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Dear Colleaggues,

The theme of the 7th International Congress of Food Technologists, Biotechnologists and Nutritionists was chosen to highlight food scientist's strategic impact on quality, diversity and sustainability of mankind's food supply.

Eminent international scientists from 9 countries will introduce you to the latest scientific achievements through 10 plenary sessions' topics, as well as original scientific research trough oral and poster presentations.

The mission of the Scientific Committee was to put together a congress programme that should have something for everyone in the world of food – scientists, technologists, industry, entrepreneurs, marketing & business managers, educators, academics and students. Thus, there should be a balance between original research in specific food sectors, commercial applications of scientific research and topics/issues with generic appeal.

We belive that You will enjoy and have the benefit from attending this 7th International Congress of Food Technologists, Biotechnologists and Nutritionists, held in this beautiful place, in Opatija, Croatia.

President of Scientific Committee

Prof.dr.sc. Rajka Božanić

Biotechnology and Bioprocess Engineering

3DIS: AN EXPERT SYSTEM FOR *IN SILICO* DRUG DESIGN AND DISCOVERY

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ABSTRACT

Bacterial natural products are important sources of chemical diversity for commercial exploitation by the pharmaceutical industry. In the past few years there has been a lot of interest in generating new compounds as potential drug candidates by manipulating the programming of biosynthetic gene-clusters *in vitro*. In order to assist this process a novel expert system for *in silico* drug design and discovery was developed (*3DIS*). The expert system has server-client architecture, with analysis being carried out on the server and a Java user interface for the client which can be a PC, Mac or Linux. *3DIS* consist of two program packages: for semi-automatic DNA sequence analysis (*ClustScan*) and for the generation of novel gene-clusters by virtual homeologous recombination (*CompGen*). *ClustScan* and *CompGen* were used to generate two specialised databases. *CSDB* is a ClustScan database of well characterised polyketide and nonribosomal peptide natural products. The database contains 170 uniformly annotated natural product gene-clusters. In contrast, *r-CSDB* is a virtual compound database for molecular modelling studies produced by the *CompGen* program package and contains more than 11,796 novel compounds. *In silico* studies are only useful if it is possible to generate strains producing them. Continuing progress in synthetic biology will improve methods to achieve that. A major issue for the pharmaceutical industry is maintaining a continuous supply of promising new leads for drug development. We propose that recombinatorial biosynthesis offers a new and exciting strategy whereby large and chemically diverse libraries of polyketides can first be screened *in silico* and then generated in the laboratory for further new lead development. Given that many polyketides are used clinically as antimicrobials, the *3DIS* comes at an important time when with ever increasing numbers of pathogens are becoming resistant to our current antibiotic armamentarium.

KEYWORDS: *natural products; gene-clusters; annotation; recombinatorial biosynthesis; databases*

INTRODUCTION

Bacterial natural products are important source of chemical diversity for commercial exploitation by the pharmaceutical industry. In the last few years there has been a lot of interest in generating new compounds as potential drug candidates by manipulating the programming of biosynthetic gene-clusters *in vitro* (1).

The modular polyketide synthases (PKS), non-ribosomal peptide synthethases (NRPS) and polyketide/peptide (PKS/NRPS) hybrid gene-clusters, which can collectively be called Thiotemplate Modular Systems (TMS) (2), are gene-clusters whose protein products are involved in the biosynthesis of very important classes of compounds that have many useful biological activities (3). These modular biosynthetic gene-clusters are particularly interesting as they function according to a "building block" principle in which each module is usually responsible for a single extension step during the synthesis of the product. DNA sequencing of PKS, NRPS and hybrid gene-clusters showed that their products are multi-functional enzymes with multi-modular organisation. The growth of the polyketide or peptide carbon chains begins with the condensation of starter unit with the first extender unit and proceeds up to the completion of the linear polyketide, peptide or hybrid chains. Each module - minimally encoding ketosynthase (KS)/condensation (C) domains, acyltransferase (AT)/adenylation (A) domains and acyl carrier protein (ACP)/peptidyl carrier protein (PCP)

domains – is responsible for the extension of a polyketide, peptide or hybrid chain by one building block and might contain additional catalytically active domains for the modification of incorporated building blocks. The last module of a PKS, an NRPS or a hybrid gene-cluster usually ends with a thioesterase (TE) domain that is responsible for release of the product and its cyclisation to form an "aglycon". After that, post-polyketide or post-peptide enzymes complete the biosynthesis of a polyketide, peptide or hybrid final structures (for review see: 4). Modularity implies that the chemical structures of the aglycon products can be predicted from the DNA sequences by analysing the specificity and functionality of the individual domains in the encoded polypeptides (3).

A number of computer programs have been developed for the analysis of new modular PKS, NRPS or hybrid gene-clusters: *SEARCHPKS* (5), *DecipherIT™* (6), *NRPSpredictor* (7), *Biogenerator* (8), *MAPSI*, (9), ClustScan (10), *CLUSEAN* (11), *NP.searcher* (12) and *SBSPKS* (13). Some of them (*SEARCHPKS*, *MAPSI* and *SBSPKS*) have been used to structure and maintain publically available databases of polyketides, peptides and hybrid compounds. The PKSDB-NRPSDB database (14; 13) holds data on publically available polyketide and peptide gene-clusters including domain and module architecture and the chemical structures of the gene-clusters products. Another useful publically available polyketide database is the ASMPKS database (9). There is also the database of non-ribosomal peptide, the Norine database (15) that does not contain information on DNA sequences. In order to use these tools and assist this process we have developed a novel expert system, *3DIS* (an acronym of the term: "*In Silico D*rug *D*iscovery and *D*evelopment"), for the *in silico* drug design and discovery which is described here.

