% of fat it reached values from 3.60 up to 7.95 $mg.kg^{-1}$of the initial sample. Based on the measured values we can state that at the end of expiration period (28th day) there was 7x higher content of DBP in the product with 50% of fat than in the product with 10% of fat. DEHP in the sample with 10% of fat reached values from 1.44 to 2.89 $mg.kg^{-1}$of the initial sample and in the sample with 50% of fat from 7.12 to 8.54 $mg.kg^{-1}$of the initial sample. At the end of expiration period there was 3x higher content of DEHP in the product with 50% of fat than in the product with 10% of fat. There was confirmed highly statistically significant evidence of migration of DBP and DEHP depending on the fat content (p=0,000**) and depending on the period of storage (p=0,000**).

According to the EU Commission Regulation No. 10/2011 there was exceeded the specific migration limit already after 1st day of storage in case of DBP in two samples with 10% of fat (4 and 5) and after 7th day of storage in one sample (1). In samples with 50% of fat there was SML exceed already after first day of storage in four samples (1, 2, 4, 5) and in one sample (3) after 14th day of storage.

In case of DEHP in the sample with 10% of fat there was SML exceeded after 1st day of storage in one sample (1), after 7th day of storage also in one sample (5) and after 21st day of storage in one sample (4). The samples (1, 2, 4, 5) with 50% of fat in case of DEHP exceeded SML already after 1st day of storage.

Based on the results (Table 1) we can state that DBP content in packages contributed by 20 % and in case of DEHP by 80% to the overall content of PAE. This finding corresponds with results of cumulation in meat products, higher concentration in meat products were found in case of DEHP (Table 2). By the comparison of PAE depending on the fat content we concluded that leaching of PAE was 2 – 21 times higher in samples with 50% of fat than in samples with 10% of fat.

Our experiment pointed at the fact that the content of plasticizers leached from packages increases with temperature, period of storage and fat content and this finding corresponds also with results of another authors. There was found increasing average concentration of DEHP in pasteurized skimmed milk (20 $\mu g.kg^{-1}$), in comparison to full fat milk (35 $\mu g.kg^{-1}$) and cream (1400 $\mu g.kg^{-1}$) **(Castle et al., 1990)**. Values of DEHP correlating with the fat content in milk were also confirmed in the study of Sharman et al. (1994). In milk with up to 1% of fat there was detected DEHP in the range of 0.02 – 0.04 $mg.kg^{-1}$, 0.05 $mg.kg^{-1}$of DEHP in milk with 1% of fat, 0.10 – 0.38 $mg.kg^{-1}$ in milk with 3% of fat and 1.06 – 1.67 $mg.kg^{-1}$ in cream with 35% of fat **(Sharman et al., 1994)**. **Shuangling and Kangquan (2009)** found that migration of DEHP from a PVC foil into meat increased with rising temperature and time.

The maximum migration was recorded at 90 ˚C and 30 minutes of effecting (75.12 mg.dm^{-2}).
The overall migration limit (10 mg.dm^{-2}) was exceeded in all observed combinations of time and temperature, except for the combination of 10 ˚C and <41 hours when migration was not observed.

Barros et al. (2011) in his study executed the analysis of food that might be contaminated by DEHP and di-etylhexyladipátem (DEHA). There were observed 18 different types of food with less than 3% of fat with possibility to be packed in plastic foils. The study proved that all food was contaminated by DEHP and DEHA while the content of observed phthalates was increasing with the period of storage.

Guo et al. (2010) proved a decreasing tendency in DEHP content with increasing distance from the surface. The authors monitored the migration of DEHP from the packaging film into ham sausages with relatively low fat content. The DEHP content in the sausages dropped significantly as the distance from the surface increased. The DEHP concentration was 8.7 mg.g^{-1} in the packaging film and 206.5 ng.g^{-1} in the first outer layer of the sausage. The first and second layer contained approximately 90 % of the total DEHP amount which migrated from the packaging. Significant levels of DEHP in the inner layers of the sausages were detected only after six months of storage.

A study by **Wang et al. (2015)** investigated the presence of phthalates in greenhouse soils and vegetables. Wang et al. monitored dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate (DnBP), butyl benzyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DnOP) content which was analysed in 44 vegetables grown in greenhouses made of plastic film and in the corresponding soil. The total phthalate content ranged from 0.51 to 7.16 mg.kg^{-1} in vegetables and from 0.4 to 6.20 mg.kg^{-1} in soils with an average concentration of 2.56 and 2.23 mg.kg^{-1}. DnBP, DEHP and DnOP contributed to the overall phthalate content in vegetables and soils in more than 90%, but the ratios of DnOP and DnBP in vegetables were significantly (p <0.05) higher than in soils. The average concentration of phthalates in mustard, celery and lettuce was >3.00 mg.kg^{-1} but <2.50 mg.kg^{-1} in the corresponding soil. Stems and leaves of the vegetables accumulated larger amounts of phthalates. No mutual relationship was detected between the phthalate content in vegetables and in the soils.

Tsumara et al. (2001) observed the content of phthalates in ten samples of lunch half-products packed in plastic packages. The quantity of DEHP in the samples moved from 45 to 517 ng.g^{-1}, with the average value of 198 ng.g^{-1}. DBP was not detected in any sample.

The main source of phthalates in food, especially in those with a high fat content, is their direct contact with surfaces of production equipment and package materials. **Tsumara et al. (2001)** demonstrated increase of DEHP in chicken. From the initial value 0.080 mg.kg^{-1} before thermal processing the content of DEHP increased to 13.10 mg.kg^{-1}after frying at a nonstick pan and further to 16.90 mg.kg^{-1} after packaging.

In a study by **Moreira et al. (2015)**, the content of 8 plasticisers in spices and in roast chicken meat stored in plastic bags was monitored. The values detected ranged between 0.01 and 0.18 g.kg^{-1}. The samples showed presence of diisobutyl phthalate and dibutyl phthalate. The highest concentration of plasticisers was detected in spice used for roasting chicken meat.

A study by **Wang et al. (2013)** discussed the migration behaviour of 9 phthalate plasticizers in food with higher fat content, and the influence of temperature on the migration amount of these substances. The studied substances were: dimethyl phthalate (DMP), diethyl phthalate (DEP), diallyl phthalate (DAP), diisobutyl phthalate (DIBP), dibutyl phthalate (DBP), benzylbutyl phthalate (BBP), bis(2-ethylhexyl) phthalate (DEHP), diisononyl ortho-phthalate (DINP) and diisodecyl ortho-phthalate (DIDP). The results have shown that the thickness of the plastic film is an essential factor in the process of phthalate migration. Another important condition in the study of the migration behaviour was

Table 2 Average concentrations of DBP and DEHP (mg.kg^{-1}) in the samples of raw meat and meat products (n = 5) with the fat content of 10 % and 50 % after 1st, 7th, 14th, 21st and 28th days of storage at 4 °C.

Sample	FAT (%)	Product		1st day		7th day		14th day		21st day		28th day	
		DBP	DEHP	DBP	DEHP	DBP	DEHP	DBP	DEHP	DBP	DEHP	DBP	DEHP
1	10	ND		0.21	2.19	0.45	3.27	0.45	3.39	0.47	3.42	0.68	3.57
	50			3.64	4.96	3.86	5.02	4.64	6.33	4.66	6.4	4.7	6.48
2	10			ND	ND	ND	ND	ND	ND	ND	ND	0.22	0.4
	50			1.18	4.81	2.37	6.45	2.42	6.65	3.08	8.21	3.61	8.34
3	10			ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	50			ND	1.26	ND	1.66	ND	1.8	ND	1.84	ND	2.5
4	10			0.72	0.77	0.8	0.98	0.83	1	1.22	1.75	1.3	2.01
	50			0.85	3.87	1.08	8.53	1.17	9.55	1.56	11.3	1.63	11.67
5	10			0.68	1.44	0.69	1.47	0.72	1.5	0.91	2.34	1.19	2.89
	50			3.6	7.12	4.92	7.94	6.31	8.04	6.68	8.5	7.95	8.54

Note: ND – the limit of detection of DBP and DEHP in fat matrices – 0.2 mg.kg^{-1}.

temperature. Measurements have proven that higher temperature accelerates the transfer and the migration of phthalate plasticisers increases. Each of the studied substances was affected differently by the increasing temperature. For instance, DINP and DIDP were affected minimally, since equilibrium was established and increasing the temperature did not change the migration amount. The migration mount measured in the temperature range of 5°C to 70°C ranged between 80 and 350 $mg.kg^{-1}$ for DMP, 75 to 375 $mg.kg^{-1}$ for DEP, 75 to 350 $mg.kg^{-1}$ for DAP, 50 to 350 $mg.kg^{-1}$ for DIBP, 75 to 325 for DBP $mg.kg^{-1}$, 100 to 275 $mg.kg^{-1}$ for BBP and 110 to 170 $mg.kg^{-1}$ for DEHP. The migration amount for DINP and DIDP reached equilibrium. This equilibrium migration amount for DINP was 140 $mg.kg^{-1}$ and for DIDP 160 $mg.kg^{-1}$. The migration values of phthalate plasticisers differ.

In dairy products, more than 80% of the total concentration of phthalates ranging from 50 to 200 $\mu g.kg^{-1}$ in ordinary milk came from suction machines. Further processing and packaging may lead to increase of the DEHP concentration in cream and cheese products **(Casajuan a Lacorte, 2004).**

CONCLUSION

The objective of the work was to observe the content of phthalates (DBP and DEHP) in packages intended for packing of meat products and observation of potential migration of phthalates from packages into products after thermal processing and storage until the expiration time depending on the fat content.

According to the EU Commission Regulation no. 10/2011 in comparison to the specific migration limits for DBP (0.3 $mg.kg^{-1}$) and DEHP (1.5 $mg.kg^{-1}$), 3 samples of packages stated in Table 1 (1, 4, 5) would not meet the regulation with respect to the specific migration limit (Table 2) after 1st day of storage. The samples of packages 2 and 3 would be suitable during the whole time of storage if it would be filled with theraw meat with the fat content of 10% (Table 2).

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Contact address:

Alžbeta Jarošová, Mendel University, Faculty of Agronomy, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: alzbeta.jarosova@mendelu.cz.

Soňa Bogdanovičová, Mendel University, Faculty of Agronomy, Department of Food Technology, Zemědělská 1, 613 00 Brno, Czech Republic, E-mail: sona.bogdanovicova@mendelu.cz.

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SWEET POTATO (*IPOMOEA BATATAS* L.) GROWING IN CONDITIONS OF SOUTHERN SLOVAK REPUBLIC

Miroslav Šlosár, Ivana Mezeyová, Alžbeta Hegedűsová, Marcel Golian

ABSTRACT

The sweet potato (*Ipomoea batatas* L.) belongs to very important crops from aspect of its world production. It is grown in large areas in Asia, on the contrary, sweet potato production in Europe presents minimal part of its total world rate. The sweet potato is less-known crop, grown only on small area in home gardens in Slovak Republic. Tubers of sweet potato are characterized by anti-diabetic, anti-oxidant and anti-proliferative properties due to the presence of valuable health-promoting components, such as carotenoids or vitamin C. The main objective of study was testing of sweet potato growing in conditions of southern Slovak Republic with focus on quantity and quality of its yield. The field trial was realised on land of the Slovak University of Agriculture in Nitra in 2015. Within trial, effect of cultivar and mulching on the selected quantitative (average tuber weight; yield per plant; yield in $t.ha^{-1}$) and qualitative (total carotenoids; vitamin C) parameters were tested. One certified cultivar of sweet potato 'Beauregard' was used as a comparative cultivar. Other two cultivars were marked according to the market place at which were purchased and sequentially used for seedling preparation. Tubers of first un-known cultivar were purchased in the Serbian market (marked as 'Serbian'). Tubers of next sweet potato cultivar were purchased on the market in Zagreb (marked as 'Zagrebian'). Outplating of sweet potato seedlings were realised on the 19th May 2015. The sweet potato was grown by hillock system. Each cultivar was planted in two variants (rows): non-mulching (bare soil) and mulching by black non-woven textile. All variants were divided to three replications with 6 plants. Difference between rows was 1.20 m and seedlings were planted in distance of 0.30 m in row. The harvested tubers were classified in two size classes: >150 g (marketable yield) and <150 g (non-marketable yield). Total carotenoid content was determined spectrophotometrically. The vitamin C content was measured chromatographically (HPLC). The highest values of average tuber weight, yield per plant and total yield ($t.ha^{-1}$) were found in cultivar 'Serbian'. Statistical analysis showed statistically significant difference in all yield quantitative parameters of cultivar 'Serbian' against cultivars 'Beauregard' and 'Zagrebian'. The highest content of total carotenoids was determined in cultivar 'Serbian' (99.52 $mg.kg^{-1}$ fresh weight) with orange-creme flesh color, followed by cultivar 'Beauregard' (94.78 $mg.kg^{-1}$) with orange flesh color and cultivar 'Zagrebian' (28.79 $mg.kg^{-1}$) with yellow-creme flesh color. Differences among all cultivars were showed as statistically significant. The highest vitamin C content was detected in tubers of cultivar 'Serbian' (155.70 $mg.kg^{-1}$), followed by cultivar 'Beauregard' (154.37 $mg.kg^{-1}$) and cultivar 'Zagrebian' (146.33 $mg.kg^{-1}$). Statistical analysis confirmed differences among cultivars as statistically non-significant. The mulching of sweet potato plants had statistically significant impact to all quantitative and qualitative characteristics of sweet potato. The application of black non-woven textile resulted in increase of average tuber weight, tuber yield and vitamin C content in sweet potato tubers. On the contrary, higher total carotenoid content was found in non-mulching variant compared to the variant with mulching.

Keywords: Slovak Republic; sweet potato; yield; carotenoids; vitamin C

INTRODUCTION

The sweet potato (*Ipomea batatas* L.) belongs to the *Convolvulaceae* family and it is original from South America. Due to Christopher Columbus, it was imported to the Europe about century earlier than classical potatoes - *Solanum tuberosum* L. **(Valíček et al., 2002)**. According to **FAOSTAT (2016)**, total world production of sweet potato tubers was more than 100 milions tones in 2014. The main production area was Asia (75.3 %), followed by Africa (20.2 %), American continents (3.7 %) and Oceania (0.8 %). Sweet potato production in Europe presented the least part of its total world value (0.1 %) and the European production was only 56 113 tones in 2014. The main European producers of sweet potatoes was Spain and Italy. From world-wide aspect, China is the main producer of sweet potatoes within recent period. The production of this commodity was more than 70 milions tones in 2014. The other important producers was Nigeria, Tanzania, Ethiopia or Indonesia. **Šlosár (2016)** state that sweet potatoes are less-known crop, grown only on small area in home local gardens in Slovak republic.

The sweet potato, known as batatas, is well known long-term species in a warm and hot climate zone and an annual plant (spring) in temperate zone. It produces moist and

delicate tubers with a sweetish taste, pleasant and aromatic smell. In addition, young leaves can also be used for consumption **(Antonio et al., 2011)**. Tubers are characterized by diverse size, shape (round, ovate, elliptic etc.), skin and flesh color (white, cream, yellow, orange, red or purple), depending on a cultivar **(Moulin et al., 2012)**.

The main nutritional compounds in tubers of sweet potato are carbohydrates (simple sugars and starches), proteins, fats and fat-soluble vitamins **(Allen et al., 2012)**. The glycemic index of sweet potatoes is quite high, thus, it is unsuitable for diabetics and overweight persons. Total carbohydrate content of this crop is 201 g. kg^{-1} of fresh weight (f. w.); starch content is 160 $g.kg^{-1}$ and soluble sugar content is 42 $g.kg^{-1}$ f. w. The proteins and fats are contained in sweet potatoes in small quantities **(Maria and Rodica, 2015)**. According to **USDA (2015)**, energetical value of fresh sweet potato is 359 kJ per 100 g. From mineral complex, potassium (337 $mg.100\ g^{-1}$), sodium (55 mg) and phosphorus (47 mg) are the most abundant in sweet potato tubers.

Tubers of sweet potato are characterized by anti-diabetic, anti-oxidant and anti-proliferative properties due to the presence of valuable nutritional and mineral components **(Abubakar et al., 2010)**. Sweet potato cultivars with an orange or yellow flesh contain significant amounts of carotenoids which are known as provitamins A **(Allen et al., 2012)**. Carotenoids show strong antioxidant capacity to scavenge free radicals because of their conjugated double bonds **(Fu et al., 2011)**. **Lichtenstein (2009)** indicates that carotenoids or their metabolites are associated with cardiovascular diseases. According to **Rao and Rao (2007)**, higher carotenoid intake in the food form helps to decrease of several cancer type risk (stomach, colon or larynx) and prevent to bone calcification, eye degeneration and neurotic diseases.

The vitamin C, also known as ascorbic acid, is another important substances within vitamin complex in sweet potato tubers **(USDA, 2015)**. Due its properties, vitamin C is characterized as very effective antioxidant. The human organism is not able to synthesize vitamin C, thus, it must be ingested in the food form, mainly vegetables and fruits **(Keresteš et al., 2011)**. The vitamin C plays an important role in immune system, stimulation of leucocytes to the increased bacteria degradation, secretion of antibodies and body resistance increase to the coldness **(Hacişevki 2009)**. According to **Feiz and Mobarhan (2002)**, sufficient vitamin C intake helps to eliminate *Helicobacter pylori* bacteria considered as important risk factor in stomach cancer formation. **Iqbal, Khan and Khan Khattak (2004)** state that vitamin C contributes to prevent human organism by elimination of nitrosamine formation which descend from nitrates contained in many food sources.

The main objective of present study was testing of sweet potato growing in conditions of southern Slovak Republic with focus on selected quantitative and qualitative parameters of its yield.

MATERIAL AND METHODOLOGY

The field trial with sweet potato was realised on the land of the Slovak University of Agriculture in Nitra in 2015. The experimental area is situated at an absolute altitude of 144 m above sea level. The climate of experimental area is characterized by warm and dry summer and slightly warm, dry or very dry winter. According to the climatic normal 1951-2000 for Nitra, annual mean temperature is 9.9 °C and mean rainfall total is 548 mm **(Šlosár and Uher, 2013)**. Within trial year 2015, the average month air temperature was 11.5 °C. The rainfall total was 418.2 mm in 2015.

Plant material

Sweet potato seedlings were purchased from Croatian producer (Ing. Darko Ďurica, Ilok). According to him, the situation with cultivar sortiment of sweet potato in Europe is often unclear and confusing. A lot of producers, including him, produces seedlings according to the tuber availability on the market. Thus, the origin of sweet potato seedlings on market is often un-known.

Within trial, one certified cultivar of sweet potato 'Beauregard' was used as a comparative cultivar. Other two cultivars were marked according to the market place at which were purchased and sequentially used for seedling preparation. Tubers of first un-known cultivar were purchased in the Serbian market. In this study, cultivar is marked as 'Serbian'. Tubers of next cultivar of sweet potato were purchased on the market in Zagreb (Croatia). Within study, the cultivar is marked as 'Zagrebian'.

Experiment organisation

The sweet potato is warm-requiring crop. It needs warm season lasting at least four month with an average temperature more than 20°C and without freeze **(Antonio et al., 2011)**. From this reason, outplanting of sweet potato seedlings was realised on the 19th May 2015 when the risk of later spring freeze is reduced.

Within soil preparation for sweet potato growing, nitrogen was only applied on the soil supply level of 60 $kg.ha^{-1}$ according to results of agrochemical soil analysis (tab. 1). The sweet potato plants were grown by hillock system, similar to the carrot growing (height of 0.30 m). The distance between hillock rows was 1.20 m. In each row, 18 sweet potato seedlings were planted in distance of 0.30 m. Rows for all tested cultivars and variant were divided to three replications with 6 sweet potato plants.