MATERIALS AND METHODS

The *3DIS* has server-client architecture, with analysis being carried out on the server and a Java user interface for the client, which can be a PC, Mac or Linux machine. The CSDB (the ClusScan DataBase) and r-CSDB (the recombinant-ClustScan DataBase) data are stored in a relational database using a PostgreSQL database system. The graphical interface has been implemented using Java Server Page (JSP) technology with the Apache tomcat server (*http://tomcat.apache.org/*). They are available at *http://bioserv.pbf.hr/cms/*. The following informatics tools and languages have been used: Universal Markup Language [UML, (16)], BioSQL v1.29 (*http://obda.open-bio.org/*), Perl (*http://www.perl.org/*), Java (*http://java. sun.com/*) and JavaScript (*http://javascript.internet.com/*). Chemical structures of the starter and extender building blocks were encoded as extended isomeric SMILES [*S*implified *M*olecular *I*nput *L*ine *E*ntry *S*ystem (17)] allowing display of product structures using Jmol v. 11.2.14, 2006 (*http://www. jmol.org/*) and/or ChemAxon (18).

RESULTS AND DISCUSSION

The *3DIS* consists of two suites of programs for the semi-automatic DNA sequence analysis (*ClustScan*) (10) and for the generation of novel gene-clusters by virtual homeologous recombination (*CompGen*) (19). *ClustScan* and *CompGen* are used for the generation of two specialised databases. The *CSDB* is a ClustScan database of well characterised polyketide, peptide or hybrid natural products. On the other hand, *r-CSDB* is a virtual compound database for molecular modelling studies developed by the use of *CompGen* program package.

The ClustScan program package

The ClustScan (the *Clus*ter *Scan*ner) is an integrated suite of computer programs that take a "top down" approach to the annotation of gene-clusters encoding TMSs. ClustScan can analyse the large amounts of data produced by sequencing projects (genome and metagenome data sets) and can generate good predictions of the most likely chemical products from these gene-clusters, allowing identification of interesting gene-clusters. Rapid progress in sequencing technology makes the use of ClustScan ever more important. Without such a tool, it is not practical to carry out sufficiently detailed analyses of the mass of data already available today. For example, even using a conservative estimate that every actinobacterial genome contains 10 TMS gene-clusters and that 1,000 sequenced genomes will soon be available, there will be 10,000 new TMS gene-clusters, potentially encoding novel chemical entities (3; 10). We have used the ClustScan program package

to analyse prokaryotic (20) and eukaryotic (21; 22) genome (3) and metagenome (10) DNA sequences.

The CompGen program package

The CompGen (the *Comp*ound *Gen*erator) is also an integrated programs suite written in Java. Like ClustScan, CompGen runs on a LINUX server with a Java client on the user's computer. The major goal of the CompGen suite of programs is structuring and maintaining a publically available database of the entirely novel chemical entities generated by the in silico modelling of homeologous recombination between sequenced TMS gene-clusters. Like ClustScan, CompGen also predicts the chemical structures from the in silico generated recombinants. The future role of CompGen will be to predict the biological activities of these chemical entities using computer-aided drug design technology (23). Pairwise recombination between 1,000 TMS gene-clusters should generate nearly 1,000,000 new gene-clusters, each potentially producing a novel chemical entity. Most exciting of all, when such a product looks promising in silico, a "designer bug" can be created in the laboratory to produce it (see below).

ClustScan and CompGen were used to structure and maintain publically available databases CSDB and r-CSDB containing genetic, biochemical and chemical information on the well known natural products synthesised by TMSs, as well as of predicted, entirely novel recombinant products.

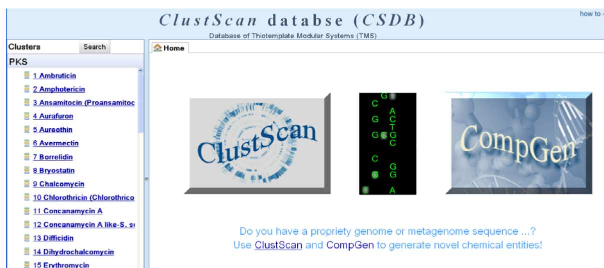
The CSDB database

The CSDB is a database containing genetic, biochemical and chemical information on natural products synthesised by TMSs and annotated using the ClustScan suite of programs (10). At present there are 57 PKS, 51 NRPS and 62 hybrid gene-clusters in *CSDB* (170 in total) (Fig. 1). The CSDB database contains all data starting with gene-clusters DNA sequences together with the DNA and protein sequences of annotated genes, modules and domains of TMS gene-clusters present in FASTA formats. It also contains all known polyketide and peptide building blocks in the form of isomeric SMILES, along with the programmed logic that allows total prediction of linear and partial prediction of cyclic polyketide and peptide chains and aglycons in the 2-D or 3-D forms suitable for further computer processing. Polyketide, peptide and hybrid linear chain and aglycons can be visualised using Jmol or ChemAxon. The CSDB database is fully searchable using ClustScan TMS gene-clusters annotations as well as TMS compound structures. The CSDB data can be manipulated using a number of conventional bioinformatic tools and programs.

The r-CSDB database

The ClustScan (10) and CompGen (Starcevic *et al.*, in preparation) suites of programs were used to structure and maintain the r-CSDB database of predicted, entirely novel recombinant products that can be used for the in silico screening with the computer aided drug design technologies (23). At present there are 47 parental PKS gene-clusters, 777 cluster pairs and 20,187 recombinant gene-clusters in r-CSDB database (Fig. 2) that generates 11,796 unique compounds. Like CSDB, r-CSDB also contains all data starting with gene-clusters recombinant DNA sequence, the DNA and protein sequences of genes, modules and domains and of the recombinant gene-clusters present in FASTA formats. It also contains all known polyketide and peptide building blocks in the form of isomeric SMILES, along with the programmed logic that allows total prediction of linear and partial prediction cyclic polyketide and peptide chains and aglycons in the 2-D or 3-D forms suitable for further computer processing. Polyketide, parental and recombinant linear chain and agycons can be also visualised using Jmol or ChemAxon. The r-CSDB database is also fully searchable using CompGen (Starcevic *et al.*, in preparation) suit of programs of TMS gene-cluster annotations as well as recombinant compound structures. As CSDB, the r-CSDB data can also be manipulated using a number of conventional bioinformatic tools.

A



B

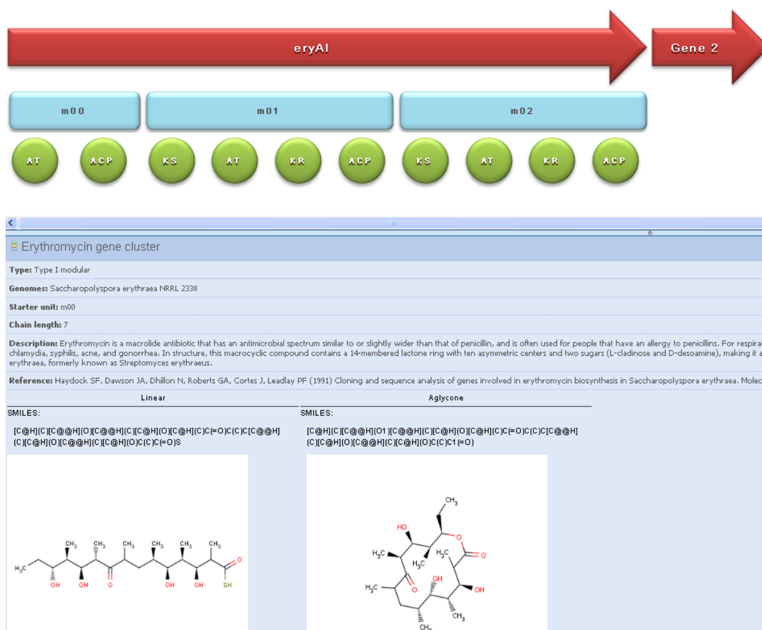
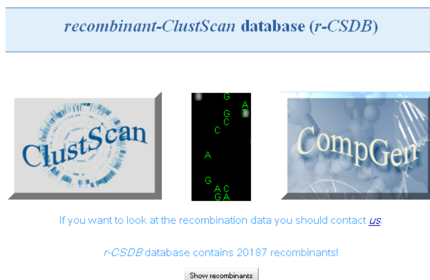


Figure 1. Screenshots of CSDB. (A) CSDB homepage and (B) the data of the erythromycin aglycon.

Construction of strains producing novel chemical structures

In silico studies are only useful if it is possible to generate strains producing them. An efficient system for the selection of recombination between gene-clusters, providing an effective method to mobilize hybrid gene-clusters onto various replicons which is necessary for successful engineering *in vivo* was described (Starcevic *et al.*, in preparation). The advantage of such approaches is that, when a gene-cluster is cloned, it can be used for recombination with all the existing cloned gene-clusters allowing the number of compounds produced to grow rapidly. Continuing progress in synthetic biology (24) should improve the methods of synthesizing long stretches of DNA and reduce the cost. PKS gene-clusters are of significant size (50-150 kb) so they would need comparable technology to that used for chromosome synthesis of *Mycoplasma* species (1.08 Mb).

A



B

recombinant-ClustScan database (r-CSDB)

C

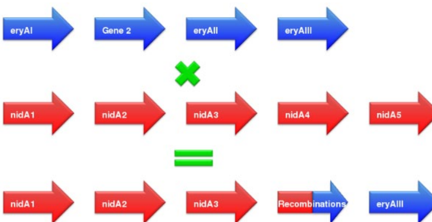


Figure 2. Screenshots of r-CSDB. (A) r-CSDB homepage, (B) the data of 7th recombinant between ery and nid gene-clusters and (C) the graphical presentation of homeologous recombination.

CONCLUSION

A major issue for the pharmaceutical industry is maintaining a continuous supply of promising new leads for drug development. We propose that recombinatorial biosynthesis offers a new and exciting strategy whereby large and chemically diverse libraries of polyketides can first be screened *in silico* and then generated in the laboratory for further new lead development. Given that many polyketides are used clinically as antimicrobials, the *3DIS* comes at an important time, with ever increasing numbers of pathogens becoming resistant to our current antibiotic armamentarium.