Within experiment, two variants for each cultivar were tested:

- non-mulching - bare soil (one row),

Table 1 Agrochemical soil characteristics before trial realisation.

pH_{KCl}	Humus (%)	Nutrient content ($mg.kg^{-1}$ of soil)					
		N_{min}*	P	K	Ca	Mg	S
7.16	3.25	19.1	245	149.5	6340	643.5	7.5

Note: *N_{min} - N mineral (N inorganic).

- mulching by black non-woven textile (one row).

The harvest of sweet potato tubers was realised on the 6th October 2015.

Morphological characteristics of cultivar

Selected morphological parameters of tubers for each cultivars were evaluated in order to its more accurate characteristics (table 2). It was realised by using of relevant international descriptor for sweet potato - *Ipomoea batatas* L. **(UPOV, 2010)**. The evaluation of morphological characteristics was done in 20 tubers of each cultivar. Following parameters of tubers were evaluated:

- shape,
- main color of skin,
- secondary color of skin,
- main color of flesh,
- intensity of main color flesh,
- secondary color of flesh,
- depth of eyes.

Quantitative parameters of sweet potato

Harvested tubers of sweet potato were classified according to average weight of tubers in two size classes:

- >150 g - marketable yield of tubers,
- <150 g - non-marketable yield of tubers.

Within experiment, average weight of tubers (g) and average yield quantity per plant (g) were evaluated. The sweet potato yield in $t.ha^{-1}$ was calculated on the basis of average plant yield. The density of plants, used for calculation, was 27 000 seedlings per hectare with using the same plant spacing as it was in realised experiment.

Total carotenoid content estimation

The estimation of total carotenoid content was realised in the laboratory of Department of Vegetable Production SUA in Nitra. The content of total carotenoids was estimated by spectrophotometric measurement of substances absorbance in petroleum ether extract on spectrophotometer PHARO 100 at 445 nm wavelengths. As an extraction reagent, acetone was used acetone **(Hegedüsová et al., 2007)**.

Vitamin C content estimation

The estimation of total carotenoid content was realised in the certified laboratory of Regional Public Health Authority in Nitra. HPLC method of vitamin C content estimation **(Stan, Soran and Marutoiu, 2014)** was used by the help of liquid chromatograph with UV detector, for separation was used RP C18 column, mobile phase was methanol : water (5:95, v/v), UV detection was adjusted to 258 nm (HPLC fy. VARIAN).

Statistical analysis

A statistical analysis was performed using Statgraphic Centurion XVII (StatPoint Inc. USA). Obtained results were evaluated by analysis of variance (ANOVA) and average values were tested by Tukey HSD test performed at the significance level of 95%.

RESULTS AND DISCUSSION

Average weight of sweet potato tubers

From aspect of marketable yield, the statistical analysis showed statistically significant differences of average tuber weight (AW) among cultivar 'Serbian' and cultivars 'Beauregard' and 'Zagrebian' (tab. 3). Difference between cultivars 'Beauregard' and 'Zagrebian' was evaluated as statistically non-significant. From aspect of marketable yield part (tuber > 150 g), values of AW were varied from 332.73 g ('Zagrebian') to 428.15 g ('Serbian'). Values in this range were similar to results in study of **Maria and Rodica (2015)** who found variability of AW from 210 g to 400 g in experiment in Romania. Similarly, **Ellong, Billard and Adenet (2014)** also found higher AW (308.91-647.75 g) in Martinique compared to our results. Thus, it is evident that locality for sweet potato growing expressively affects the awerage tuber weight, one of the

Figure 1 Sweet potato cultivars in realised treatment.

most important parameters of sweet potato from aspect of its total production.

Regarding to non-marketable part of sweet potato yield (<150 g), AW was ranged from 36.00 g ('Serbian') to 58.94 g ('Beauregard'). Statistical analysis of results showed statistically significant differences of its values among all tested cultivars.

Yield of sweet potato tubers per plant

Values of yield/plant (marketable yield) were increasing from 1185.62 g 'Zagrebian' to 1455.54 g 'Serbian'. Difference between mentioned values was evaluated as statistically significant. On the contrary, statistical analysis showed statistically non-significant difference between cultivars 'Beauregard' and 'Zagrebian'. **Yildirim, Tokuşoğlu and Öztürk (2011)** tested the impact of genotype on the yield of sweet potato per plant (13 genotypes) in Turkey. Its values, found by authors, were ranged from 210.5 g 621.8 g. It means markedly lower range of values compared to our trial results. The lower yield of sweet potato per plant (380-460 g) was also presented in study of **Uwah et al. (2013)**. On the contrary, **Maria and Rodica (2015)** reached comparable tuber yield per plant (1071-1600 g) to obtained trial results.

The yield of non-marketable tubers per plant was varied from 106.07 g ('Zagrebian') to 241.96 g ('Beauregard'). According to the statistical analysis, statistically significant differences between cultivars 'Beauregard' and 'Serbian'/'Zagrebian' were found.

Yield of marketable sweet potato tubers per hectare

The total marketable yield of sweet potato, in dependency on cultivar, was ranged from 32.01 $t.ha^{-1}$ ('Zagrebian') to 39.30 $t.ha^{-1}$ ('Serbian'). Statistical analysis confirmed statistically significant difference of yield between mentioned cultivars, similarly as between 'Beauregard' and 'Serbian'. Yield difference between cultivars 'Beauregard' and 'Zagrebian' was evaluated as statistically significant. **Maria and Rodica (2015)** found expressively higher yield in sweet potato cultivar 'Pumpkin' (53.3 $t.ha^{-1}$) compared to our results. On the contrary, total yield of cultivar 'Chestnut' (35.6 $t.ha^{-1}$) was comparable to results in our study. Comparable values of sweet potato yield were presented by **Jian-wei et al.**

Table 2 Evaluated morphological characteristics of sweet potato tubers.

Tuber parameters	Beauregard	Serbian	Zagrebian
Shape	oblong	ovate	ovate
Main color of skin	brownish orange	purple red	medium purple
Secondary color of skin	pink	orange	orange
Main color of flesh	orange	orange	yellow-creme
Main flesh color intensity	medium	medium	light
Secondary color of flesh	absent	beige	orange
Depth of eyes	shallow	medium	shallow

Table 3 Effect of cultivar on quantitative parameters of sweet potato yield.

Cultivar	Marketable tubers (>150 g)			Non-marketable tubers (<150 g)		Ratio of marketable tubers
	AW* (g)	Yield/plant (g)	Yield ($t.ha^{-1}$)	AW* (g)	Yield/plant (g)	(%)
Beauregard	348.54^{a}	1213.34^{a}	32.76^{a}	58.94^{c}	241.96^{b}	81.58^{a}
Serbian	428.15^{b}	1455.54^{b}	39.30^{b}	36.00^{a}	116.63^{a}	92.82^{b}
Zagrebian	332.73^{a}	1185.62^{a}	32.01^{a}	48.36^{b}	106.07^{a}	91.31^{b}

Note: * AW - average weight of sweet potato tubers.
Different letters (a; b; c) within the same column means statistically significant difference (at 95.0 % confidence level).

Table 4 Effect of mulching variant on quantitative parameters of sweet potato yield.

Cultivar	Marketable tubers (>150 g)			Non-marketable tubers (<150 g)		Marketable share of tubers
	AW* (g)	Yield/plant (g)	Yield ($t.ha^{-1}$)	AW* (g)	Yield/plant (g)	(%)
Non-mulching	232.24^{a}	981.05^{a}	26.49^{a}	52.03^{b}	164.42^{a}	85.46^{a}
Mulching	351.76^{b}	1588.62^{b}	42.89^{b}	43.51^{a}	145.53^{b}	91.68^{b}

Note: * AW - average weight of sweet potato tubers.
Different letters (a; b; c) within the same column means statistically significant difference (at 95.0 % confidence level).

(2001) who grown this crop in nine localities in China. The average yield of sweet potatoes, found in Chinese study, was 36.7 $t.ha^{-1}$. The marketable yield of sweet potato cultivar 'Beauregard' (22.5 – 36.8 $t.ha^{-1}$ in depdendency on vegetation period) presented by **Bonte and Wilson (2008)** was also comparable to the yield of this cultivar found in our trial. Within study of **Uwah et al. (2013)**, total yield of sweet potato was varied from 20.8 $t.ha^{-1}$ to 25.5 $t.ha^{-1}$ in dependence on the cultivar and experimental year. The markedly lower yield of sweet potato tubers (3.4 –14.4 $t.ha^{-1}$), compared to obtained results, was reached within study of **Yildirim, Tokuşoğlu and Öztürk (2011)**. The lower yield of marketable sweet potato tubers, compared to our trial, was also found in studies of other authors **(Hartemink, 2003**; **Oliveira et al., 2010**; **Sowley, Neindow and Abubakari, 2015)**.

Ratio of marketable yield

The highest ratio of marketable sweet potato tubers from total yield was found in cultivars 'Serbian' (92.82%), followed by cultivars 'Zagrebian' (91.31%) and 'Beauregard' (81.58%). Statistically non-significant difference was between cultivars 'Serbian' and 'Zagrebian'. Other differences between cultivars were evaluated as statistically significant. **Sokoto, Magaji and Singh (2007)** examined the effect of various intra-row spacing on the ratio of marketable yield in trial with sweet potato. Within variant, in which the same spacing was used as in our trial (0.30 m), marketable ratio of sweet potato was 48.99 %. It was expressively under value found in our trial. **Hartemink (2003)** found that marketable ratio of sweet potato tubers was varied from 83.53% to 89.56%, dependent on the growing year. It is comparable to results obtained in our trial.

Total carotenoid content

According to **Sebuliba, Nsubuga and Muyonga (2001)**, orange-fleshed sweet potato tubers are rich sources of carotenoids. Compared to cultivars with yellow and white flesh color, orange-fleshed sweet potato has expressively higher content of total carotenoids.

The content of total carotenoids was increasing in following cultivar order: 28.79 $mg.kg^{-1}$ fresh weight ('Zagrebian') <94.78 $mg.kg^{-1}$ f. w. ('Beauregard') <99.52 $mg.kg^{-1}$ f. w. ('Serbian'). The statistical analysis of results showed statistically significant differences among cultivars with orange flesh color ('Beauregard' and 'Serbian') and cultivar 'Zagrebian' with yellow-creme color of tuber flesh. Difference between cultivars with orange flesh color was not statistically significant.

Kammona et al. (2015) found the significant variability of carotenoid content in dependency on the color of sweet potato flesh. Total carotenoid content in orange-fleshed tubers was more than three-fold higher compared to sweet potatoes with yellow, purple and white flesh color. The strong interaction between flesh color and total carotenoid content in sweet potato was also presented in study of **Ellong, Billard and Adenet (2014)**, **Grace et al. (2014)** and **Hussein et al (2014)**. Within trial in China**, Tang, Cai and Xu (2015)** found higer total carotenoid in sweet potato tubers compared to results showed in our trial. The total carotenoid content in cultivar with orange flesh color was 157.9 $mg.kg^{-1}$ f. w. Cultivars with yellow-creme (75.4 $mg.kg^{-1}$), light-purple (5.19 $mg.kg^{-1}$), white (4.46 $mg.kg^{-1}$) and deep-purple color of tuber flesh had several-fold lower content of total carotenoid compared to orange sweet potato cultivar.

The most important and predominant carotenoid substance in sweet potatoes is β-carotene (**USDA, 2015**). According to study of **Kammona et al. (2015)**, β-carotene ratio from total carotenoid content is variable in relation with flesh color of sweet potato. The highest β-carotene ratio was found in purple-fleshed tubers (97.9%), followed by tubers with orange (93.8%), yellow (84.1%) and white (79.0%) flesh color.

Within study with ten cultivars of sweet potato, **Yildirim, Tokuşoğlu and Öztürk (2011)** found marked variability of β-carotene content in cultivars with yellow-creme color of tuber flesh (50.1 – 70.3 $mg.kg^{-1}$ f. w.). The β-carotene content in orange tubers was 70.3 $mg.kg^{-1}$. This results were in contrast to our study where expressive difference between orange and yellow-creme sweet potato was found. **Suparno, Prabawardani and Pattikawa (2016)** found similar β-carotene content values in yellow-creme sweet potato (62.98 – 64.69 $mg.kg^{-1}$ f. w.) compared

Table 5 Effect of cultivar on qualitative parameters of sweet potato tubers (marketable yield).

Cultivar	Total carotenoids ($mg.kg^{-1}$ fresh weight)	Vitamin C ($mg.kg^{-1}$ fresh weight)
Beauregard	94.78^{b}	154.37^{a}
Serbian	99.52^{b}	155.70^{a}
Zagrebian	28.79^{a}	146.43^{a}

Note: Different letters (a; b; c) within the same column means statistically significant difference (at 95.0 % confidence level).

Table 6 Effect of mulching variant on qualitative parameters of sweet potato tubers (marketable yield).

Cultivar	Total carotenoids ($mg.kg^{-1}$ fresh weight)	Vitamin C ($mg.kg^{-1}$ fresh weight)
Non-mulching	77.81^{b}	143.23^{a}
Mulching	70.92^{a}	161.10^{b}

Note: Different letters (a; b; c) within the same column means statistically significant difference (at 95.0 % confidence level).

to study of previous authors. Results in study of **Aywa, Nawiri and Nyambaka (2013)** also confirmed fact that orange flesh cultivars of sweet potato have markedly higher content of β-carotene (46.19 – 48.89 mg.kg^{-1} f. w.) compared to the tubers with yellow flesh color (20.17 – 26.28 mg.kg^{-1} f. w.).

Obtained results confirmed that cultivar is important factor influencing on the content of carotenoids in sweet potatoes. The expressive impact of cultivar to the total carotenoid content was found in the experiments with tomato **(Mendelová et al., 2012)**, bell pepper **(Ignat et al., 2013)** or sea buckthorn **(Mendelová et al., 2016)**.

Vitamin C content

Compared to total carotenoid content, variability of vitamin C content among cultivars was not marked. The vitamin C content was from 146.43 mg.kg^{-1} ('Zagrebian') to 155.70 mg.kg^{-1} f. w. ('Serbian'). According to statistical analysis, differences of vitamin C among cultivars were evaluated as statistically non-significant. Comparable values of vitamin C content (129-142 mg.kg^{-1} f. w.) in sweet potato were presented by **Maria and Rodica (2015)** in field trial in Romania.

Within trial in Poland, **Krochmal-Marczak et al. (2013)** found higher vitamin C in tubers of sweet potato compared to our trial results. Its values were varied, dependent on cultivars, from 202.6 mg.kg^{-1} to 242.0 mg.kg^{-1} f. w. The higher content of vitamin C in sweet potato tubers, compared to our study, was also presented in study of **Suparno, Prabawardani and Pattikawa (2016)**. Authors found variable content of vitamin C in dependency on the flesh color of sweet potato tubers. The highest vitamin C content was determined in purple cultivars (727.1 mg.kg^{-1}), followed by cultivars with white (672.2 mg.kg^{-1}) and yellow flesh color (204.7-254.4 mg.kg^{-1}). According to study of **Ellong, Billard and Adenet (2014)**, determined vitamin C content in yellow or creme sweet potatoes varied from 177.5 mg.kg^{-1} to 290.5 mg.kg^{-1} f. w. It meant higher values of vitamin C content in comparison with our trial. **Yildirim, Tokuşoğlu and Öztürk (2011)** also stated that cultivar and flesh color had an significant impact on the vitamin C content in sweet potato tubers. Within yellow-creme flesh cultivars, vitamin C content was ranged from 237 mg.kg^{-1} to 386 mg.kg^{-1} f. w. The cultivar with orange flesh color ('Regal') showed lower vitamin C content than most of yellow-creme cultivars.

On the contrary, markedly lower content, compared to trial results, was presented in study of **Gichuhi, Kokoasse Kpomblekou and Bowel-Benjamin (2014)**. In cultivar 'Beauregard' (the same cultivar as in our study), value of vitamin C content was 64.3 mg.kg^{-1} f. w. Similarly, lower values of vitamin C content were found (48.5 – 57.3 mg.kg^{-1} f. w.) in the field trial realised in different localities of Western Kenya **(Aywa, Nawiri and Nyambaka, 2013)**.

Compared to obtained results, more expressive and statistically significant impact of cultivar to the vitamin C content was found in studies with vegetable pepper and tomatoes **(Valšíková et al., 2010)**, broccoli **(Koh et al., 2009)** potatoes **(Mareček et al., 2016)**.

Effect of mulching on sweet potato yield

According to **Novak et al. (2007)**, sweet potato [*Ipomoea batatas* (L.) Lam] needs a yearly minimum of three month with air temperatures above 15 ºC for its growth and development. For the purpose of achieving the highest possible sweet potato yield during a relatively short vegetation period in middle Europe, using of mulching material (PE foil, non-woven textile or organic materials) is necessary for succesfull growing. **Wees, Seguin and Boisclair (2016)** similarly emphasize that use of black mulch to heat the soil can markedly improve and optimize yields of sweet potato and attain its market quality standards in cooler climate.

The statistical analysis showed statistically significant increase of particular quantitative parameters of sweet potato marketable yield in mulching variant (black non-woven textile) compared to variant without mulching (tab. 6). Between mentioned variants, increase of average tuber weight and total sweet potato yield (t.ha^{-1}) was presented by values of 51.5% and 61.8%. In mulching variant, the marketable ratio of tubers was higher about 7.3% compared to the non-mulching variant (bare soil). According to study of **Novak, Zutić and Toth (2007)**, mulching with black PE-film had a significant effect on the yield and average weight of sweet potato tubers. Within study, higher yield about 5.3 kg.m^{2} was found compared to non-mulching variant.**Novak et al. (2007)** found a significantly higher yield of marketable tubers of sweet potato in mulching variant by black PE film mulch compared to uncovered soil. In mentioned study, realised in Croatia, marketable yield was increased from 1.16 to 2.53 kg.m^{-2} (118 %). **Aregheore and Tofinga (2004)** tested effect of mulching by using of organic materials (guinea grass, dadap leaves) on the yield of sweet potato tubers. In all tested mulching treatments, sweet potato yield icncrease was reached compared to the treatment without mulch, varying from 4.6% to 12.2%. According to **Laurie et al. (2015)**, using of organic (grass straw) and inorganic (black plastic foil) mulching materials resulted in higher total and marketable yield of sweet potato tubers compared to the un-control treatment. The application of grass straw was showed by increase of total yield about 33.3% and marketable yield about 63.5%. In the treatment with black plastic foil, total and marketable yield of sweet potato were higher about 77.7% and 69.4% subsequently. Positive impact of mulching on the sweet potato yield was also presented in study of **Ossom et al. (2001)**.

Compared to experimental results, **McKinley Sullen (2010)** found minimal and non-significant impact of mulching by variously colored plastic material on the total carotenoid content in sweet potato (cultivar Beauregard). Its value was varying in this treatment order: black PE foil (159.4 mg.kg^{-1} f. w.) < red PE foil (159.6 mg.kg^{-1} f. w.) < control - bare soil (159.9 mg.kg^{-1} f. w.) < silver PE foil (160.1 mg.kg^{-1} f. w.) < white PE foil (160.4 mg.kg^{-1} f. w.) < blue PE foil (160.6 mg.kg^{-1} f. w.). The effect of mulching on the total carotenoid content (TCC) in sweet potato is not well-documented. On the other side, TCC in dependency on mulching was examined in studies with other crops. **Siwek, Libik and Zawiska (2012)** tested various biodegradable mulching materials and their impact on the TCC in butterhead lettuce. Authors found non-statistically significant increase of TCC in treatments with applied mulching materials compared to the control

treatment. Differences to control treatment in TCC were varying from 1.5% to 6.7%. **Szafirowska and Elkner (2009)** found variable effect of mulching on the content of β-carotene, main carotenoid substance, in sweet pepper by using of various materials. The application of cloves (organic mulching) resulted in statistically significantly higher TCC in pepper fruits compared to the control treatment. On the other hand, TCC decrease was detected in treatment with black polypropylene foil used for mulching in comparison with control. According to **Moreno et al. (2014)**, lycopene is the main carotenoid susbtance in tomato fruits. The using of different mulching materials (papers; biodegradable foil; black PE foil; straw) was showed by increase of lycopene content in tomato fruits in the range from 1.6% to 15.1% compared to control treatment.