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OPPORTUNITIES IN SUCCINIC ACID PRODUCTION BY YEAST

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ABSTRACT

Succinic acid biosynthesis has been investigated using the yeast strains *Candida catenulata*, *Candida zeylanoides*, and *Yarrowia lipolytica*. The strain *Candida catenulata* produced succinic acid up to 5.2 g/L with mass yield of 0.33 g/g; the strain *Candida zeylanoides* excreted 9.4 g/L of succinic acid with mass yield of 0.39 g/g. We developed the promising process for industrial application, which includes the intensive microbial synthesis of alpha-ketoglutaric acid by the yeast *Yarrowia lipolytica* (step 1) and subsequent oxidation of the acid by hydrogen peroxide to succinic acid (step 2). The maximum concentration of succinic acid and its yield were found to be 80 g/L and 0.70 g/g of the ethanol consumed, respectively. The purity of the succinic acid isolated from the supernatant reached 100 %.

KEYWORDS: *Yarrowia lipolytica; microbial production; succinic acid; alpha-ketoglutaric acid; Yarrowia lipolytica; ethanol*

INTRODUCTION

Succinic acid (SA) is a common metabolite formed by microorganisms, plants, and animals. Industrially, SA is used as a flavouring agent for food and beverages, as an intermediate for dyes, insecticides, perfumes, lacquers, and in vehicle water cooling systems and the manufacture of clothing, paint, links, and fibers. In recent years, the commercial demand for SA has been expanding because of its use as a starting material for the synthesis of 1,4-butanediol, adipic acid, tetrahydrofuran, γ-butyrolactone, N-methylpyrrolidone and other four-carbon compounds widely applied in chemical industry, and for the manufacture of easily biodegradable new packing materials (Zeikus et al., 1999). Monoglyceride derivative of SA is useful as emulsifying and ameliorative agents in food, pungent taste-controlling agents for food, its oil-in-water type emulsion is markedly stabilized by lecithin and succinic acid monoglycerides which inhibit feathering, oil-off and had high organoleptic test scores when added to coffee. SA with other composition is used as an additive in meat treatment to make the meat soft, juicy, and tasty after cooking or frying. Addition of aconitic acid, gluconic acid and/or SA, optionally with sclareolides, improves the organoleptic properties of foods, especially by imparting the "Umami" effect and full-mouth feel, in place of sulphur containing amino acids and their salts, pyrrolidonecarboxylic acids and their salts, and nucleotides. A chelant selected from SA, and some other classes are provided as a bactericidal compound for use in cosmetic and food products, dioctylsodium sulfosuccinate is used as a mycotoxin inhibitor in human and animal foodstuffs. Prof. Maria Kondrashova from Institute of Theoretical and Experimental Biophysics of Russian Academy of Sciences and her team developed a special SA preparation to aid the citizens of Tomsk, Siberia in recovering from the devastating effects of a nuclear accident at the Siberian chemical combine. SA was also prescribed to victims of Spitak earthquake in Armenia. SA is used in medicine for therapeutic purposes as an antistress, an antihypoxic, and as immune active agents. About ten food additives and several medicine preparations containing SA are manufactured in Russia. These means are elaborated on the basis of the revealing physiological role of SA as the stimulator of energy processes during active functioning. This effect of SA was called the signal. It is mediated by adrenaline through sympathetic nervous system (Kondrashova, 2002; Kondrashova et al., 2005).

On an industrial scale, SA is produced through synthesis from butane *via* maleic anhydride presumably to

satisfy the chemical market. For food and pharmaceutical industries, bio-based SA, produced through the sublimation of amber or by microbiological synthesis, is recommended (Carole et al., 2004).

The advantages of microbiological production of SA over the chemical method have been noted in reviews (Zeikus et al., 1999; Carole et al., 2004). In addition, the substances obtained from biological sources are generally recognised as safe (GRAS). This is attributed either to the absence of toxic contaminations, introduced by chemical synthesis, or to the presence of both natural and artificial optical isomers. The first defect is quite probable for widely used chemical synthesis of SA. SA has no optical isomers. However, this simple molecule can exist in three different structures, named conformers which formation is determined by isolation conditions (Tadataka, 1967). The cited patent has shown that only one of conformers is identical to the natural form. This natural conformer possesses considerably greater biological activity than others. Besides more complicated methods including toxic substance for spectroscopic differentiation, the authors detected the valid difference between conformers in the melting point. The conformer with higher biological activity has a lowest melting point.

The microbial production of SA is commonly based on the use of anaerobic bacteria, such as *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, and *Escherichia coli*. Less is known about the synthesis of SA by yeasts and fungi. In particular, SA synthesis was reported for the fungus *Penicillium simplicissimum* (Gallmetzer et al., 2002) grown on glucose, for yeasts of the *Candida* genus grown on n-alkanes (Sato et al., 1972) and ethanol (Mandeva et al., 1981), and for the yeast *Saccharomyces cerevisiae* grown on glucose (Arikawa et al., 1999).

The aim of this work was to present the methods of SA production from ethanol by different yeasts. Ethanol as the substrate for the microbial production of SA possesses some advantages over other possible carbon sources. First, ethanol can be produced from renewable resources (sugar cane, sugar beet, corn, or lignocellulose). Second, this substrate facilitates the isolation and purification of SA because of the low content of by-products produced. Third, the products manufactured from ethanol are permissible for usage in the food industry and medicine.

MATERIALS AND METHODS

Candida catenulata VKM Y-5, *Candida zeylanoides* VKM Y-2324 and *Yarrowia lipolytica* VKM Y-2412 were obtained from the collection of the Laboratory of Aerobic Metabolism of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The strain was maintained at 4 °C on agar slants with n-alkanes as carbon source.

Candida catenulata and *Candida zeylanoides* were cultivated in a 10-litre ANKUM-2M (SKB, Pushchino, Russia) fermentor with an operating volume of 5 L. The medium contained (in g/L): $MgSO_4 \cdot 7H_2O$ - 1.4, NaCl - 0.5, $Ca(NO_3)_2$ - 0.8, KH_2PO_4 - 2.0, K_2HPO_4 - 0.2, and Burkholder's trace element solution (Burkholder et al., 1944). The mixture of vitamins contained 1.0 mg/L of thiamine-HCL and 0.1 mg/L of biotin. Pulsed addition of ethanol was performed as the pO_2 value increased by 5 % indicating a decrease in respiratory activity of cells due to the total consumption of carbon sources. Fermentation conditions were maintained automatically at the constant level: temperature 28 °C, pH=5.5 was adjusted with 5-15 % NaOH, dissolved oxygen concentration (pO_2) was 80 % (from air saturation), agitation was 800 rpm. Cultivation was performed as indicated in the text.

Yarrowia lipolytica were cultivated in a 10-litre fermentor with an operating volume of 5 L in the medium containing (g/L): $(NH_4)_2SO_4$ – 12; $MgSO_4 \cdot 7H_2O$ - 1.4; NaCl - 0.5; $Ca(NO_3)_2$ - 0.8; KH_2PO_4 - 2.0; K_2HPO_4 - 0.2; Burkholder's trace element solution. The thiamine concentration was 2.0 µg/L. The fermentation conditions were maintained automatically at the constant level: temperature 28 °C; pH=3.5 was adjusted with 20 % KOH; dissolved oxygen concentration (pO_2) was 60 % (air saturation); agitation was 800 rpm. Pulsed addition of ethanol was performed as the pO_2 value increased by 5 %. Cultivation was performed as indicated in the text.

Yeast growth was followed by measuring the absorbance of the culture at 540 nm with a Spekol 221 spectro-

photometer (Germany). The biomass dry weight was estimated from the absorbance of the cell suspension using a calibration curve.

To analyze organic acids, the culture broth was centrifuged (8000 g, 20 °C, 3 min); 1 ml of the supernatant was diluted with an equal volume of 6 % $HClO_4$ and the concentration of organic acids was measured on an HPLC-chromatograph (LKB, Sweden) on an Inertsil ODS-3 reversed-phase column (250 x 4 mm, Elsiko, Russia) at 210 nm; 20 mM phosphoric acid was used as a mobile phase with the flow rate of 1.0 mL/min; the column temperature was maintained at 35 °C. Quantitative determination of organic acids was carried out using calibration curves constructed with the application of SA, alpha-ketoglutaric acid, citric acid, threo-Ds-(+)-isocitric acid, acetic acid, maleic acid, aconitic acid, and fumaric acid (Boehringer Manheim, Germany) as standards. Additionally, SA was analysed enzymatically using biochemical kit (Boehringer Mannheim/R-Biopharm). The determination of SA was based on the measurement of the NAD^+ produced during conversion of SA acid to L-lactate in coupled reactions, reactions are catalysed by succinyl-CoA-synthetase, pyruvate kinase and lactate dehydrogenase.

For the amino acid assay, the biomass was freeze-dried. 2 ml of 80 % ethanol acidified with 0.1 N HCl was added to 20–30 mg of dry biomass and held for 24 h at room temperature. The extract was centrifuged, the residue was discarded and the supernatant was assayed on a Biotronic LC2000 amino acid analyzer (Germany) for free amino acids by the method (Spackman et al., 1958).

For the bound amino acid assay, the biomass residue after extraction of free amino acids with ethanol was additionally washed with 80 % ethanol and dried at 65–70 °C. 10–15 mg of the sample was hydrolyzed with 6 N HCl at 110 °C for 24 h. Excess HCl was removed from the extract using a rotary evaporator. 2 ml of 0.2 N Na-citrate buffer (pH = 2.2) was added to the dry residue. The amino acid content was determined using a Biotronic LC2000 automatic amino acid analyzer (Germany).

RESULTS AND DISCUSSION

Succinic acid synthesis by ***Candida zeylanoides*** and ***Candida catenulata***

Time courses of yeast growth, ammonium consumption, and SA synthesis are shown in Fig. 1. By the end of exponential growth phase, biomass of *C. catenulata* and *C. zeylanoides* reached up to 20 g/L. Intense excretion of SA was observed in the stationary phase and reached 5.2 g/L in *C. catenulata* and 9.4 g/L in *C. zeylanoides* in 68 h of cultivation. As compared to literature data, SA production by hydrocarbon-grown yeasts was 23.6 g/L in mutant strain *C. brumptii* IFO 0731 in 9 days of cultivation (Sato et al., 1972).