In realised trial, positive impact of mulching on the vitamin C content in sweet potato was found. It is consistent with study results presented by **McKinley Sullen (2010)** who tested variously colored plastic foil on the sweet potato yield. Author found higher vitamin C content in sweet potato tubers compared to the control treatment (bare soil), presenting increase of its value in the range from 4.6% (blue foil) to 22.3% (black foil). Positive impact of mulching on vitamin C content was also found in trial with chilli pepper (*Capsicum annuum* L.) realised by **Ashrafuzzaman et al. (2011)**. Authors found higher vitamin C content in chilli pepper fruits in all treatments with variously-colored plastic mulching foil (transparent, black and blue) compared to the control treatment. The most significant increase of its value was reached by using of black plastic foil. **Franczuk et al. (2009)** tested effect of different types of organic mulching (phacelia, vetch, serradella, oat) on the yield quality of tomato and onion. Authors also presented increase of vitamin C content in both vegetables as the results of all used mulching materials during growing period. According to **Dvořák et al. (2012)**, using of black textile mulch resulted in statistically non-significant increase of vitamin C content (9,8%) in potato tubers compared to the treatment with bare soil.

CONCLUSION

The sweet potato is marked warm-requiring crop, grown mainly in Asia. The mulching is important intesification factor of production influencing yield of sweet potato. From aspect of quantitative parameters of sweet potato yield, all tested cultivars were showed significant increase of selected quantitative parameters (yield per plant; average tuber weight; yield in $t.ha^{-1}$; marketable tuber ratio) in mulching treatment compared to the control treatment. The highest yield of tubers (39.30 $t.ha^{-1}$) was showed in sweet potato cultivar 'Serbian' . The cultivar and mulching had also expressive impact on the quality of sweet potato tubers (total carotenoids; vitamin C). The highest content of total carotenoids (99.52 $mg.kg^{-1}$ fresh weight) and vitamin C (155.70 $mg.kg^{-1}$ f. w.) was also found in tubers of cultivar 'Serbian'. Results gained in presented study indicate that sweet potato can be succesfully grown in conditions of Southern Slovak Republic.

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Contact address:

Ing. Miroslav Šlosár, PhD., Slovak University of Agriculture in Nitra, Horticulture and Landscape Engineering Faculty, Department of Vegetable Production, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: miroslav.slosar@uniag.sk.

Ing. Ivana Mezeyová, PhD., Slovak University of Agriculture in Nitra, Horticulture and Landscape Engineering Faculty, Department of Vegetable Production, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, E-mail: ivana.mezeyova@uniag.sk.

prof. RNDr. Alžbeta Hegedüsová, PhD., Slovak University of Agriculture in Nitra, Horticulture and Landscape Engineering Faculty, Department of Vegetable Production, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: alzbeta.hegedusova@uniag.sk.

Ing. Marcel Golian, PhD., Slovak University of Agriculture in Nitra, Horticulture and Landscape Engineering Faculty, Department of Vegetable Production, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xgolian@is.uniag.sk.

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HISTOLOGICAL ANALYSIS OF FEMORAL BONES IN RABBITS ADMINISTERED BY AMYGDALIN

Veronika Kováčová, Radoslav Omelka, Anna Šarocká, Patrik Šranko, Mária Adamkovičová, Róbert Toman, Marek Halenár, Adriana Kolesárová, Monika Martiniaková

ABSTRACT

Cyanogenic glycosides are present in several economically important plant foods. Amygdalin, one of the most common cyanoglucoside, can be found abundantly in the seeds of apples, bitter almonds, apricots, peaches, various beans, cereals, cassava and sorghum. Amygdalin has been used for the treatment of cancer, it shows killing effects on cancer cells by release of cyanide. However, its effect on bone structure has not been investigated to date. Therefore, the objective of this study was to determine a possible effect of amygdalin application on femoral bone microstructure in adult rabbits. Four month old rabbits were randomly divided into two groups of three animals each. Rabbits from E group received amygdalin intramuscularly at a dose 0.6 mg.kg^{-1} body weight (bw) (group E, n = 3) one time per day during 28 days. The second group of rabbits without amygdalin supplementation served as a control (group C, n = 3). After 28 days, histological structure of femoral bones in both groups of rabbits was analysed and compared. Rabbits from E group displayed different microstructure in middle part of the compact bone and near endosteal bone surface. For endosteal border, an absence of the primary vascular longitudinal bone tissue was typical. This part of the bone was formed by irregular Haversian and/or by dense Haversian bone tissues. In the middle part of *substantia compacta*, primary vascular longitudinal bone tissue was observed. Cortical bone thickness did not change between rabbits from E and C groups. However, rabbits from E group had a significantly lower values of primary osteons' vascular canals and secondary osteons as compared to the C group. On the other hand, all measured parameters of Haversian canals did not differ between rabbits from both groups. Our results demonstrate that intramuscular application of amygdalin at the dose used in our study affects femoral bone microstructure in rabbits.

Keywords: amygdalin; femoral bone; rabbit; histomorphometry

INTRODUCTION

Cyanogenic glycosides are natural plant toxicants **(Bolarinwa et al., 2015)**. All cyanogenic glycosides are potentially dangerous due to production of hydrogen cyanide (HCN) by their hydrolysis **(Vetter, 2000)**, known as prussic acid **(Francisco and Pinotti, 2000)**. Consumption of cyanogenic plants may cause acute and chronic toxicity in both animals and humans **(Yildirim and Askin, 2010)**.

Amygdalin, D-mandelonitrile-β-D-gentiobioside, $C_{20}H_{27}NO_{11}$, is one of the most frequently occurring cyanogenic glycosides **(Bolarinwa et al., 2014)**. It is especially presented in fruit kernels of peaches, apricots, bitter almonds **(Blaheta et al., 2016)**, pears, plums and apples **(Kolesár et al., 2015)**. Amygdalin is composed of two molecules of glucose, one of benzaldehyde and one of hydrocyanic acid **(Chang et al., 2006; Abdel-Rahman, 2011).**

Amygdalin itself is non-toxic, but it is able to generate toxic hydrogen cyanide (HCN) **(Bolarinwa et al., 2014)** which is decomposed by some endogenous plant enzymes **(Song and Xu, 2014; Kolesár et al., 2015)**. Amygdalin has been used as a one of the most popular alternative treatments of cancer, asthma, atherosclerosis, hypertension, migraine and chronic inflammation **(Cheng et al., 2015)**. However, the Food and Drug Administration (FDA) has not approved amygdalin as a therapeutic agent owing to insufficient clinical evidence of its positive efficacy and potential toxicity **(Zhou et al., 2012; Halenár et al., 2015)**. The acute lethal dose of peroral application of HCN for human ranges between 0.5 and 3.5 mg.kg^{-1} bw **(Speijers, 1993; Bolarinwa et al., 2014)**. The maximum tolerance dose of intramuscular injection of amygdalin is 3 g.kg^{-1} bw in rabbits **(Song and Xu, 2014)**.

Amygdalin has a stimulating effect on the growth of skeletal muscle cells (line C2C12) which is dose-dependent **(Yang et al., 2014)**. However, the impact of amygdalin on bone microstructure is still unknown.

Therefore, the aim of our study was to investigate the effect of intramuscular application of amygdalin on femoral bones microstructure in adult male rabbits.

MATERIAL AND METHODOLOGY

Adult male rabbits (n = 6) of outbred line P91 (Californian broiler line) were used in the experiment. The animals (at the age of 4 months, weighing 4 ±0.2 kg) were obtained from an experimental farm of the Animal Production Research Centre in Nitra, Slovak Republic.

Males were housed in individual flat-deck wire cages (area 0.3 m^2) under standard conditions (temperature 20 – 22 °C, humidity 55 ±10%, 12/12 h cycle of light and darkness) with access to food (feed mixture) and drinking water *ad libitum*.

Clinically healthy animals were randomly divided into two groups of three individuals each. In the experimental group (E), adult rabbits were intramuscularly injected with amygdalin (99% purity, Sigma-Aldrich, St. Louis, MO, USA) at the dose 0.6 $mg.kg^{-1}$ bw one time per day during 28 days. The second group (C; n = 3) without amygdalin addition served as a control. All experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic, no. 3398/11-221/3 and Ethic Committee.

After 28 days, all the rabbits were euthanized and their femurs were collected for microscopical analysis. Right femurs 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 tissue 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 primary osteons' vascular canals, Haversian canals and 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.

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 both groups of rabbits.

RESULTS AND DISCUSSION

Femoral diaphysis of rabbits from the C group had the following microstructure in common. Primary vascular longitudinal bone tissue, as a basic structural pattern of rabbit's bones, formed the endosteal and periosteal borders. This tissue contained vascular canals which ran in a direction essentially parallel to the long axis of the bone. Near endosteal and periosteal borders, primary vascular radial bone tissue occurred (mainly in anterolateral and anteromedial views). It was created by branching or non-branching vascular canals radiating from the marrow cavity or *periosteum*. The middle part of the compact bone was composed by dense Haversian bone tissue with a high concentration of secondary osteons. Several secondary osteons were also observed near the endosteal surface (especially in the anterior and posterior views) (Figure 1).

Rabbits intramuscularly administered by amygdalin (E group) had different bone microstructure in the middle part of the *substantia compacta* and near endosteal bone surface. For endosteal border, an absence of the primary vascular longitudinal bone tissue was typical (mainly in anterolateral and anteromedial views). Endosteal surface was formed by irregular Haversian tissue (characterized by occurrence of scattered secondary osteons) and/or by dense Haversian bone tissue. In the middle part of *substantia compacta*, primary vascular longitudinal bone tissue was observed (it extended there from *periosteum*). Also, no secondary osteons were presented there. The periosteal surface was composed of primary vascular longitudinal bone tissue (Figure 2).

The results of qualitative histological analysis in rabbits from the C group corresponded with previous studies **(Enlow and Brown, 1956; Martiniaková et al., 2003; Martiniaková et al., 2009)**. However, intramuscular application of amygdalin (in rabbits from E group) induced changes near endosteal bone surface and also in the middle part of the compact bone. Central area of the compact bone was composed of primary vascular longitudinal bone tissue. Secondary osteons were found only near endosteal border. This phenomenon can be associated with adaptation of bone tissue to amygdalin exposure. This mechanism might prevent towards bone cells apoptosis and bone tissue necrosis. The experiment by **Shou et al., (2000)** has shown that cyanide treatment induced apoptosis by inducing oxidative stress in cortical neurons. It is known that bone is richly innervated **(Marenzana and Chenu, 2008)**. Nerve endings are in direct contact with bone cells **(He et al., 2013)**, indicating that nerve fibers may regulate growth and remodeling of the bone. It was found that sympathetic denervation induced abnormal formation and resorption of bone **(Chenu and Marenzana, 2005)**. Also, increased bone resorption was observed in rats after removal of sympathetic nerve supply **(He et al., 2011)**.

Therefore, the absence of primary vascular longitudinal bone tissue near endosteal surface in rabbits from E group can be connected with intensive endosteal resorption due to amygdalin administration. Generally, skeletal system is a dynamic organ and is constantly undergoing remodeling, which is enabled by osteoblasts and osteoclasts **(Guntur and Rosen, 2012; Arakaki et al., 2013)**. The formation and activation of osteoclasts to enhance bone resorption have shown to be associated with the higher generation of oxygen-derived free radicals **(Garrett et al., 1990; Baek et al., 2010)**. **Gunasekar et al., (1998)** reported that excessive production of reactive oxygen species (ROS) might be cyanide-induced. With accordance with this finding, **Daya et al., (2000)** reported that cyanide led to generation of oxidative stress and also lipid peroxidation which are associated with hypoxia **(Gunasekar et al., 1998)**. **Chang et al., (2014)** found that hypoxia reduced proliferative activity of osteoblasts and inhibited mineralization and bone formation. On the other hand, hypoxia increased formation of osteoclasts **(Patntirapong and Hauschka, 2007; Arnett, 2010)** and activity of these cells **(Knowles and Athanasou, 2009)**. The authors **(Patntirapong and Hauschka, 2007)** further stated that

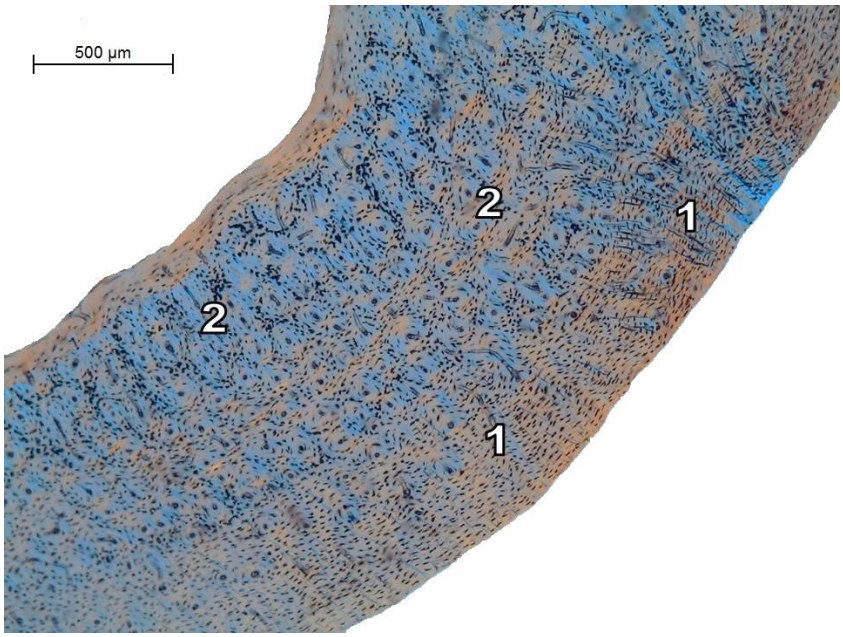

Figure 1 Microscopical structure of femoral bone in rabbits from the C group:
1 – primary vascular longitudinal bone tissue,
2 – dense Haversian bone tissue.

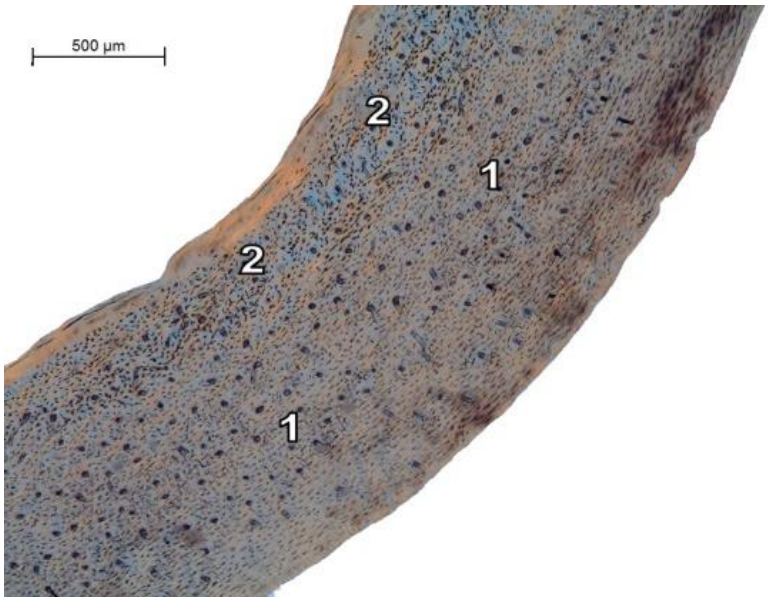

Figure 2 Microscopical structure of femoral bone in abbits from the E group:
1 – primary vascular longitudinal bone tissue,
2 – dense Haversian bone tissue.

low concentration of oxygen may change homeostasis of bone, leading to osteolysis, osteonecrosis **(Martiniaková et al., 2013b)** and osteoporosis **(Alagiakrishnan et al., 2003)**. Our results also showed increased periosteal bone apposition due to amygdalin administration which can serve as a compensatory mechanism to enormous endosteal bone reduction **(Szulc et al., 2006)**.

Our results showed an – non-significant effect of amygdalin application on cortical bone thickness in male rabbits (1085.15 ±145.54 mm and 1053.90 ±153.17 mm in rabbits from E and C groups, respectively).

For the quantitative histological analysis, 196 vascular canals of primary osteons, 98 Haversian canals and 98 secondary osteons were measured in both groups of rabbits. The results are summarized in Tables 1, 2 and 3. We have found that intramuscular application of amygdalin significantly affected sizes of the primary osteons' vascular canals and secondary osteons. Primary osteons' vascular canals and secondary osteons were significantly lower (p <0.05) in males from the E group. On the other hand, the size of Haversian canals did not differ between rabbits from both groups.

Rabbits injected with amygdalin had significantly lower values of primary osteons' vascular canals. This finding could be connected with a negative impact of amygdalin on blood vessels which are situated in vascular canals of primary osteons **(Currey, 2002; Greenlee and Dunnell, 2010)**.

Table 1 Data on primary osteons' vascular canals in rabbits from E and C groups.

Rabbit´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
E	96	290.67 ±48.08	61.40 ±5.40	10.76 ±1.31	8.66 ±0.99
C	100	314.33 ±63.56	63.73 ±6.67	11.14 ±1.51	9.01 ±1.18
T-test		p <0.05	p <0.05	NS	NS

Note: N: number of measured structures; NS: non-significant changes.

Table 2 Data on Haversian canals in rabbits from E and C groups.

Rabbit´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
E	49	338.25 ±73.78	66.05 ±6.83	11.64 ±1.30	9.23 ±1.29
C	49	343.88 ±58.93	67.18 ±5.63	11.93 ±1.40	9.24 ±1.22
T-test		NS	NS	NS	NS

Note: N: number of measured structures; NS: non-significant changes.

Table 3 Data on secondary osteons in rabbits from E and C groups.

Rabbit´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
E	49	4887.19 ±2408.89	245.70 ±62.40	43.42 ±11.55	33.95 ±9.16
C	49	6849.68 ±2795.79	291.70 ±57.75	50.66 ±9.85	41.54 ±9.50
T-test		p <0.05	p <0.05	p <0.05	p <0.05

Note: N: number of measured structures**.**

Waypa et al., (2001) reported that cyanide caused vasoconstriction. According to **Hamel (2011)** hypoxia has a negative effect on cardiovascular system and induced constriction of pulmonary arteries and dilatation in systemic arteries **(Weir and Archer, 1995)**. Higher levels of ROS production are associated with endothelial dysfunction like atherosclerosis and hypertension **(Guzik et al., 2011)**. **Cheng et al., (2015)** found that amygdalin increased the intracellular calcium level which can be associated with atherosclerosis **(Henry, 1985; Orimo and Ouchi, 1990)**. According to many authors **(Baum and Moe, 2008; Yarema and Yost, 2011)** glucocorticoid hormones have essential roles in homeostatic regulation and stress adaptation. These types of hormones cause vasoconstriction of blood vessels and mediate hypertension **(Saruta, 1996; Ponticelli and Glassock, 2009)**. According to **Ullian (1999)** application of hydrocortisone or corticosterone into rabbit aortic strips leads to greatly potentiated contractile responses to norepinephrine. Also **Berecek and Bohr (1978)** mentioned that deoxycorticosterone application stimulated vasoconstrictive effect of norepinephrine and angiotensin II in pigs. Based on these findings, we assume that the significant reduction of the size of primary osteons' vascular canals could be associated with these aspects.