Data on the efficiency of the growth and SA synthesis by *C. catenulata* and *C. zeylanoides* are given in Table 1. The mass cell yield ($Y_{X/S}$) was o.67 g/g in *C. catenulata* and 0.63 g/g in *C. zeylanoides*. The maximum specific growth rate (μ_{max}) calculated from the linear segment in the semilogarithmic plot of the growth curve amounted to 0.16 h^{-1} for *C. catenulata* and 0.31 h^{-1} for *C. zeylanoides*. As seen from Table 1, both strains produced SA and malic acid approximately at the same level, which is indicative of a high activity of the glyoxylate cycle. The mass SA yield (Y_{SA}) was 0.33 g/g for *C. catenulata* and 0.39 g/g for *C. zeylanoides*.

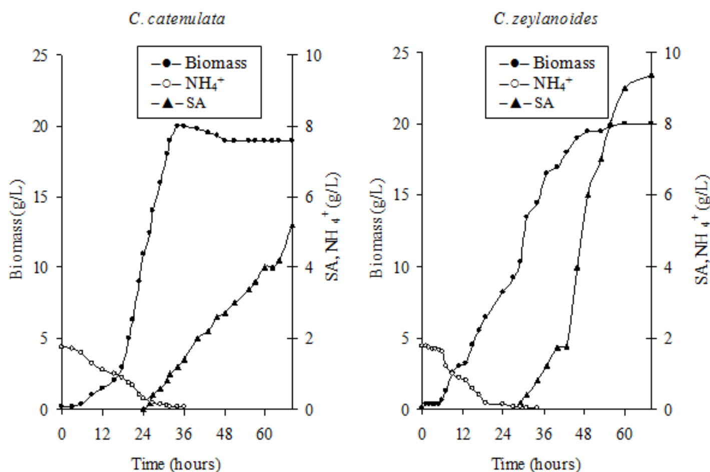

Figure 1. Time courses of growth, ammonium consumption, and SA synthesis by C. catenulata and C. zeylanoides

Table 1. Growth parameters and organic acid production by *C. catenulata* and *C. zeylanoides*

Parameters	C. catenulata	C. zeylanoides
$Y_{X/S}$ (g/g)	0.67	0.63
μ_{max} (h^{-1})	0.16	0.31
SA (g/L)	5.2	9.4
Malic acid (g/L)	4.0	8.0
Other acids (g/L)	2.3	2.5
Y_{SA} (g/g)	0.33	0.39

*Microbial production of succinic acid by **Yarrowia lipolytica***

A new two-step process of SA production has been developed, which includes the microbial synthesis of α-ketoglutaric acid (KGA) by the yeast *Yarrowia lipolytica* (step 1) and subsequent oxidation of KGA by hydrogen peroxide to SA (step 2).

I step - microbial synthesis of alpha-ketoglutaric acid

The yeast *Yarrowia lipolytica* is not capable of synthesizing the pyrimidine moiety of the thiamine molecule. During the cultivation of *Y. lipolytica* under thiamine limitation conditions, cells convert the source of carbon into an incomplete oxidation product KGA. The acid is excreted into the medium and can accumulate in large amounts. The regulatory mechanism of the TCA cycle key enzymes involved in processes of KGA superproduction in the *Y. lipolytica* yeast has been also studied (Lozinov et al., 1974; Morgunov et al., 1995; Chernyavskaya et al., 2000; Stottmeister et al., 2005). At a thiamine limitation, the activity of α-ketoglutarate dehydrogenase containing thiamine pyrophosphate as a cofactor has been shown to sharply decease; as the result, a major part of KGA formed is excreted from the cell into the medium.

A batch cultivation of *Y. lipolytica* VKM Y-2412 in a 10-litre ANKUM-2M fermentor under optimal conditions was carried out. The data on the growth dynamics and KGA accumulation are given in Figure 2. In the first 48 h, cells multiplied and biomass increased up to 10 g/L, after which the culture passed into the growth decline phase (up to 96 h) caused by the depletion of thiamine in the medium. Intensive synthesis of KGA

was observed in the stationary phase (after 96 h). By the end of the cultivation (240 h), KGA accumulated in the supernatant in the amount of 98.98 g/L. The productivity is on average 0.759 g/L·h at a yield of 0.70 g/g of ethanol consumed.

II step - oxidation of alpha-ketoglutaric acid by hydrogen peroxide to succinic acid

In this experiment, aliquots of the supernatant containing 98.98 g/L of KGA were incubated for 1 h with 2750 mM H_2O_2. After 1 h of incubation with H_2O_2, the supernatant contained SA and not KGA.

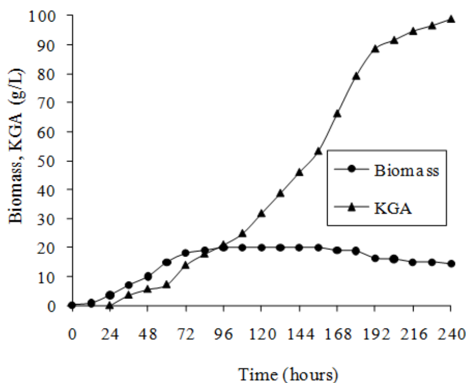

Figure 2. Time courses of growth and KGA synthesis by Y. lipolytica

The HPLC-evaluation of peak areas in this case showed that 98.98 g/L KGA was completely oxidized by 2750 mM H_2O_2 to give rise to 80 g/L SA. The isolation procedure involved some preliminary steps; namely, the decomposition of hydrogen peroxide in the filtrate, followed by filtrate bleaching and acidification with a mineral acid. After such preliminary treatment, the filtrate was concentrated, and SA from lyophilized residue was extracted with ethanol. Ethanol was evaporated, and the residue was crystallized. The yield of crystalline SA was found to be 82 % of its amount in the supernatant. The purity of SA was 100 %.