On the other hand, non-significant changes in the size of Haversian canals were observed in rabbits from E group. It is generally known that the structure of primary and secondary osteons is different. Secondary osteons and also Haversian canals are surrounded by a cement line which is not found in primary osteons **(Currey, 2002; Martiniaková et al., 2013b)**. We suppose that the cement line is a main reason for different results in histomorphometry of both canals.

We have also found significantly lower secondary osteons in rabbits from E group. It is known that collagen type I (the major organic component of mineralized bone matrix, **Buchwald et al., 2012**) is produced by osteoblasts **(Bosetti et al., 2003; Wang et al., 2012)**. Secondary osteons are formed by lamellae in which collagen fibers run parallel to each other **(Martiniaková et al., 2013b)**. The arrangement of collagen fibers in lamellaes of secondary osteons ensures strength **(Dylevský, 2009)** and biochemical properties of the compact bone **(Martiniaková et al., 2013a)**. According to **Wang et al., (2012)** osteoblasts also synthesize alkaline phosphatase (ALP) which promotes mineralization of bone matrix. **Mody et al., (2001)** observed increase of intracellular oxidative stress and inhibition of differentiation markers in osteoblasts. The same findings have also been documented in the study of **Bai et al., (2004)**. In addition, these authors observed a reduction in the expression of collagen type I and ALP in rabbits calvarial osteoblasts. The experiment by **Arai et al., (2007)** has shown that bone mineralization significantly decreased by hydrogen peroxide (the most important ROS) exposure. Therefore, changes in the size of secondary osteon's may be associated with the inhibitory effect of oxidative stress (caused by amygdalin) on the osteoblastic activity which is connected with a reduction of collagen **(Patntirapong and Hauschka, 2007)**.

CONCLUSION

Natural plant substances like amygdalin are still a major part of traditional medicine. However, its effect on animal and human organisms is still not clear. Our results demonstrate that administration of amygdalin at the dose 0.6 $mg.kg^{-1}$ bw one time per day during 28 days induced changes in femoral bone microstructure of rabbits. Primary vascular longitudinal bone tissue was not found near endosteal surface. On the other hand, it was observed near *periosteum* and also in the middle part of *substantia*

compacta. Moreover, an absence of secondary osteons in the central area of the bone was identified in amygdalin-injected rabbits. Also, rabbits from E group had significantly lower (p <0.05) values of primary osteons' vascular canals and secondary osteons as compared to the C group.

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Contact address:

Veronika Kováčová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra, Slovakia, E-mail: veroni.kovacova@gmail.com.

Radoslav Omelka, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Botany and Genetics, 949 74 Nitra, Slovakia, E-mail: romelka@ukf.sk.

Anna Šarocká, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra, Slovakia, E-mail: sarocka.anna@gmail.com.

Patrik Šranko, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Botany and Genetics, 949 74 Nitra, Slovakia, E-mail: patriksranko@gmail.com.

Mária Adamkovičová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Botany and Genetics, 949 74 Nitra, Slovakia, E-mail: madamkovicova@ukf.sk.

Róbert Toman, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Veterinary Sciences, 949 76 Nitra, Slovakia, E-mail: robert.toman@uniag.sk.

Marek Halenár, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, 949 76 Nitra, Slovakia, E-mail: halenarmarek@gmail.com.

Adriana Kolesárová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, 949 76 Nitra, Slovakia, E-mail: adriana.kolesarova@uniag.sk.

Monika Martiniaková, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra, Slovakia, E-mail: mmartiniakova@ukf.sk.

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MINOR LIPOPHILIC COMPOUNDS IN EDIBLE INSECTS

Monika Sabolová, Anna Adámková, Lenka Kouřimská, Diana Chrpová, Jan Pánek

ABSTRACT

Contemporary society is faced with the question how to ensure suffiecient nutrition (quantity and quality) for rapidly growing population. One solution can be consumption of edible insect, which can have very good nutritional value (dietary energy, protein, fatty acids, fibers, dietary minerals and vitamins composition). Some edible insects species, which contains a relatively large amount of fat, can have a potential to be a „good" (interesting, new) source of minor lipophilic compounds such as sterols (cholesterol and phytosterols) and tocopherols in our diet. For this reason, the objective of this work was to characterize the sterols and tocopherols composition of fat from larvae of edible insect *Zophobas morio* L. and *Tenebrio mollitor* L. Cholesterol and three phytosterols (campesterol, stigmasterol and β-sitosterol) were reliably identified and quantified after hot saponification and derivatization by GC-MS. Other steroid compounds, including 5,6-trans-cholecalciferol were identified only according to the NIST library. Cholesterol was the predominant sterol in all analysed samples. Both types of larvae also contained high amount of phytosterols. Different region of origin had a no significant impact on sterols composition, while the effect of beetle genus was crucial. Tocopherols were analysed by reverse phase HPLC coupled with amperometric detection. Tocopherols content in mealworm larvae was lower than content in edible oils, but important from the nutritional point of view. Change of tocopherols composition was not observed during the storage under different conditions. Larvae of edible insect can be a potential good dietary source of cholesterol, but also vitamin D_3 isomers, phytosterols and tocopherols.

Keywords: sterol; tocopherol; *Tenebrio mollitor*; *Zophobas morio*

INTRODUCTION

The large group of minor lipophylic compounds include higher hydrocarbons, alcohols, ketones and diketones, steroids, lipophilic vitamins, pigments and other compounds. Steroids and lipophilic vitamins are most important compounds from this group.

Several steroid compounds are usually present in plants and organisms, but the major steroids are phytosterols and cholesterol. Steroids are synthesised in organisms via complex mechanisms from isoprene units, isopentenyl diphosphate and dimethylallyl diphosphate. Reaction gives an important intermediate, farnesyl diphosphate. Two molecules of farnesyl phosphate give rise to triterpenic hydrocarbon squalene, which in the body of animals yields triterpenic alcohol lanosterol and the triterpenic alcohol cycloartenol in plants. Lanosterol in animals is a precursor for the biosynthesis of the most important zoosterol cholesterol. An intermediate in the biosynthesis of cholesterol is 7-dehydrocholesterol, which is a precursor of vitamin D_3. Cholesterol in the body is used for the biosynthesis of steroid hormones and bile acids **(Velíšek, 2014)**.

Insects also need cholesterol for the synthesis of vitamin D_3 and also for the synthesis of insect steroid hormones called as ecdysteroids (e.g. ecdysone, 20-hydroxy-ecdysone, makisterone A etc.). But, insects are not able to synthesize cholesterol *de novo* and they have to use plant phytosterols (β-sitosterol, campesterol, stigmasterol) for cholesterol synthesis by the side dealkylation on the C-24 alkyl group of dietary phytosterols. Cholesterol and 7-dehydrocholesterol are formed during synthetic pathway. For example, the beetle *Tenebrio molitor* produces about 17% of 7-dehydrocholesterol and about 67% of cholesterol from sitosterol **(Leclerq, 1948; Svoboda and Feldlaufer, 1991; Ikekawa et al., 1993; Svoboda and Lusby, 1994; Ikekawa et al., 2013)**.

Cholesterol is mainly found in animal fats and in human tissues. In lower animals other sterols, known collectively as zoosterols may also be present. Cholesterol and its esters are present in all membranes and in blood lipids, but particularly rich sources are nervous tissues, especially the brain. Egg yolk is other very important source of dietary cholesterol. Other sources include meat, milk and cheeses, but also animal fats, lard and butter to a greater extent **(Velíšek, 2014)**. Sterols are essential components of lipoproteins and lipid membranes in animals. They are particularly important in nerve tissues and in the transport of lipids, which are bound in lipoproteins. In humans, dietary cholesterol intake is lower than the daily requirement, therefore the body synthesises (in the liver) the majority of cholesterol that is needed. Cholesterol in the diet is easily absorbed, but problems may occur during transport of cholesterol from the intestinal wall during lymph and blood circulation. Excessive cholesterol transport in low-density lipoproteins may cause cardiovascular diseases. It is therefore recommended that the intake of dietary cholesterol should not exceed 300 mg per day **(Dinh et al.,**

2011; Golebiowski et al., 2014; Velíšek, 2014).

Vitamin D_3 is formed from 7-dehydrocholesterol after UV irradiation (wavelength 280 – 320 nm) through the intermediate precholecalciferol. Calcitriol (1α,25-dihydroxycholecalciferol) is the active form of vitamin D_3 that is created in oxidation in the liver and subsequently in the kidney. Along with two other hormones, calcitonin (from thyroid gland) and parathormone (parathyroid gland) act in the resorption, metabolism and excretion of calcium and phosphorus **(Lawson, 1971; Velíšek, 2014; Finke, 2015)**. Two geometrical isomers of the vitamin D_3 and its 25-hydroxyderivative can be formed during synthesis from 7-dehydrocholesterol and consecutive oxidation in the liver. 5,6-*cis* isomer, after oxidation in the kidney, stimulates intestinal calcium transport. Vitamin activity of 5,6-*trans* isomer is significantly lower, but this compound shows another biological activity – mainly antiproliferative activity **(Holick et al., 1972; Chen et al., 2000; Filip et al., 2010)**.

Plants synthesise a number of steroid substances from cycloartenol, mainly demethylsterols (campesterol and other phytosterols) and 4,4-dimethylsterols (mainly as saponins – betulinic acid etc.) or 4-methylsterols as minor compounds **(Kuksis, 2001; Velíšek, 2014)**.

Vitamin E, formerly also known as antisterile vitamin, has eight basic structurally-related derivatives of chroman-6-ol (2H-1-benzopyran-6-ol). Structural bases common to all compounds with the reported activity of vitamin E are tocol and tocotrienol, which contain a hydrophobic chromane ring with a saturated or unsaturated isoprenoid side chain of 16 carbon atoms **(Velíšek, 2014)**. Vitamin E, especially α-tocopherol is the most important lipophilic antioxidant that acts in eucaryotic cells to protect (poly)unsaturated lipids against free radical damage. Tocopherols show the antioxidant activity *in vivo* and also *in vitro*. It protects the structure and integrity of biomembranes, such as the cytoplasmic cell membrane (or plasmolema) and intracellular membranes of organelles (nucleus, mitochondria, lysosome and endoplasmic reticulum). It is also employed in the protection of lipoproteins present in plasma. It is transported in the bloodstream by association with the lipid phase of low density lipoprotein (LDL) particles. Each LDL particle contains six molecules of vitamin E **(Li et al., 1996; Munné-Bosch and Alegre, 2002; Hofius and Sonnewald, 2003; Velíšek, 2014)**.

The aim of this study was to characterize the profile of minor lipophilic (unsaponifiable) compounds (mainly steroids and vitamin E) in three types of the edible insect.

MATERIAL AND METHODOLOGY

Analyzed samples

Insect species used for analysis were larvae of mealworm (*Zophobas morio* L.) and larvae of superworm (*Tenebrio mollitor* L.). The stage of development of analyzed insect was suitable for culinary preparation. *Zophobas morio* and *Tenebrio mollitor* were purchased lyophilized in local market in Sumatra. *Zophobas morio* sample were also purchased in Radek Frýžela Company, Brno, Czech Republic. Insects were starved for 48 hours, killed with boiling water (100 °C) and dried at 105 °C to constant weight. These samples were homogenized and stored at 4 – 7 °C until analysis. Sampels of *Zophobas morio* (from Czech Republic) used for tocopherols analysis were stored at two different conditions: at 5 – 6 °C and at 25 °C (room temperature).

Fat extraction

Fat from analysed samples was extracted by Soxtherm® apparatus (Gerhardt, Königswinter, Germany). Approximately 5 g of homogenized sample was weight into extraction cartridge and extracted by petroleum ether 120 min at 70 °C. Fat content was measured gravimetrically after drying to the constant weight at 103 °C. Extracted fat was used for sterols and tocopherols analysis.

Sterols analysis

Sterols content was determined according to AOCS Official Method Ch 6-91, American Oil Chemists´ Society, USA, 1997. Approximately 0.5 g of fat was boiled by 50 ml ethanolic KOH (2 $mol.L^{-1}$) for 1 hour. The unsaponifiable fraction was extracted with diethylether. The solvent was evaporated using rotary vacuum evaporator. Dried samples were silylated by pyridine and BSTFA (Bis(trimethylsilyl)-trifluor-acetamide; Merck, Czech Republic). Sterols were determined by GC Agilent 7820A coupled with mass detector Agilent 5975 Series MSD (Agilent Technologies, Palo Alto, CA, USA). Analytes were separated by capillary column Supelco (SACTM5, 22 m x 025 mm I.D. x 0.25 µm film). High purity helium was used as carrier gas at a flow rate of 20 $mL.min^{-1}$. Column temperature program started at

245 °C for 1 min, than heated at a rate 10 °C/min to 290 °C for 33 min, than increased by 5 °C/min to 310 °C for 15 min. The injector temperature was set to 280 °C. Samples were injected (1µL) in a split mode (20:1). 5α-cholestane was used as internal standard for quantification of cholesterol, campesterol, stigmasterol and ß-sitosterol in SCAN mode. Peaks were identified by their retention time compared with pure standard, comparison of their mass spectra with the NIST library spectra and also by comparison with literature.

Tocopherols analysis

Tocopherols were determined by reverse phase HPLC with amperometric detection The analysis was performed under the following conditions: a mobile phase - mixture of acetonitrile/methanol (1:1, v/v) with $LiClO_4$ (0.02 $mol.L^{-1}$) and NaCl (0.005 $mol.L^{-1}$); a flow rate 1 ml/min (LCP 4020.31 nonmetallic pump Ecom, Prague, Czech Republic); injected volume 20 µL. Separation was performed by steel column (4 x 250 mm, a particle size 5 µm, Tessek, Prague, Czech Republic); column temperature 28 °C (LCO 101 column heater, Ecom, Prague, Czech Republic); detection potential - +0.7 V (HP 1049A amperometric detector with a glassy – carbon working and solid state Ag/AgCl reference electrode (Agilent Technologies, St. Clara, USA). The quantification of tocopherols was provide by external calibration. For the determination of tocopherols insect fat samples were prepared as follows. Approximately 0.25 g of fat extracted from insect was weighed into 25 mL volumetric flask and filled to the mark with acetone.

RESULTS AND DISCUSSION

Amounts of sterols in samples of edible insects are shown in Table 1.

Table 1 Sterol composition of edible insect species.

Insect	Sterol			
	Cholesterol (mg.kg^{-1}±SD)	**Campesterol (mg.kg^{-1}±SD)**	**Stigmasterol (mg.kg^{-1}±SD)**	**ß–sitosterol (mg.kg^{-1}±SD)**
Zophobas morio	1784.1 ±30.4	227.6 ±19.9	79.3 ±9.4	344.1 ±35.8
Zophobas morio*	1594.9 ±164.1	169.2 ±8.45	unquantified	260.2 ±12.3
Tenebrio mollitor	669.4 ±34.7	350.5 ±56.0	71.9 ±2.5	244.7 ±12.0

*Czech Republic

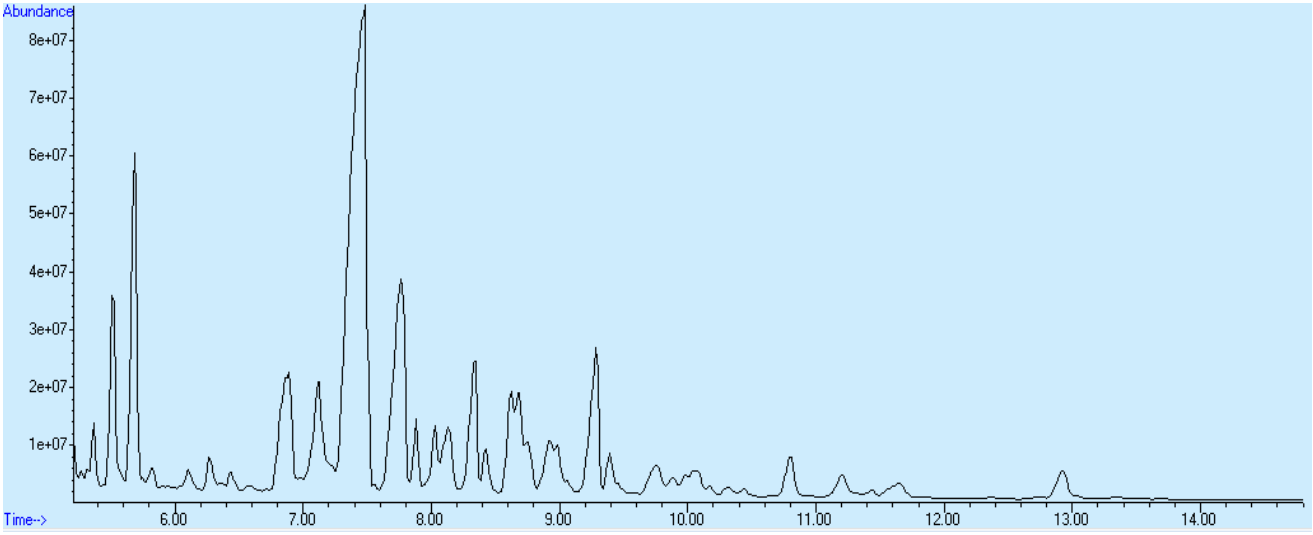
Table 2 Tocopherols composition of *Zophobas morio* (Czech Republic) stored at different conditions.

Conditions (°C)	α – tocopherol mg.kg^{-1} ±SD	ß + γ – tocopherol mg.kg^{-1} ±SD	δ – tocopherol mg.kg^{-1} ±SD
5 – 6	75.7 ±3.2	5.3 ±0.1	LOQ
25	77.2 ±0.1	5.3 ±0.3	LOQ

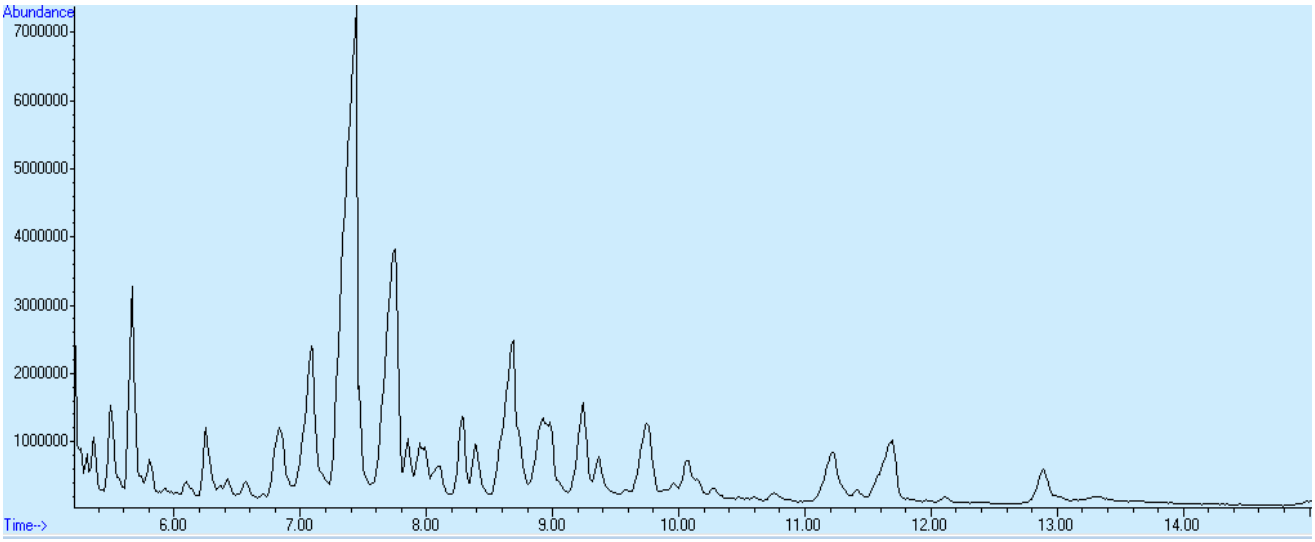
Cholesterol, typical animal sterol, was the most abundant sterol found in analysed samples. There are only minimum amount of cholesterol in food source of plant origin. Negative health impact (metabolic syndrome) of cholesterol is well known for a long time **(WHO, 2004).** On the other hand, cholesterol lowering effect of phytosterols is well known as well **(Peterson, 1951)**. Phytosterols reduce intestinal cholesterol absorbtion **(Normén et al., 2000)** and plasma LDL-cholesterol **(Piironen et al., 2000)**. Phytosterols (namely ß-sitosterol, campesterol and stigmasterol), typical plant origin sterols, were also determined in insects' samples. Identity of these compounds was confirmed by comparison with standards.