Biodiethyl succinate

Besides its direct applications, SA produced by *Y. lipolytica* yeasts can be used in the form of diethyl succinate as a flavouring for food products and as a perfume component ("flower fragrance").

Diethyl succinate was obtained by direct etherification of SA, produced by the method described in the previous section, with excess of absolute ethanol. Sulphuric acid (3 % of succinate) was used as a catalyst. Water formed was distilled off as an azeotropic mixture with toluene. Upon elimination of the solvents, diethyl succinate was distilled at a reduced pressure. Boiling temperature, 105 °C at 15 mm Hg.

Characteristics of amino acid composition of superproducer biomass

Production of KGA by *Y. lipolytica* cells grown on ethanol under thiamine deficiency conditions is accompanied with the formation of a large pool of amino acids (314.19 mg/g dry weight). The concentrations of glutamic acid, asparagine, lysine, leucine and valine are the highest in the amino acid composition. Taken together, they account for 50 % of the total amount of amino acids. The pool of free acids is 4.81 % of the total amount of amino acids in the cell. γ-aminobutyric acid (GABA) – the only amino acid not occurring in proteins – makes a significant part of the free acid pool (37.3 %).

Table 2. Amino acid (AA) composition of superproducer biomass

AAX	Bound AA/ (mg/g dry weight)	Free AA/ (mg/g dry weight)	Total amount of bound and free AA/ (mg/g dry weight)
Glutamic acid	45.89 ± 0,13	0.27 ± 0,1	46.16
Asparagine	35.25 ± 3.48	0.88 ± 0.38	36.13
Lysine	28.34 ± 4.07	1.22 ± 0.03	29.56
Leucine	27.30 ± 0.81	1.84 ± 0.33	29.14
Valine	21.57 ± 2.6	0.63 ± 0.25	22.2
Threonine	19.44 ± 1.77	0.30 ± 0.12	19.74
Serine	18.10 ± 1.56	0.30 ± 0.12	18.4
Tyrosine	17.78 ± 2.96	0	17.78
Alanine	17.45 ± 0.79	2.02 ± 1.47	19.47
Isoleucine	17.16 ± 1.77	0.42 ± 0.24	17.58
Phenylalanine	16.51 ± 3.34	0	16.51
Glycine	16.32 ± 0.79	0.63 ± 0.39	16.95
Histidine	8.58 ± 1.27	0.30 ± 0.07	8.88
Methionine	7.05 ± 0.52	0.14 ± 0.10	7.19
Cysteine	3.06 ± 0	0.55 ± 0.28	3.61
GABA	0	5.65 ± 0.23	5.65

GABA is known to be the main inhibitory neurotransmitter of the nervous system, whose receptors are widespread in the brain structures, virtually in all neuronal groups, in contrast with glutamate and aspartate, which belong to excitatory neurotransmitters. Disrupted biosynthesis and secretion of GABA in humans leads to various disorders, including epilepsy and Alzheimer's disease.

The function of GABA has been less studied in plants and microorganisms. It has been found that GABA is produced in yeast cells in response to various stresses, such as hypoxia, exposure to cold, oxidative stress, as well as at thiamine limitation (Ilchenko et al., 2010).

The diverse amino acid composition of the producer makes it possible to use this biomass in parenteral nutrition mixtures and as the basis for neuroleptics.

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WASTEWATER DISCHARGE FROM FISH PROCESSING PLANTS IN CROATIA

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ABSTRACT

The global increase in fish consumption tallies with trends in food consumption in general. Following this trend the global fish production has grown impressively and it is believed that the increasing trend of animal protein demand will continue in both developing and developed countries. Over 90% of fish products on the international market are traded in the processed form. Fish processing industry is one of the most important industrial sectors in Croatia. Despite this, effective regulations regarding emission limit values for wastewater (NN 087/2010) do not specify values for fish processing industry. Moreover, monitoring reports from Croatian fish processing plants suggest that wastewaters have significantly high concentrations of chlorides and very high concentration of biological and chemical oxygen demand. For this reason it is necessary to perform detailed study in order to establish specific effluent values for different parameters, considering effective emission standards set by EU Directives (76/464/EEC, 91/271/EEC and 2000/60/EC) and international emission guidelines for the management of wastewaters from the fish processing plants. Fish processing plants are situated on islands, in coastal and hinterland region including sensitive water protected areas, which requires specific solutions for each one. This paper reviews this problem and suggests its possible solution through microzonation and elaboration of specific studies for every single area, in order to consider all peculiarities of different plant locations. Helping the fish processing facilities to meet the environmental management objectives for sustainable development and good status of wastewater, values for some parameters are suggested as suitable limits to be considered for the complementation of the regulations, taking into account the location and wastewater discharges in urban wastewater treatment plants, specific discharge recipients and receiving local water bodies.