Cholesterol content was quite similar for the same insect species which came from different regions *Z. morio* from the Czech Republic (1594.9 mg.kg^{-1}) and *Z. morio* from Sumatra (1784.1 mg.kg^{-1}). The results indicate, that the extent of cholesterol synthesis is probably determined genetically and the influence of climate or type of feed is negligible. But larger set of samples would be needed to confirm these conclusions. However, sterols composition differences were much bigger between various insect species. *Z. morio* contained more cholesterol (1594.9 – 1784.1 mg.kg^{-1}) than *T. mollitor* (669.4 mg.kg^{-1}). An interesting trend can be seen in the content of phytosterols, which is the opposite content ratios of campesterol and β-sitosterol of mealworm larvae (228 and 344 mg.kg^{-1}) and superworm larvae (350 and 246 mg.kg^{-1}). This could indicate a different mechanism of cholesterol biosynthesis. Mealworm probably favors a demethylation of β-sitosterol (C_{29}-C_{28}), superworm favors easier demethylation of campesterol (C_{28}). The cholesterol content of the *T. mollitor* was comparable with content in meat of common livestock. Larvae of *Z. morio* synthesized much larger amount of cholesterol than there is in eggs – cholesterol content in the whole eggs is 2000 – 3500 mg per kg **(Velíšek, 2014)**. High cholesterol level could mean a nutritional problem.

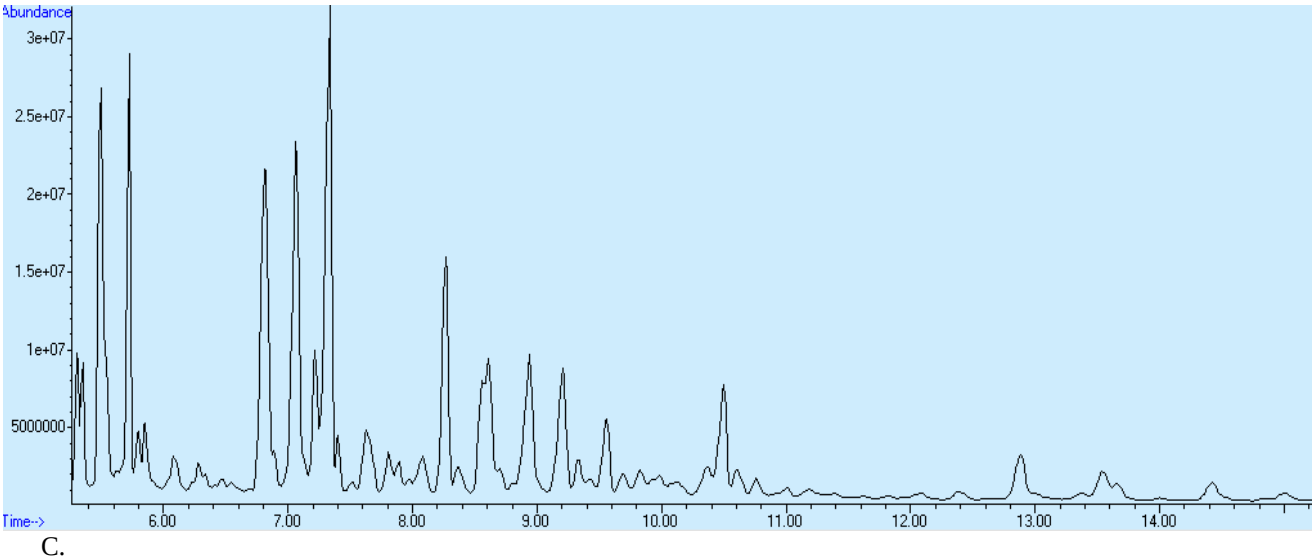
Chromatograms from sterols analysis in fat from different edible insect are shown in Figure 1. According to mass spectra, the following sterols and stanol (saturated sterol) were identified by the NIST library: lanosterol (RT 7.9), ergosterol (RT 8.1) and stigmastanol (RT 9.4). Ergosterol was found as pro-vitamin D_2 in food from plant and microbial origin **(Gorman et al., 1987; Tsiaras et al., 2011)**. All analyzed samples contained significant amount of the compound, that was identified as cholecalciferol (vitamin D_3). The main food sources of cholecalciferol are fish (mainly fatty fish such as herring, mackerel and salmon), meat, eggs, milk and dairy products **(Schmid and Walther, 2013; Velíšek, 2014)**. The compound with retention time 7.7 was identified as cholecalciferol according to the NIST library. However, retention time of cholecalciferol standard was different (6.1 to 7.1). Comparison of standard, real sample and the NIST library mass spectra of cholecalciferol is shown in Figure 2. As shown in this figure, the spectra are practically identical. Similarity of mass spectra and differences in retention times of these compounds suggests that these compounds are probably different cholecalciferol isomers. This compound is probably 5,6-*trans*-cholecalciferol, which is described in literature. This isomer was identified with high probability only at *Z. morio* samples. This isomer was identified with high probability only at *Z. morio* samples. 5,6-t*rans* vitamin D_3 does not have the same biological activity as 5,6-*cis* vitamin D_3, but it has other biological activities **(Borsje et al., 1977; Chen et al., 2000; Filip et al., 2010)**. 5,6-*cis* vitamin D_3 plays the crucial role in calcium and phosphorus metabolism (bone development and maintenance) and is also important for cell differentiation process and immune system **(Ovesen et al., 2003; Velíšek, 2014)**. Concerning other peaks of analytes, they were not identified with sufficient reliability.



A.

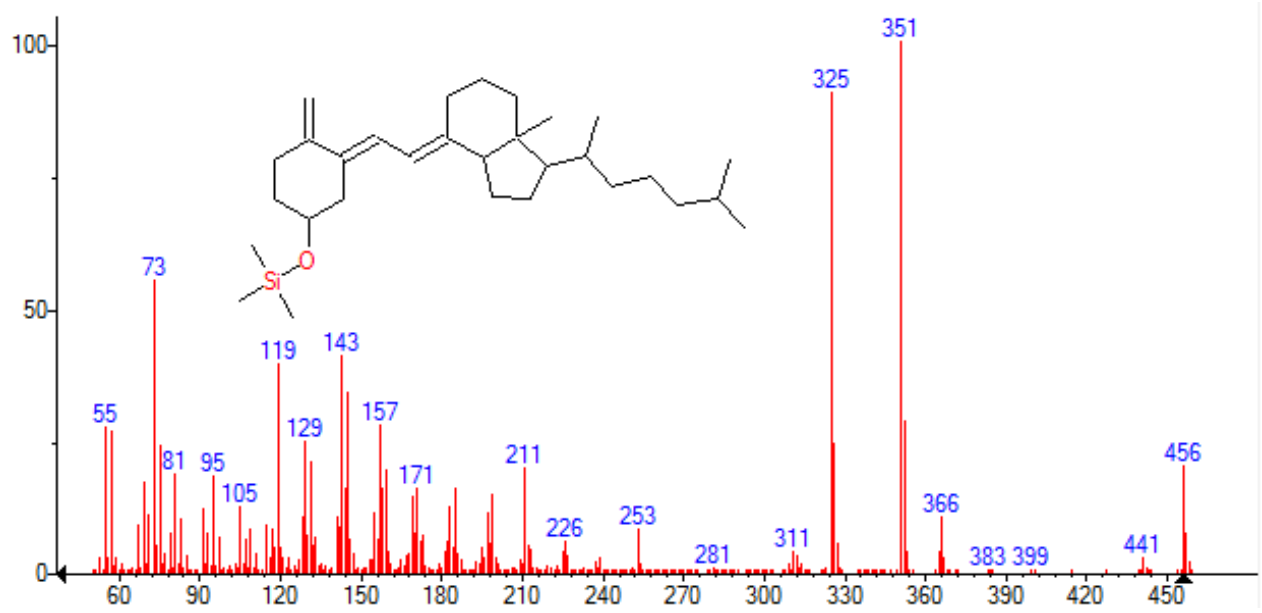


B.

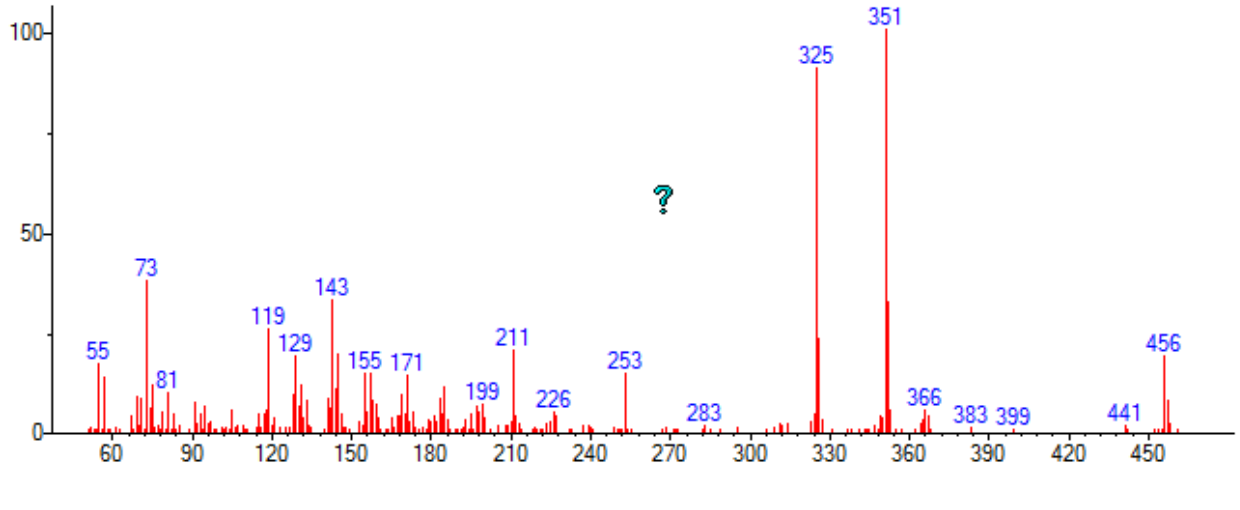


C.

Figure 1 Chromatograms – sterols composition of A - *Zophobas morio* (Sumatra), B - *Zophobas morio* (Czech Republic), C - *Tenebrio mollitor* (Sumatra); retention times: 5α-cholestane 5.4, cholesterol 7.4, 5,6-*trans*-cholecalciferol 7.7, lanosterol 7.9, ergosterol 8.1, campesterol 8.3, stigmasterol 8.6, ß-sitosterol 9.2, stigmastanol 9.4.



A.



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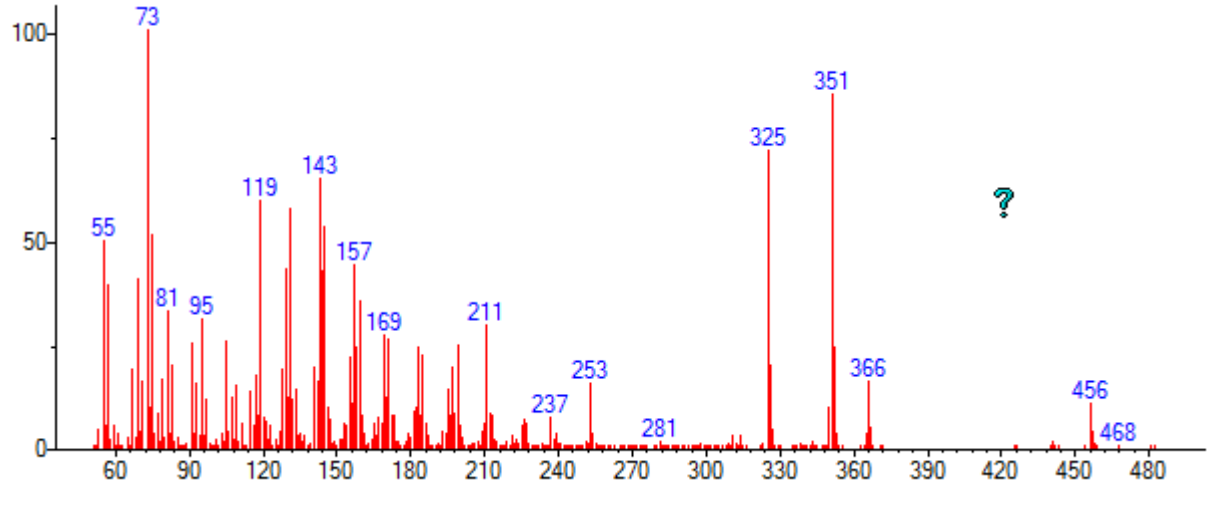

C.

Figure 2 Mass spectra of cholecalciferol: A – NIST libary, B – standard, C – analyzed sample.

Tocopherols content in edible insect samples is shown in Table 2. The α-tocopherol was the most abundant tocopherol analyzed in larvae of mealworm *Zophobas morio*, followed by β- and γ-tocopherols. Δ-tocopherol content was under the limit of quantification (LOQ). The content of α-tocopherol can be interesting from the nutritional point of view. The requirement of α-tocopherol for adults is about 15 mg of tocopherol equivalent per day. In this case, the consumption of about 50 g of edible insect brings approximately 25% of recommended daily intake. The storage temperature had no impact to the tocopherols content in analyzed insect samples.

The content of α-tocopherol was 75.7 mg per kg and 77.2 mg per kg in sample stored at 5 – 6 °C and 25 °C, respectively.

CONCLUSION

Larvae of beetle *Zophobas morio* L. and *Tenebrio mollitor* L. had a relatively high content of minor lipophilic compounds, which can be interesting from a nutritional point of view. The cholesterol content at superworm larvae was comparable with its content in meat, mealworm larvae had its content significantly higher. Both types of larvae contain approximately the same amount of phytosterols, their total content was comparable with cholesterol in superworm larvae, and in case of mealworm larvae. Mealworm larvae contained significant amounts of α-tocopherol, other tocopherols content was insignificant.

Relatively high level of phytosterols and α-tocopherol may be one of the major benefits of this new food commodity.

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Contact address:

Monika Sabolová, University of Chemistry and Technology Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 5, 166 28 Prague 6 Czech Republic, E-mail: monika.sabolova@vscht.cz.

Anna Adámková, Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Quality of Agricultural Products, Kamycka 129, 165 21 Prague 6 Czech Republic, E-mail: adamci@centrum.cz.

Lenka Kouřimská, Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Microbiology, Nutrition and Dietetics, Kamycka 129, 165 21 Prague 6 Czech Republic, E-mail: kourimska@af.czu.cz.

Diana Chrpová, University of Chemistry and Technology Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technická 5, 166 28 Prague 6 & Secondary Nursing School and Nursing College, 5. května 51, 140 00 Prague 4 Czech Republic, E-mail: dianach@centrum.cz.

Jan Pánek, University of Chemistry and Technology Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 5, 166 28 Prague 6 Czech Republic, E-mail: jan.panek@vscht.cz.

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MICROBIOLOGICAL EVALUATION OF FISH

Olga Cwiková

ABSTRACT

Fish meat has a specific composition that positively influences human health. Thanks to this composition, it is an excellent nutritional medium for growth and reproduction of undesirable microorganisms, which may cause spoilage and they can also lead to alimentary illnesses. Microbiota of fish is dominated by Gram negative and psychrophilic bacteria**.** Microbial contamination causes fish deterioration and leads to the end of its shelf-life when reaches levels between 10^7 and 10^9 CFU.g^{-1}. The most appropriate temperature for storage of fish is between -1 °C and 4 °C and the ideal relative air humidity is 80 to 85%. The objective of the work was to evaluate microbiological quality of fresh fish (Rainbow Trout, Atlantic Salmon, Atlantic Cod) bought in various types of stores in the Czech Republic and to evaluate if different storage temperatures have influence on the quantity of microorganisms. The following microorganisms were monitored: the total aerobic count (TAC), coliform bacteria, *E. coli*, *Salmonella* spp. and *Vibrio parahaemolyticus.* Based on the obtained results it is possible to state that difference between individual stores (p >0.05) in the total aerobic count and the quantity of *E. coli* (except for cod) was not proven. After 2 days of storage there was increase (p <0.05) of the total aerobic count in case of all monitored fish species from all stores. In case of coliform bacteria and *E. coli* there was increase (p <0.05) of their quantity in a majority of the analysed samples. Different storage temperature (4 °C and 8 °C) did not have influence (p <0.05) on the TAC, the quantity of coliform bacteria (except for cod) and the quantity of *E. coli* (except for trout).

Keywords: fish; TAC; *E. coli*; *Salmonella* spp.; *Vibrio parahaemolyticus*; storage temperature

INTRODUCTION

Fish plays an important role in the human diet and there is an observed increase in the consumption of fish per capita in Europe **(Novoslavskij et al., 2016)**. Consumption of fish meat in the Czech Republic is low in comparison to another EU countries **(Buchtová 2001)**. Consumption of two portions of fish per week is recommended as prevention of cardiovascular and oncological diseases **(Clonan et al., 2012)**. Fish is a source of many beneficial substances such as vitamins, polyunsaturated fatty acids, mineral substances and in some countries of the world they are practically the only source of animal protein **(Matyáš et al., 2002)**. Generally, fish meat is sterile while it is alive. However, a large number of bacteria are found on the outer surface, scales, gills and intestine **(Hempel et al., 2011)**. Microbiota of fish is dominated by Gram negative and psychrophilic bacteria **(Görner, Valík, 2004)**. During the capture and handling of fish, the muscle is colonized by these microorganisms. Microbial contamination causes fish deterioration and leads to the end of its shelf-life when reaches levels between 10^7 and 10^9 CFU.g^{-1} **(Scheleguedaa et al., 2016)**. Fish meat is a very suitable substrate for growth and reproduction of microorganisms due to high water content **(Pipová et al., 2006)**. Most fish contain only very little carbohydrate (<0.5%) in the muscle tissue and only small amounts of lactic acid are produced post mortem. This has important consequences for the microbiology of fish **(Gram, Huss, 1996)**.

Safety of fish products and their quality assurance is one of the main problems of food industry today. The presence or absence of foodborn pathogens in a fish product is a function of the harvest environment, sanitary conditions, and practices associated with equipment and personnel in the processing environment **(Grigoryan, Badalyan Andriasyan, 2010)**.

The most suitable storage temperature for fish is between -1 °C and 4 °C. The ideal relative air humidity is 80 to 85%. Chilled fish stays "fresh" approximately for 4 days (in crushed ice), if fish is sliced, the shelf-time decreases to two days at 4 °C **(Buchtová, 2001)**.

The objective of the work was to evaluate microbiological quality of meat of Rainbow Trout, Atlantic Salmon and Atlantic Cod, and to find out if storage temperature and the type of store, where fish was bought, have impact on the quantity of monitored microorganisms.

MATERIAL AND METHODOLOGY

Samples of fresh fish bought in the regular sales network in the Czech Republic were observed. 3 representative samples of every fish species were analysed. They included samples of Rainbow Trout (*Oncorhynchus mykiss*), Atlantic Salmon (*Salmo salar*) and Atlantic Cod *(Gadus morhua*). Samples were bought in the store focusing on sale of fresh fish and fish products, it has several branches in the Czech Republic and it guarantees fast transport of fish from a place of catching and thus also

freshness of freshwater as well as saltwater fish (denoted as the store 1). The second set of samples was bought in one of the store chains that offers food as well as non-food products (denoted as the store 2). The third set of samples was bought in a small store that is specialized on sales of fish and it is owned by private persons (denoted as the store 3).

Samples were transported into the microbiological laboratory of the Institute of Food Technology at MENDELU in Brno in a cooling box, stored at the temperature of melting ice and the microbiological analysis was carried out on the day of purchasing. The subsequent microbiological analysis was carried out after 2 days of storage at different temperature conditions at 4 °C and 8 °C.

For every sample of fish the following microbiologic indicators were determined:

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

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

Quantity of bacteria *E. coli*. Cultivation on Chromocult Coliform agar (Merck, Francie) for 24 to 48 hours at 37 C.

Vibrio parahaemolyticus. Pre-reproduction in peptone water with higher salt content at 41.5 °C for 18 hrs. Cultivation on Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Biokar Diagnostics, Francie) for 24 hrs at 37 °C. Biochemical confirmation, oxidase test.

***Salmonella* Species.** Cultivation after pre-reproduction on Salmonella Enrichment with addition of IRIS Salmonella selective supplement (18 hrs at 41.5 °C) in IRIS Salmonella® Agar (Biokar Diagnostics, Francie) for 24 hrs at 37 °C. Biochemical confirmation.

Sampling and processing was carried out based on ČSN ISO 7218 and ČSN 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 (ANOVA, Turkey test). Evaluation was performed using the programme STATISTICA CZ, version 10.

RESULTS AND DISCUSSION

The total aerobic count

The total aerobic count (Figure 1) found in the samples of Rainbow Trout bought in the individual stores was comparable (p >0.05). In the store 1, the total aerobic count was 6.1 log CFU.g^{-1} (1.3 x 10^6 CFU.g^{-1}), in the store (2) 6.4 log CFU.g^{-1} (2.2 x 10^6 CFU.g^{-1}) and in the store (3) 6.6 log CFU.g^{-1} (3.7 x 10^6 CFU.g^{-1}).

Also in the samples of Atlantic Salmon the total aerobic count was comparable (p >0.05). The highest values of the total aerobic count were found in the sample from the store (3), specifically 6.6 log CFU.g^{-1} (3.6 x 10^6 CFU.g^{-1}), the lowest in the store (1) 6.1 log CFU.g^{-1} (1.2 x 10^6 CFU.g^{-1}).

Regarding cod, the highest (p >0.05) total aerobic count was recorded in the sample bought in the store (1) specifically 7.0 log CFU.g^{-1} (10.7 x 10^6 CFU.g^{-1}), the lowest quantity (p <0.05) was found in the sample from the store (2), specifically 6.3 log CFU.g^{-1} (1.9 x 10^6 CFU.g^{-1}). As it is stated by **Ingr (2010)**, according to currently invalid Notice no. 132/2004 Coll., fish and its parts intended for heat treatment may contain the total aerobic count 6 log CFU.g^{-1} (10^6 CFU.g^{-1}), according to **ČSN 56 9609 (2008**) the quantity of 6.7 log CFU.g^{-1} (5.10^6 CFU.g^{-1}) is permissible. The total aerobic count in the observed samples was lower in all fish samples bought in all stores, except for cod bought in the store (1). If we compare the found values of TAC and results of **Kordiovská et al. (2004)**, then the quantities found by our research are higher. In the above mentioned experiment of **Kordiovská et al. (2004)**, the total aerobic count fresh fish bought in the sales network was 6.2 x 10^4 CFU.g^{-1} (4.8 log CFU.g^{-1}). On the contrary **Nespolo et al. (2012)** present in salmon TAC comparable with our study (1.1 x 10^3 to 3.9 x 10^6 CFU.g^{-1}).

During capture and storage fish are almost invariably come into contact with nets, decks, ropes, boxes, human hands and clothing. These contacts are introducing microorganisms from other sources such as humans, birds and soil **(Fernandes, 2009).**

During storage (Figure 2) after 2 day there was increase (p <0.05) of the total aerobic count in the all observed species of fish from all stores. Considering storage of fish at different temperatures, there was no difference in TAC recorded (p <0.05). TAC was comparable after two days of storage at the temperature 4 °C as well as 8 °C. The highest TAC was found in the sample of trout, specifically 9 log CFU.g^{-1}. With this high total aerobic count there already occurs spoilage and sensory changes **(Miks-Krajnik et al., 2016)**. **Parlapani and Boziaris (2016)** also carried out monitoring spoilage microbiota, where the total aerobic count detected at the beginning of storage was 3.5 log CFU.g^{-1}, and after storage at 5 °C then 8.1 log CFU.g^{-1}, which is lower than we found during the analyses.

The quantity of coliform bacteria

The quantity of coliform bacteria (Figure 3) in the samples of Rainbow Trout bought in the individual stores was comparable (p >0.05), only in the store (1) there was higher quantity of coliforms recorded (p >0.05) in comparison to the store (2), specifically 4.7 log CFU.g^{-1} (4.8 x 10^4 CFU.g^{-1}); respectively 4.4 CFU.g^{-1} (2.3 x 10^4 CFU.g^{-1}) in the store (2).

In case of Atlantic Salmon higher (p <0.05) quantity of coliform bacteria was recorded in the store (2) in comparison to the store (1), 4.5 log CFU.g^{-1} (3.2 x 10^4 CFU.g^{-1}) and 3.5 log CFU.g^{-1} (3.2 x 10^3 CFU.g^{-1}) respectively.

Regarding the store (3), 3.7 log CFU.g^{-1} (5.10^3 CFU.g^{-1}) was found in salmon, which is comparable quantity of coliforms as in the store (1). In comparison to **Kordiovská et al. (2004)**, higher quantities of coliform bacteria were recorded in our experiment. In the above mentioned experiment there was found the quantity 2.9 log CFU.g^{-1} (7.6 x 10^2 CFU.g^{-1}) of coliform bacteria. Lower quantities were also recorded in fresh fish before storage by **Miks-Krajnik (2016)**, specifically up to 4 log CFU.g^{-1}.

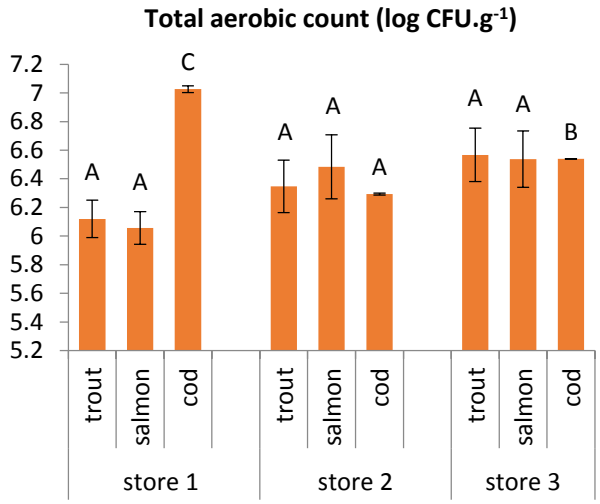

Figure 1 Comparison of the total aerobic count (log CFU g^{-1}) in the samples of fresh fish from three different stores (1 = specialized wholesale of fish, 2 = chain store, 3 = fishmonger´s). The averages marked with different letters are statistically different (p <0.05); n = 3 within the observed factor (store).

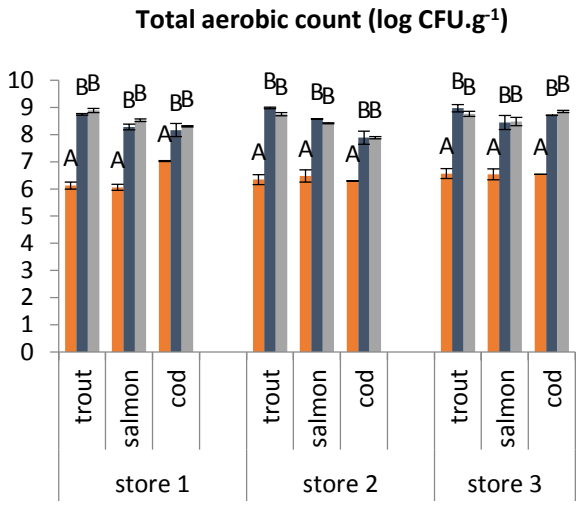

Figure 2 Comparison of the total aerobic count (log CFU g^{-1}) in the samples of fresh fish (marked red) and fish stored 2 days at the temperature 4°C (marked blue) and at the temperature 8°C (marked greed) from three different stores (1=specialized wholesale of fish, 2=chain store, 3=fishmonger´s). The averages denoted by different letters are statistically different (p < 0.05); n = 3 within the observed factor (storage temperature).

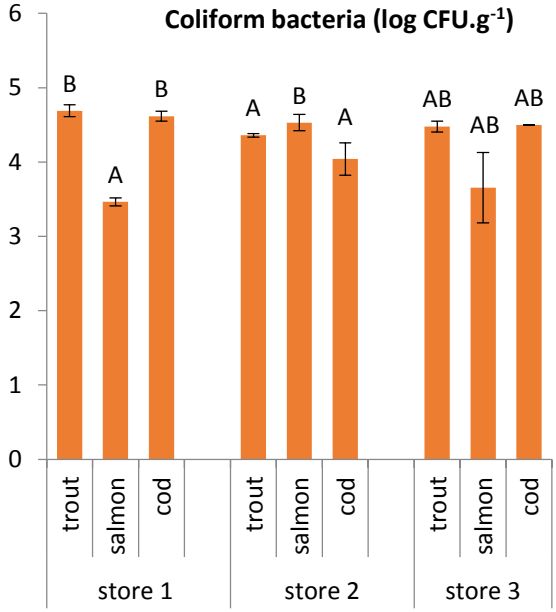

Figure 3 Comparison of the quantity of coliform bacteria (log CFU.g^{-1}) in the samples of fresh fish from three different stores (1 = specialized wholesale of fish, 2 = chain store, 3 = fishmonger´s). The averages denoted by different letters are statistically different (p <0.05); n = 3 within the observed factor (store).

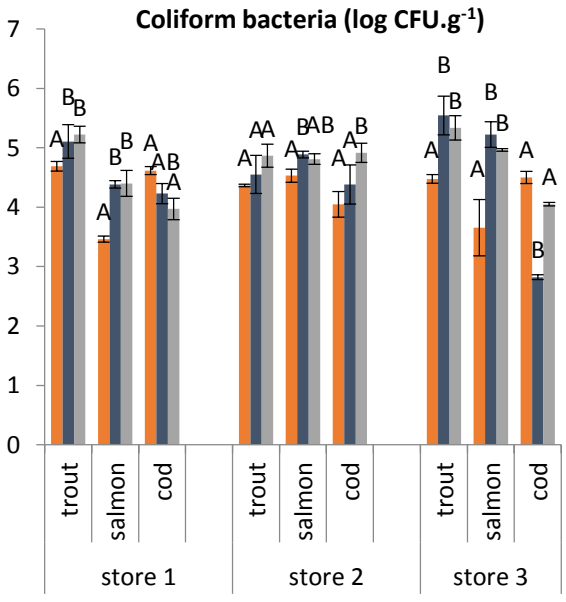

Figure 4 Comparison of the quantity of coliform bacteria (log CFU.g^{-1}) in the samples of fresh fish (marked red) and fish stored for 2 day at the temperature 4 °C (marked blue) and at the temperature 8 °C (marked greed) from three different stores (1 = specialized wholesale of fish, 2 = chain store, 3 = fishmonger´s). The averages denoted by different letters are statistically different (p <0.05); n = 3 within the observed factor (storage temperature).

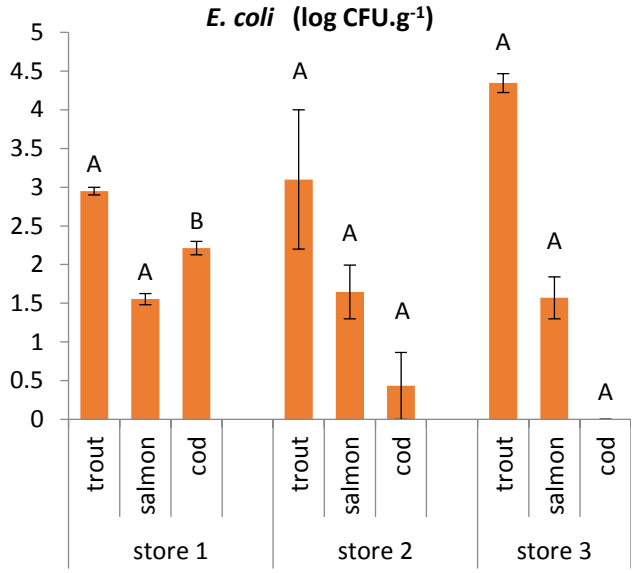

Figure 5 Comparison of the quantity of *E. coli* (log CFU.g^{-1}) in the sample of fresh fish from three different stores (1 = specialized wholesale of fish, 2 = chain store, 3 = fishmonger´s). The averages denoted by different letters are statistically different (p <0.05); n = 3 within the observed factor (store).

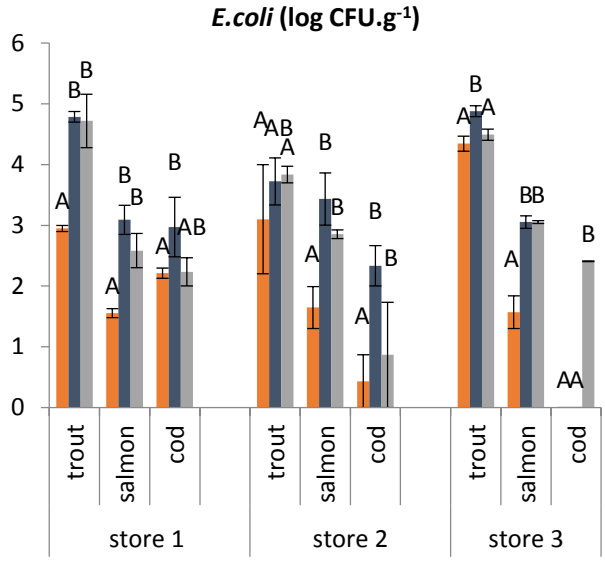

Figure 6 Comparison of the quantity of *E. coli* (log CFU.g^{-1}) in samples of fresh fish (marked red) and fish stored for 2 days at the temperature 4 °C (marked blue) and at the temperature 8 °C (marked greed) from three different stores (1 = specialized wholesale of fish, 2 = chain store, 3 = fishmonger´s). The averages denoted by different letters are statistically different (p <0.05); n = 3 within the observed factor (storage temperature).

Coliform bacteria belongs to the family of *Enterobacteriaceae*, which can play a key role in food spoilage due to their ability to metabolize amino acids to malodorous volatile compounds **(Remenanta et al., 2015)**. Higher quantity of coliforms in food is attributed especially to incorrect treatment of food and to not keeping of storage temperatures (**Görner, Valík, 2004)**.

During storage (Figure 4) there was increase (p <0.05) of the quantity of coliform bacteria in a majority of the observed samples. The highest value was recorded in trout bought in the store (3), specifically 5.5 log CFU.g^{-1}. Different storage temperature did not have (except for the cod samples) impact on the quantity of coliform bacteria (p >0.05).

The* quantity of bacteria *E. coli

In case of Rainbow Trout and Atlantic Salmon (Figure 5) the individual stores were not different in the quantity of *E. coli* (p >0.05). The highest values were recorded in the sample of trout bough in the store (3), specifically 4.4 log CFU.g^{-1} ($2.2 \cdot 10^4$ CFU·g^{-1}). Considering salmon, the quantities of *E. coli* were comparable and they were from 1.6 log CFU.g^{-1} (3.5×10^1 CFU.g^{-1}) to 1.7 log CFU.g^{-1} (4.5×10^4 CFU.g^{-1}).

In the samples of Atlantic cod, higher quantity (p <005) of *E. coli* in the sample from the store (1) was recorded: 2.2 log CFU.g^{-1} (1.6×10^2 CFU.g^{-1}), in the samples from the store (3) bacteria *E. coli* were not detected.

According to **ČSN 56 9609 (2008)** the highest permitted quantity for *E. coli* in fresh fish and their parts intended for heat processing is 2 log CFU.g^{-1}, respectively 2.7 log CFU.g^{-1} (5×10^2 CFU.g^{-1}) in two samples from five. In our experiment the maximal legislative limit was not kept only in case of the trout samples. Despite norms do not have a binding character, it is necessary to take these values as limiting in case of missing legislative requirements. **The Commission Regulation (ES) 2073 (2005)** states the highest permitted values of *E. coli* only for products of boiled crustaceans and molluscs.

During storage (Figure 6) there was increase (p <0.05) in the quantity of *E. coli* a majority of the observed fish samples, expect for trout (the store 2). The highest quantity of *E. coli* was recorded in trout bought in the store (3), specifically 4.9 log CFU.g^{-1}. Different storage temperature did not have (except for the samples of trout from the store 3) impact on the quantity of *E. coli* (p >0.05). The highest permitted quantity (2.7 log CFU.g^{-1}) was exceeded by all fish samples at the storage temperature 4 °C, except for two cod samples. At the storage temperature 8 °C it was 4 samples. If we would take 2 log CFU.g^{-1} as the highest threshold, then only two analysed samples would meet this requirement.

Vibrio parahaemolyticus

Bacteria *Vibrio parahaemolyticus* were detected in 4 samples of fresh fish, especially in the samples of trout from the store (2) 0.9 log CFU.g^{-1}, in the samples of salmon from the stores (2) or (3) 1.8 log CFU.g^{-1}, and 0.5 log CFU.g^{-1} respectively, and in the sample of cod from the store (1) 0.3 log CFU.g^{-1}.

Vibrio parahaemolyticus is widely distributed in the marine environments and considered as the leading cause of human gastroenteritis **(Alaboudi et al., 2016)**. This pathogen is connected especially with fish products and sea products **(Komprda, 2004)**. Higher quantities of the species *Vibrio* were proved in fish by **Aagesen and Häse (2014)**, especially in case of violation of cooling chain or repeated temperature fluctuations. Major outbreaks are associated with the warmer month. Control of *Vibrio parahaemolyticus* growth in shellfish meats is temperature

depended in the first place **(Fernandes, 2009)**. The negative criteria in 25 grams is stated in fish in case of *V. parahaemolyticus* **(ČSN 56 9609, 2008)**.

***Salmonella* species**

Bacteria of the Salmonella species were detected in 4 samples. It included two samples of trout from the store (1), one sample of salmon from the store (1) and one sample of salmon from the store (2). **The Commission regulation (ES) 2073 (2005)** on microbiologic criteria for food requires no presence of salmonella in 25 g of a sample. The above stated samples thus do not correspond with the legislative limit. On contrary **Patil et al. (2013)** during observing microbiological quality of fish stored at 4 °C did not detect salmonella in samples.

The presence of *Salmonella* in seafood may derive from contamination occurring in the natural aquatic environment, in aquaculture or cross-contamination during store, transportation and processing **(Amagliani et al., 2012)**. Salmonella was not isolated also from the freshwater fish in the study of Terenjeva et al**.** According this study salmonella-negative results are in agreement with those of previous reports for France, Great Britain, Portugal, Czech Republic, Slovakia, and the United States for marine and freshwater fish. But, the presence of *Salmonella* in fish was detected in several countries of Asia and Africa **(Terenjeva et al., 2015)**. **Yang et al. (2015)** study implied that pollution by human or animal feces and sewage may be a major reason for the high prevalence of *Salmonella* in freshwater fish samples.

CONCLUSION

Ensuring health safety of food is a basic responsibility of all producers. Controlling bacterial contamination is important all the way from catching and handling to processing. In processing and producing operation there are therefore implemented HACCP systems, which are responsible not only for analysing danger, but especially for eliminating this danger at an acceptable level and to choose effective preventive measures.

Microbial criteria and monitoring of microbial levels are important part of food inspection and determine acceptability or unacceptability of fish and seafood for the consumers.

In conclusion, it is possible to state that quality of fish bought by us was in all types of stores comparable and that a consumer does not have to be afraid to buy fish also in stores that are not exclusively specialized on sale of these products. Despite our results of the quantity of observed microorganisms in comparison to results of another studies were higher, they met legislative requirements (except for five analysed samples). Obviously, pathogens (*Salmonella* spp., *V. parahaemolyticus*) should not occur in products offered to consumers. Considering fish storage, it is not possible to recommend storage at fridge temperatures because this temperature is often higher than 8 °C and fish stored like this would reach limits of spoilage after 2 days (8 log CFU.g^{-1}). Based on the obtained results it is possible to recommend consumption of fish on the day of purchase or storage at the temperature of melting ice, specifically from -1 °C to 2 °C.

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Contact address:

Olga Cwiková, Mendel University in Brno, Faculty of Agronomy, Department of Food Technology, Zemědělská 1, 61300 Brno, Czech Republic, E-mail: cwikova@mendelu.cz.

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EFFECT OF FARMING SYSTEM ON COLOUR COMPONENTS OF WHEAT NOODLES

Magdalena Lacko-Bartosova, Lucia Lacko-Bartosova

ABSTRACT

Colour of noodles is definitely a key element of a consumer's buying decisions. It can be influenced by many factors. Conditions, under which is winter wheat grown, can be considered as one of these factors. The aim of this work was to evaluate colour of noodles that were prepared from winter wheat grown in ecological and integrated arable farming systems, after different forecrops with two levels of fertilization (fertilized and unfertilized) during the years 2009, 2010 and 2011. Winter wheat noodles were prepared from white flour and wholegrain flour and its colour was evaluated using the spectro-colorimeter. Colour was measured by three coordinates: lightness L*, red/ green value a* and yellow/ blue value b*. Wholegrain noodles had lower L* value, so they were darker than white flour noodles, with higher redness and higher yellowness. Colour of white flour noodles and wholegrain noodles was significantly influenced by crop nutrition (fertilized and unfertilized variants), farming system and meteorological conditions during experimental years. Wholegrain noodles from ecological system were darker, with lower lightness and higher redness compared to noodles from integrated system. White flour noodles from ecological system were also darker compared to noodles from integrated system. Fertilization decreased lightness of white flour noodles, on the contrary, fertilization increased the lightness and decreased the redness of wholegrain noodles. In non-fertilized treatment, ecological wheat noodles were darker, with higher redness and yellowness than noodles prepared from winter wheat grown in integrated arable farming system.

Keywords: ecological arable system; integrated arable system; wheat noodle colour

INTRODUCTION

The key quality attributes in the evaluation of noodles include texture and colour, which are important quality factors since they are associated with flour **(Chang and Wu, 2008)**. Flour colour influences the quality of end-use products of common wheat. It is determined by the content of yellow pigment in the endosperm. A high yellow pigment content is preferred for yellow alkaline noodles. A minimal addition of alkaline salts leads to an improved texture and yellowness, as well as the antioxidant functions, derived from its main component carotenoids during the aging process **(Zhang et al., 2009)**. Flour colour is mainly controlled by genetic factors, several studies mentioned the effects of the 1B.1R translocation on flour colour and yellow pigment content in common and durum wheat **(Liu et al., 2005)**.

Flour extraction rate has an important influence on noodle attributes, especially colour. Studies on dried noodles showed a decline in brightness and increase in yellowness with increased extraction rate **(Kruger et al., 1994; Lee et al., 1987)**. Noodle darkening increases with the increase of flour extraction rate. This is due to the action of polyphenol oxidase enzymes which are largely located in the bran layer **(Fuerst et al., 2006)**. Low flour extraction and ash levels are preferred for the manufacture of noodles with a clean and bright appearance. Low ash content (1.4% and less) in flour is always an advantage for noodles since flour ash is traditionally viewed as causing noodle discoloration. For white salted noodles, a white or creamy white colour is desirable with firm texture. The level of natural yellow pigment levels in flour is highly correlated with noodle colour, which is wheat variety dependent. For yellow alkaline noodles, a bright yellow colour is required. The primary component of yellow colour development in alkaline noodles is due to a pH dependent, chemically induced colour shift in water-soluble flour flavonoids, with a secondary effect due to flour xanthophylls **(Fu, 2008; Asentorfer et al., 2006)**. It is well known that the amber-yellow colour of semolina is due to the presence of natural pigments from carotenoid and xanthophyll families in wheat. As these pigments increase, the yellow colour detected by the human eye becomes brighter and more vivid. It is also known that semolina with a high pigment content do not always produce very yellow pasta. This is because carotenoids and xanthophylls have components which are affected by several oxidizing enzymes. It is important to have a durum wheat with a low content of the enzymes, exercising this negative action **(Landi, 1995)**. Since colour is a key element of a consumer's buying decisions, it is important to ensure noodle colour options availability on the market. The influence of farming system and plant nutrition pattern on colour components of wheat noodles on the basis of CIE L*a*b* readings is not broadly documented. In this study, the relationship between winter wheat noodle colour components, obtained from different farming systems,

nutrition treatment and flour extraction rate was investigated.

MATERIAL AND METHODOLOGY

Materials

Field experiments of ecological and integrated arable farming systems were conducted at the Research experimental station of the Faculty of Agrobiology and Food Resources in Nitra during 2009, 2010 and 2011 growing periods. Experiments were established on a Haplic Luvisol developed at proluvial sediments mixed with loess. The altitude of the experimental field is 178 m. The location has a continental climate with an average temperature 19.7 °C in July and -1.7 °C in January, an average annual precipitations are 561mm. Both arable farming systems were composed of six-course crop rotations. The ecological system was composed of the crop rotation: beans + alfalfa – alfalfa – winter wheat – peas – maize – spring barley. The integrated system consisted of the crop rotation: winter wheat – peas – winter wheat — maize – spring barley – alfalfa (3 years at the same plot). Subplots were fertilized (F) and unfertilized (NF). The fertilized variant in ecological system was based on 40 t of manure while the integrated system also received 40 t of manure plus synthetic fertilizers (Table 1), treatments were replicated four times. Sowing and harvesting dates, rainfall and average temperature calculated for vegetative period of the crop, synthetic fertilizer inputs ($kg.ha^{-1}$) applied in the integrated system are shown also in the Table 1. Nitrogen fertilizers were applied in three split applications. Winter wheat was grown within both farming systems, after different forecrops, fertilized and unfertilized variants. Winter wheat noodles were prepared from white flour and from wholegrain flour. Egg noodles with moisture of 30.5% were produced on the apparatus for pasta producing P3 (La Monferina) and were dried at 50 °C for 12 hours.

Milling

Winter wheat samples were milled without further conditioning on a Quadrumat Senior Mill (Brabender, Germany). Combined fractions I. + II. are referred to here as „white flour". Wholegrain flour was obtained by grinding on the special mill PSY MP (Mezos, s.r.o, Czech Republic.

Colour Analysis

Noodle colour was evaluated using the spectrophotometer SP60 (X-Rite, Inc., Germany) which allows very accurate measurement of the basic optical properties of the surface. The International Commission on Illumination (CIE, Commission Internationale l'Eclairage) has standardized colour order system to derive values for describing colour. The CIE Colour System utilize three coordinates to locate a colour in a three-dimensional colour space and is used to compare the colours of two objects. Colour of an object is defined by three coordinates (L*a*b*). CIE L* values represent lightness, a* denotes the red/green value and b* the yellow/blue value. CIE L*a*b* uses Cartesian coordinates to calculate a colour in a colour space. The a* axis runs from left to right, from -60 (pure green) to +60 (pure red), + value indicates a shift toward red. The b* axis is at an angle of 90 degrees to a* axis. Values on the b* axis range from -60 (pure blue) to +60 (pure yellow), so + value represents a shift toward yellow, - value means a shift toward blue. The L* axis range from 0 (pure black) at the bottom to 100, which represents pure white.

Colour evaluation of cooked noodles was carried out in triplicate; analysis of variance was used for statistical evaluation. The statistical tests were performed with the software STATISTICA version 10.0.

RESULTS AND DISCUSSION

L*values

CIE L* values of wheat white flour noodles and wholegrain noodles were significantly ($p \leq 0,05$) influenced by farming system (Table 2). Average lightness of white flour noodles was 75.92 and wholegrain noodles 56.46. There was significant variation between ecological and integrated system in both, white flour and wholegrain noodles. Noodles prepared from w. wheat grown in ecological system were darker with significantly lower CIE L* values. Crop nutrition also significantly influenced the lightness of noodles.

a*values

The mean CIE a* value for the white flour noodles was 0.92, for wholegrain noodles 9.8, what means that the redness (a*) of wholegrain noodles was almost ten times higher compared to white flour noodles. Significant differences were observed for a* value in wholegrain noodles between farming systems, the redness of ecological wholegrain noodles was higher than integrated noodles. However, there was no significant difference in the a* values in white flour noodles. Crop nutrition significantly influenced the redness of wholegrain noodles, with higher CIE a* value for non-fertilized variant. Variation between a* values for white flour noodles and two crop nutrition treatments was not significant.

b* values

The mean CIE b* value for wholegrain noodles was 20.52, for white flour noodles 18.83, the yellowness (b*) of wholegrain noodles was higher than white flour

Table 1 Crop management data for winter wheat 2009-2011.

Growing season	Sowing date	Harvest date	Rainfall (mm)	Average temperature (°C)	Nitrogen ($kg.ha^{-1}$)	Phosphorus ($kg.ha^{-1}$)	Potassium ($kg.ha^{-1}$)
2008 – 2009	13/10/08	15/07/09	426	9.6	82.5	37.5	20.0
2009 – 2010	07/10/09	28/07/10	610	8.8	62.5	7.5	40.0
2010 – 2011	12/10/10	13/07/11	326	8.6	76.0	30.0	120.0

Table 2 Effect of farming system, crop nutrition and year on colour evaluation of wheat noodles.

white flour noodles				
		lightness L*	**colour a***	**colour b***
Crop nutrition	F	74.49 a	0.95 a	19.14 a
	NF	77.34 b	0.88 a	18.51 a
Farming system	ES	75.58 a	0.92 a	19.05 a
	IS	76.26 b	0.92 a	18.60 a
Year	2009	79.92 c	1.75 c	22.54 c
	2010	74.95 b	0.88 b	20.03 b
	2011	72.89 a	0.12 a	13.91 a
Standard error		±3.479	±0.717	±3.869
wholegrain noodles				
		L*	**a***	**b***
Crop nutrition	F	57.45 b	8.82 a	20.35 a
	NF	55.47 a	9.35 b	20.69 a
Farming system	ES	55.61 a	9.24 b	20.65 a
	IS	57.32 b	8.93 a	20.39 a
Year	2009	57.06 a	10.15 c	21.01 b
	2010	55.42 b	8.44 a	19.58 a
	2011	56.91 a	8.67 b	20.97 b
Standard error		±2.594	±0.974	±0.897

Legend: F = fertilized; NF = non-fertilized; ES = ecological system; IS = integrated system.

noodles. The difference was significant (Table 3). Variations between CIE b* values for farming systems and crop nutrition, both white flour and wholegrain flour noodles, were not significant (Table 2).

There was significant variation for all colour components L*a*b* caused by variable meteorological conditions during three growing seasons (years 2009 – 2011).

Effect of fertilisation on colour components was significant (Table 3). CIE L*a*b* values were significantly different for farming system in non-fertilized treatment. Ecological wheat noodles were darker (lower L*), with higher redness (a*) and yellowness value (b*). No significant effect of farming system on CIE L*a*b* values was recorded under fertilized conditions. The effect of wholegrain flour and white flour on noodle colour components showed the same tendency under both, fertilized and non-fertilized treatment. Wholegrain noodles were darker, with higher redness and yellowness.

The correlation between the L* value and a* value, but also b* value, was positive, significant (r^2 = 0.79;

Table 3 Winter wheat noodle colour evaluation, effect of fertilisation.

non-fertilized treatment				
		lightness L*	**colour a***	**colour b***
Farming system	ES	65.82 a	5.32 b	20.08 b
	IS	66.99 b	4.92 a	19.11 a
Noodles	W	55.47 a	9.35 b	20.68 b
	Fl	77.34 b	0.88 a	18.51 a
Standard error		±11.426	±4.344	±2.876
fertilized treatment				
		L*	**a***	**b***
Farming system	ES	66.04	4.84	19.61
	IS	65.91 n.s.	4.93 n.s.	19.88 n.s.
Noodles	W	57.45 a	8.82 b	20.35 b
	Fl	74.49 b	0.95 a	19.14 a
Standard error		±3.479	±0.716	±3.869

Legend: ES = ecological system; IS = integrated system; W = wholegrain flour noodles; Fl = white flour noodles.

Table 4 Correlation analysis of CIE L*a*b* values.

white flour noodles			wholegrain noodles		
	a*	**b***		**a***	**b***
L*	0.79 **	0.71 **	**L***	-0.39 *	0.02
a*		0.89 **	**a***		0.58 *

Marked values are significant at ** p <0.01, * p <0.05.

$r^2 = 0.71$) for white flour noodles. Low CIE a* values in white flour noodles are strongly correlated with lightness (L*). For wholegrain noodles the correlation between L* and a* value was negative, but not strong ($r^2 = -0.39$). The correlation between L* and b* value was not significant (Table 4).

Miskelly (1984) studied the influence of components contributing to the colour and brightness of flour, flour paste, and Chinese and Japanese style noodles. Differences in brightness and yellowness were attributable to a multitude of factors including wheat cultivar, milling extraction rate, protein content, starch damage, and brown and yellow pigments. Most of the variation was attributed to genetic factors, but growing environment and milling procedures were also important. Noodle brightness is related inversely to protein content and to flour-grade colour. Lutein is a yellow plant pigment that belongs to the carotenoid family, namely to Xanthophylls. It acts as an effective antioxidant, it protects the organism against heart diseases and cancer **(Šivel et al., 2014)**.

Humphries et al., (2004) analysed whole-meal wheat, including common, durum varieties, and triticale samples for their carotenoid content and colour. A positive correlation between CIE b* (yellowness) and lutein concentration was shown, there was little correlation between CIE L* or CIE a* (redness) and lutein, α or β carotene. In contrast, the b* value correlated well with the concentration of α and β carotene, but those wheat groups with the lowest CIE b* values did not have a strong correlation. Study has identified CIE b* as a useful diagnostic for rapid screening of wheat varieties for lutein content and was also indicative of the provitamin A carotenoid content. In our experiment, variation in CIE b* values caused by farming system, crop nutrition treatment and flour extraction rate was lower than variation in CIE a* value. We assumed, that differences in CIE L* and CIE a* values can't be attributable to one factor – the concentration of carotenoids.

Ma et al., (2007) showed that N fertilizer increased the redness and yellowness, while brightness decreased. **Wang et al., (2004)** reported that wheat grain protein content strongly correlated with noodle colour.

In small quantities, flavonoids are also present in cereals, located in the pericarp **(Dykes and Rooney, 2007)**. In our previous study, higher concentration of total flavonoid and free flavonoid content was found in wholegrain flour compared to white flour. Free flavonoids represented 77.9% of the total content for wholegrain flour and 68.7% for white flour. Significant effect of farming system and fertilization treatment on free and total flavonoid contents was recorded for wholegrain flour. Concentrations of phenolic compounds were significantly higher in wholegrain flour in all, free, bound and total content. In contrary, farming system showed significant differences in white flour, when total, free and bound phenolic contents were higher in ecological system. Fertilization treatment was significant also for white flour **(Kosík et al., 2014)**. It is estimated that flavonoids account for approximately two thirds of the phenolics in our diet and the remaining one third are from phenolic acids **(Liu, 2004)**.

Large controlled studies with a more factorial approach to the effect of the different components involved in cultivation systems have shown that the ecological production is more likely to favour the synthesis of secondary compounds in food plants. Both environmental factors and production methods have been shown to affect plant growth and composition including the content of secondary bioactive metabolites that may be important to health **(Holmboe-Ottesen, 2010)**.

Bran is a key factor in determining wholegrain products health benefits. Bran has higher vitamin and mineral contents than endosperm, high antioxidant activity and higher secondary metabolites content. These characteristics give wheat bran and wholegrain food very interesting nutritional properties, by reducing the risks of developing chronic diseases **(Liu, 2007; Li et al., 2007; Kosík et al., 2014)**.

Bednářová et al., (2015) concluded that breads produced of blue coloured wheat wholemeal flour were not below the average in sensory properties and its market position could be very high in the future, due to the content of health promoting substances.

Variation in concentration of phytochemicals caused by growing environment and milling extraction rate may affect the colour of end product. A brown or red hue in pasta is detrimental to consumer acceptance in many countries where a bright amber colour is preferred **(Owens, 2011)**.

CONCLUSION

Colour of noodles is an important quality factor influencing the decision of a consumer. Farming system, fertilization, flour extraction rate, forecrop and weather conditions during growing period of winter wheat may have significant effect on the colour of wheat noodles. Colour was measured by three coordinates: lightness L*, red/ green value a* and yellow/ blue value b*. Wholegrain noodles had lower L* value, so they were darker than white flour noodles, with higher redness and higher yellowness. Wholegrain noodles from ecological system were darker, with lower lightness and higher redness compared to noodles from integrated system. White flour noodles from ecological system were also darker compared to noodles from integrated system. Fertilization decreased lightness of white flour noodles, on the contrary, fertilization increased the lightness and decreased the redness of wholegrain noodles. In non-fertilized treatment, ecological wheat noodles were darker, with higher redness

and yellowness than noodles prepared from winter wheat in integrated arable farming system. Since colour is a key element of buying preferences, increased interest of consumers for more healthy, wholegrain food may shift their acceptance of darker colour of pasta.

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Contact address:

Magdalena Lacko-Bartosova, Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Sustainable Agriculture and Herbology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: magdalena.lacko-bartosova@uniag.sk.

Lucia Lacko-Bartosova, University of Economics in Bratislava, Faculty of National Economy, Department of Applied Informatics and Computing Technology, Dolnozemská cesta 1, 852 35 Bratislava, Slovakia, E-mail: lucia.lacko-bartosova@euba.sk.

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CHEMICAL AND PHYSICAL PARAMETERS OF DRIED SALTED PORK MEAT

Petronela Cviková, Juraj Čuboň, Simona Kunová, Miroslava Kačániová, Lukáš Hleba, Peter Haščík, Lenka Trembecká, Gabriela Bartošová

ABSTRACT

The aim of the present study was analysed and evaluated chemical and physical parameters of dried salted pork neck and ham. Dried salted meat is one of the main meat products typically produced with a variety of flavors and textures. Neck (14 samples) and ham (14 samples) was salted by nitrite salt mixture during 1week. The nitrite salt mixture for salting process (dry salting) was used. This salt mixture contains: salt, dextrose, maltodextrin, flavourings, stabilizer E316, taste enhancer E621, nitrite mixture. The meat samples were dried at 4 °C and relative humudity 85% after 1 week salting. The weight of each sample was approximately 1 kg. After salting were vacuum-packed and analysed after 1 week. The traditional dry-cured meat such as dry-cured ham and neck obtained after 12 – 24 months of ripening under controlled conditions. The average protein content was significantly (p <0.001) lower in dried pork neck in comparison with dried salted pork ham. The average intramuscular fat was significantly (p <0.001) lower in dried pork ham in comparison with dried salted pork neck. The average moisture was significantly lower (p ≤0.05) in dried salted ham in comparison with dried pork neck. The average pH value was 5.50 in dried salted pork ham and 5.75 in dried salted pork neck. The content of arginine, phenylalanine, isoleucine, leucine and threonine in dried salted ham was significantly lower (p <0.001) in comparison with dried salted pork neck. The proportion of analysed amino acids from total proteins was 56.31% in pork salted dried ham and 56.50% in pork salted dried neck.

Keywords: 5 pork; neck; ham; chemical quality; intramuscular fat; moisture; amino acids; proteins; pH value

INTRODUCTION

Meat quality has always been important to the consumer, and it is an especially critical issue for the meat industry in the 21st century **(Joo et al., 2013)**. In the course of production, the nutritional composition of commercial meat products undergoes changes due to variations in the meat and non-meat ingredients and the processing conditions. Animal production practices (genetic and dietary strategies) play an important role in the nutritional quality of meat raw materials **(Jiménez-Colmenero, 2011)**. Consumers require meat products with high quality, health benefits and high safety. Nevertheless, processing of the muscle into ready-to-eat products with acceptable and high nutritional value, convenience and palatability to use is indispensable to add value to this muscle much higher it´s conventional profitability. The pork dry-cured quality is defined as a combination of different characteristics of raw and processed meat **(Joo et al., 2013)**.

The characteristics like water-holding capacity (WHC) of muscle and pH value are important because it affects both qualitative and quantitative aspects of meat and meat products **(Van der Wal et al., 1997)**. These important factors have the biggest influence on biochemical changes in the muscle *post mortem*. The most popular meat products processed mostly from pork muscle is dry-cured meat according consumers expressions due its typical flavour and palatability **(Ventanas et. al., 2007)**.

Dry-cured ham is one of the main meat products typically produced with a variety of flavors and textures. The processing of dry-cured ham is based on traditional manufacturing practices consisting primarily of salting and drying steps, followed by a more or less extensive ripening period, which is dependent on the desired final product quality **(Toldrá, 2015)**. The dry – cured meat is produced salting, drying and ripening. The ripening process of dry-cured meat involves complex of biochemical and chemical changes **(Ruitz et. al., 2002)**.The ripening, is the processing step that develops the unique and characteristic aroma and flavor. The intensity of the dry- cured aroma and flavor is a result of extensive lipolysis and proteolysis that are proportional to the length of the aging time. The quality characteristics of dry-cured meat products are related to the raw material and processing technology **(Jurado et al., 2007)**.

Dry-aging is typically the aging of premium meat under critically controlled ambient conditions of temperature, relative humidity and airflow. These parameters need to be carefully balanced and monitored to inhibit microbial growth and minimise weight loss while producing excellent eating quality resulting from tenderisation and enhanced flavour **(Yuan et al., 2016)**. The acceptance of dry-cured hams by consumers is mainly determined by their sensory quality. The aroma is perhaps the most important quality parameter and it is markedly affected by the raw material and the processing of the dry- cured meat.

In the case of dry-cured hams, the aroma is due to the presence of many volatile compounds, most of them produced by chemical and enzymatic mechanisms during the post-mortem process **(Sánchez-Peña et al., 2005)**.
Flavor and aroma are key attributes that impact the overall acceptance of dry-cured hams and are markedly affected

by raw material processing techniques and aging time. The flavor and aroma of dry-cured ham can be determined by sensory descriptive analysis and the composition of aroma impact compounds, most of which are produced *post-mortem* by chemical and enzymatic mechanisms **(Pham et al., 2008)**. Flavour is a very important attribute contributing to the sensory quality of meat and meat products. Although the sensory quality of meat includes orthonasal and retronasal aroma, taste as well as appearance, juiciness and other textural attributes **(Neethling et al., 2016)**. Flavour affects on the customer acceptance **(Ruiz et al., 2002)**. In general, important reductions in both moisture content and water activity take place during the production process of dry-cured meat products. This reduction depends on the drying conditions and the decreasing water activity may affect enzyme activity which influences the sensory characteristics of the final product **(Jiménez-Colmenero et al., 2010)**.

Sodium chloride is the very important ingredient, its manufacturing process begins with a salting step during which salt and other curing ingredients (nitrate or nitrite) and additives (ascorbic acid) slowly diffuse into the meat followed by brushing or washing of hams to remove the excess of salt, a post-salting step and a ripening or drying stage **(Martínez-Onandi et al., 2016)**. Salt, nitrate and nitrite are the major ingredients in the cure mix. Salt inhibits the growth of spoilage microorganisms by reducing the water activity and solubilizing some of the myofibrillar proteins. Nitrate is reduced to nitrite and then nitric oxide by nitrate reductase a natural enzyme in the ham. The typical red/pinkish color of ham is due to the reaction of nitric oxide and myoglobin which forms nitrosyl myoglobin **(Zhao et al., 2016)**.

Lipid oxidation is a very important biochemical reaction in dry-cured meat products. Many studies have investigated the relationship between the muscle lipid oxidation and flavor formation in dry-cured meat products and proved that lipid oxidation plays an important role in the formation of the final flavor of dry-cured meat products **(Guofeng et al., 2015)**.

The effect of environmental, nutritional and production factors on intramuscular fat have formerly been quantified as well as their consubsequent influence on meat quality. Genetic factors also influence intramuscular fat deposition **(Pannier et al., 2014)**. Intramuscular fat of dry-cured meat contributes to odour and flavour impression during mechanisms such as lipid oxidation. Fat content is believed to be one of the most crucial quality traits of cured hams (the higher the fat content, the greater the acceptability of cured hams) but what most affects the appearance, texture (juiciness) and intensity and persistence of flavour of dry-cured hams is the intramuscular fat content **(Jiménez-Colmenero et al., 2010)**. Intramuscular fat plays one of important role in the impression of the texture of dry-cured meat, especially in juiciness **(Ventanas et al., 2005)**. Maillard reactions concerned creation of volatile compounds formation **(Ruiz et al., 2002)**.

Proteolysis is one of the most important biochemical processes during the ripening of ham and neck. This biochemical process influences texture and flavour due to the formation of free amino acids and other low-molecular weight compounds. Free amino acids influence directly in taste. The major ways for generation of volatile compounds from amino acids in ham and neck are Maillard and Strecker reactions **(Jurado et al., 2007)**. The sensory quality depends not only of the curing process but also on factors such as the age, breed and feeding of pigs. The chemical changes occurring in different muscles during the ripening of hams and necks influence the ham and neck aroma and flavour **(Diego et al., 2008)**. The flavour of high quality is the result of enzymatic reactions (proteolysis and lipolysis) and chemical processes (lipid autooxidation, Strecker degradation and Maillard reactions) **(González et al., 2008)**.

According to the nutrition importance for human the amino acids are divided into essential: valine (Val), leucine (Leu), isoleucine (Ile), threonine (Thr), methionine (Met), lysine (Lys), phenylalanine (Phe) and tryptophan (Trp); semi-essential: arginine (Arg) and histidine (His); and nonessential ones: glycine (Gly), alanine (Ala), serine (Ser), cysteine (Cys), aspartic acid (Asp), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), tyrosine (Tyr) and proline (Pro) **(Belitz et al., 2001)**.

Passi and de Luca (1998) stated that in the human nutrition it is possible to consider only 10 amino acids as principal, i.e. essential nutrients, which the humans must obtain from various diets. The remaining amino acids may be synthesized from the products of metabolism and of essential amino acids.

The aim of the study was analysed basic chemical and aminoacids composition of dry-cured pork ham and neck salted and matured during 1week. After salting were vacuum-packed and analysed after 1 week.

MATERIAL AND METHODOLOGY

The aim of the present study was to determine and evaluate chemical parameters of dried, salted pork neck and ham. Neck (14 samples) and ham (14 samples) was matured and salted by nitrite salt mixture during 1week. The nitrite salt mixture was used for salting process (dry salting). This salt mixture contains: salt, dextrose, maltodextrin, flavourings, stabilizer E316, taste enhancer E621, nitrite mixture. The meat samples were matured at 4 °C and relative humudity 85% after 1 week of salting. The samples were vacuum-packed and storage 1 week after salting. The weight of each sample was approximately 1 kg.

Determination of chemical composition analysis and amino-acids analysis

The chemical composition and amino-acids composition of the ham and the neck (50 g) was measured by the device Nicolet 6700 (Thermo Scientific, USA). The intramuscular fat content in $g.100g^{-1}$, total proteins in $g.100g^{-1}$, total water in $g.100g^{-1}$, amino-acids in $g.100g^{-1}$ were analysed by the FTIR method. FTIR spectroscopy provides information about the secondary structure content of proteins. This spectroscopy works by shining infrared radiation on a sample and seeing which wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample. Each compound has a characteristic set of absorption bands in its infrared spectrum. The infrared spectrum of the muscular

homogenate analysis was transferred out by molecular spectroskopy method.

Determination of NaCl (salt content)

Samples (approximately 2 g) with 2 mL of indicator were titrated by solution of silver nitrate by using the indicator potassium chromate. This suspension was titrated by solution of silver nitrate until a light orange colour. The amount of chloride ions was evaluated. The titration amount of silver nitrate was divided by weight of sample.

Determination of pH value

The pH value was measured using the pH meter Gryf 209L (Sigma-Aldrich, Czech Republic). The pH value of dried neck and ham at different ripening periods were measured.

Determination of water activity a_w

Water activity of salted and dried neck and ham was determined at 25 °C by using the device FA-st lab (GBX advanced technology, Switzerland).

Statistical analyse

The data were subjected to statistical analysis using the SAS (Statistical Analysis System) package SAS 9.3 using of application Enterprise Guide 4.2. Differences between groups were analysed by t-test.

RESULTS AND DISCUSSION

The traditional dry-cured meat such as dry-cured ham and neck obtained after 12 – 24 months of ripening under controlled conditions **(Dall´asta et al., 2010)**. Nowadays is tendency to make aging time shorter. Chemical parameters of dried salted pork neck and ham were analysed in this article.

Table 1 shows basic chemical parameters of dried salted pork neck and ham. The moisture content ranged from 61.11% to 67.13% in dried salted pork ham and from 52.98% to 64.18% in dried salted pork neck. The average moisture content was 63.52% in dried salted pork ham and 58.88% in dried salted pork neck. There was found significantly lower ($p \leq 0.05$) moisture content in dried salted pork neck in comparison with dried salted pork ham. **Benedini et al., (2012)** found out similar results of moisture in ham (61.2%) in dried salted *biceps femoris* muscle.

The protein content ranged from 22.91% to 26.34% in dried salted pork ham and from 18.41% to 22.22% in dried salted pork neck. The average protein content was 23.37% in dried salted pork ham and 19.98% in dried salted pork neck. There was found significantly lower ($p < 0.001$) protein content in dried salted pork neck in comparison with dried salted pork ham.

Kunová et al., (2015) found out similar our results protein content 24.87% in dried salted pork ham and 20.51% in dried salted pork neck. **Lorido et al., (2015)** found out higher protein content (39.26%) in *musculus semimembranosus.* **Benedini et al., (2012)** found out protein content 27.00% in *biceps femoris* muscle.

The intramuscular fat ranged from 3.38% to 8.99% in dried salted pork ham and from 7.26% to 20.80% in dried salted pork neck. The average intramuscular fat was 4.05% in dried salted pork ham and 14.11% in dried salted pork neck. There was found significantly higher ($p < 0.001$) intramusculat fat in dried salted pork neck in comparison with dried salted pork ham. **Lorido et al., (2015)** found out higher content of intramuscular fat (10.62%) in *semimembranosus.* **Jiménez-Colmenero et al., (2010)** fond out in Iberian ham content of intramuscular fat in range 2.6 – 9.5%.

The salt content ranged from 3.01% to 6.68% in dried salted pork ham and from 4.35% to 6.05% in dried salted pork neck. The average salt content was 4.85% in dried salted pork ham and 4.41% in dried salted pork neck. There wasn't found statistical difference between salt content in dried salted pork neck in comparison with dried salted pork ham.

Matínez – Onandi et al., (2016) found out salt content in average 5.49% and ranged from 2.87% to 7.91% in

Table 1 The basic chemical composition of dried salted pork ham and neck.

	Moisture (%)	Proteins (%)	Intramuscular fat (%)	Salt (%)
		Ham		
x	63.52	23.37	4.05	4.85
s	2.09	0.68	2.30	1.19
s_x	0.75	0.21	0.65	0.36
min.	61.11	22.91	3.38	3.01
max.	67.13	26.34	8.99	6.68
v%	3.09	2.79	40.10	21.90
		Neck		
x	58.88	19.98	14.11	4.41
s	4.42	0.88	4.55	0.55
s_x	1.64	0.22	1.64	0.19
min.	52.98	18.41	7.26	4.35
max.	64.18	22.22	20.80	6.05
v%	7.55	4.33	32.90	14.00
t-test	+	+++	+++	-

Note: -$p > 0.05$; +$p \leq 0.05$, ++$p < 0.01$, +++$p < 0.001$.

Table 2 Physical parameters of dried salted pork ham and neck.

Parameters	pH value	Water activity (a_w)
Ham		
x	5.50	0.899
s	0.06	0.019
s_x	0.03	0.003
min.	5.75	0.822
max.	6.05	0.945
v%	0.75	1.235
Neck		
x	5.75	0.935
s	0.25	0.035
s_x	0.03	0.005
min.	5.42	0.888
max.	6.10	0.955
v%	1.62	2.236
t-test	-	-

Note: -p >0.05; +p ≤0.05, ++p <0.01, +++p <0.001.

Table 3 Content of amino acids of dried salted pork neck and ham (g.100g^{-1}).

Prarameters	x	s	s_x	v%	x	s	s_x	v%	t-test
	neck				ham				
arginine	1.72	0.13	0.05	7.69	1.44	0.10	0.04	7.09	+++
cysteine	0.44	0.03	0.01	6.71	0.47	0.06	0.02	11.9	-
phenylalanine	1.09	0.08	0.03	7.48	0.92	0.07	0.03	7.07	+++
histidine	1.17	0.12	0.05	10.2	0.99	0.13	0.05	13.4	+
isoleucine	1.04	0.09	0.03	8.44	0.84	0.08	0.03	9.23	+++
leucine	2.09	0.17	0.06	7.96	1.72	0.15	0.06	8.44	+++
lysine	2.35	0.18	0.07	7.51	2.01	0.12	0.05	6.06	++
methionine	0.94	0.06	0.02	6.17	0.91	0.04	0.02	4.60	-
threonine	1.19	0.09	0.03	7.42	1.02	0.05	0.02	4.97	+++
valine	1.12	0.11	0.04	9.83	0.97	0.09	0.04	10.2	+

Note: -p >0.05; +p ≤0.05, ++p <0.01, +++p <0.001.

Serrano hams. **Lorido et al., (2015)** found out similar salt content (4.38%) in *musculus semimembranosus.*

Table 2 shows the change in pH value and water activity (a_w) of dried salted pork neck and ham. The pH value ranged from 5.75 to 6.05 in dried salted pork ham and from 5.42 to 6.10 in dried salted pork neck. The average pH value was 5.50 in dried salted pork ham and 5.75 in dried salted pork neck. The pH value of both products showed that meat has not been ripened. **Bednářová et al., (2014)** found out in *musculus semimembranosus* pH values in range from 5.56 to 5.63.

The water activity ranged from 0.822 to 0.945 (a_w) in dried salted pork ham and from 0.888 to 0.955 (a_w) in dried salted pork neck. The average water activity was 0.899 in dried salted pork ham and 0.935 (a_w) in dried salted pork neck. **Bjarnadottit et al., (2015)** found out similar value of water activity in dried ham.

Table 3 shows contein of amino acids composition of dried salted pork neck and ham. The average content of arginine in dried salted pork ham was 1.44 ±0.10 g.100g^{-1} and 1.72 ±0.13 g.100g^{-1}. There was found significantly higher (p <0.001) content of arginine in dried salted pork neck in comparison with dried salted pork ham. The average content of lysine was 2.01 ±0.12 g.100g^{-1} in dried salted pork ham and 2.35 ±0.18 g.100g^{-1} in dried salted pork neck. There was found significantly higher (p <0.01) content of lysine in dried salted pork neck in comparison with dried salted pork ham. The average content of leucine was 1.71 ±0.15 g.100g^{-1} in dried salted pork ham and 2.09 ±0.17 g.100g^{-1} in dried salted pork neck. There was found significantly higher (p <0.001) content of leucine in dried salted pork neck in comparison with dried salted pork ham. The average content of methionine was 0.91 ±0.04 g.100g^{-1} in dried salted pork ham and 0.94 ±0.06 g.100g^{-1} in dried salted pork neck. There wasn't found statistical difference between content of methionine in dried salted pork neck in comparison with dried salted pork ham.

Bučko et al., (2015) found out similar content of aminoacids in the pork *musculus longissimus dorsi* but with lower intramuscular fat content (1.19 g.100g^{-1}). Contents of arginine found out 1.50 g.100g^{-1}, cysteine 0.36 g.100g^{-1}, lysine 2.01 g.100g^{-1} and histidine 1.09 g.100g^{-1}. **Wilkinson et al., (2014)** and **Okrouhlá et al., (2006)** found out the proportion of amino acid from total amount of amino acids. **Wilkinson et al., (2014)** found out content

of arginine 7.16 g.100g^{-1}, lysine 8.64 g.100g^{-1}, leucine 8.68 g.100g^{-1} and methionine 2.97 g.100g^{-1}. **Okrouhlá et al., (2006)** found out content of arginine 7.3 g.100g^{-1}, lysine 9.71 g.100g^{-1} and leucine 8.38 g.100g^{-1}. The proportion of analysed amino acids from total proteins was 56.31% in pork salted dried ham and 56.50% in pork salted dried neck. **Wilkinson et al., (2014)** found out proportion of amino acids from total proteins 66.42%, but they analysed more amino acids in comparison with our experiment.

CONCLUSION

The aim of this article was to determine physical and chemical parameters of dried salted pork neck and ham. The protein content in dried salted pork ham was significantly higher in comparison with dried salted pork neck. The value of intramuscular fat in dried salted pork neck was significantly higher in comparison with dried salted pork ham. The moisture was significantly lower in neck in comparison with dried salted pork ham. The salt content was comparable in neck with ham. The pH value was similar in dried salted pork neck as in dried salted pork ham. The value of water activity (a_w) was similar in ham as in neck. The content of arginine, phenylalanine, isoleucine, leucine and threonine in dried salted ham was significantly lower (p <0.001) in comparison with dried salted pork neck. The proportion of analysed amino acids from total proteins was 56.31% in pork salted dried ham and 56.50% in pork salted dried neck.

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Contact address:

Petronela Cviková, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Products Evaluation and Processing, Tr. A. Hlinku 2, 94976 Nitra, Slovakia, E-mail: alenortepcc@gmail.com.

Juraj Čuboň, Peter Haščík, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: juraj.cubon@uniag.sk.

Simona Kunová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: simona.kunova@uniag.sk.

Miroslava Kačániová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: miroslava.kacaniova@gmail.com.

Lukáš Hleba, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: lukas.hleba@gmail.com.

Peter Haščík, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: peter.hascik@uniag.sk.

Lenka Trembecká, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: lenka.trembecka@uniag.sk.

Gabriela Bartošová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Evaluation and Processing of Animal Products, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: bartosovagabriela031@gmail.com.

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