KEYWORDS: *Wastewater; Fish Processing; Legislation; Croatia.*

INTRODUCTION

Per capita fish consumption has been increasing steadily, from an average of 9.9 kg in the 1960s to 16.7 kg in 2006. Following this trend the global fish production has grown impressively in the last decades, thus over 90% of processed products on the international market are fish products. Due to increasing global competition, economic pressure and environmental awareness, the industries constantly improving its production procedures to make them more sustainable and competitive, (Uttamangkabovorn et al., 2005); however for fish processing industry environmental impact is a ongoing problem. Nowadays, the fish processing industries are facing problems of waste disposal and more stringent pollution prevention regulations, therefore right solutions for waste handling must be found through a good waste management and waste treatment technology. Production of fish processing wastes and their discharge into the coastal and nearshore environment have not been quantified in details and little is known about the probable role of this industry in polluting the coastal and marine environment (Islam et al., 2004). Water consumption and production of large wastewater volumes in a fish processing industry are of great concern and liquid effluent regulations for this type of industry are becoming more stringent (Chowdhury et al., 2010). Fish processing requires large amounts of water for storage and refrigeration of fish products before and during processing, but also for washing and cleaning purposes throughout the plant. Wastewater quality and degree of water contamination are highly dependent upon the type of processing operation and type of fish being processed, as contact between water and processing wastes (fish heads, guts, scales, blood, fats and oils) generate effluents rich

with high organic loads. Different authors reported different examples of waste handling in fish processing industry, together with

effluents problems, cleaner production and possible future strategies (Islam et al., 2004; Espósito et al., 2009; Thrane et al., 2009; Chowdhury et al., 2010).

The Croatian seafood industry consists of many small processing plants and represents one of the most important industrial sectors of the country. In the last decade, this industry gave main importance to technical improvements, efficiency of processing methods and quality of final products, without considering preventive solutions to reduce the impact on the environment. In fact, no measures were taken in order to reduce production of solid waste and wastewaters generated as by-products of processing operations. Existing national effective regulations do not specify how to treat and manage solid waste produced in fish processing industry, while the regulations regarding emission limit values wastewater are not specified for this industry. The lack of specific waste management in this industry resulted with discharge of solid waste and wastewaters into coastal waters and other receiving bodies, presenting a potential hazard to the receiving environment. Moreover, it is important to notice that processing plants are situated on islands, in coastal and hinterland regions, including sensitive water protected areas, with different ways of solid waste disposal and wastewater discharges. Numerous types of seafood are processed and processing operations vary between plants depending on raw material, source of utility water and unit processes. Dry salting, freezing, canning and marination are main processes in Croatian seafood industry, while the most represented type of processing is salting of small pelagics anchovies (Engraulis encrasicolus) and sardines (Sardine pilchardus). Most of the water consumed during fish salting ultimately becomes effluent containing high concentration of chlorides and organic loads. As the effective Croatian regulations regarding emission limit values for wastewater (NN 087/2010) do not specify values for fish processing industry, a national expert group was established in 2010. in order to identify specified emission limit values in fish processing industry to be included in existing regulations. The present paper attempts to discuss the characteristics of domestic seafood production wastes and current situation observed in the seafood industry regarding the effluents in the wastewater. The objective of this study was to implement analyses of fish processing wastewaters in order to obtain an overview of problem related to wastewater handling and to provide a base of recommendations for future sustainable strategies in wastewater management.

MATERIALS AND METHODS

Data were collected from 28 official monitoring analyses in nine different fish processing plants. The values of 4 different parameters were considered: biological oxygen demand (BOD5), chemical oxygen demand (COD), chlorides and pH, in order to obtain an overview of the actual state and review the characteristics of pollutant concentrations in wastewaters generated in processing plants, together with problems in their removal within the possible treatment methods of wastewaters.

Moreover, emission limit values for wastewater were analyzed taking into account effective Croatian regulations regarding (NN 087/2010), together with emission standards set by EU Directives (76/464/EEC, 91/271/EEC and 2000/60/EC) and international emission guidelines for the management of wastewaters from the fish processing plants.

RESULTS AND DISCUSSION

The water consumption from nine analysed fish processing plants ranged from 10 to 140 m^3 per day, depending on the size of the processing plant and quantity of produced processed fish products. The wastewater from all processing steps of these plants included: faecal waste water, cooling water (fish can production), brines of different concentrations (marinated and salted fish production), waste water from freezing processes (brines from freezing process using brine immersion, glazing water ect.), water from thawed ice, cleaning water ect. The data obtained from the monitoring analyses of the wastewaters showed high concentrations of chlorides from all fish processing plants which affected the concentrations and determination methods for biological and chemical oxygen demand. Existing water permits and authorizations are established specifi-

cally for each plant, considering effective regulations for general emission limit values (not specified for fish processing industry) and based on the characteristics of receiving body and the effluent conditions. The type of the processing procedures and used technology were not considered when the water permit was issued. The result of these specific authorizations resulted in different water permits, water sampling plans and effluent parameters to be considered in monitoring programs of individual plants. For example, in plants situated on the coast or near the sea, with discharge of

wastewaters in coastal waters, monitoring measures of chlorides was not obliged so the plant number 4 had no obligation to analyse it (Fig. 1). On contrary, for processing plants in other areas limit values for chlorides are prescribed by law (1000 mg/L) and applied in specific authorization for each plant (Fig. 1). In this way plants situated on the coast are usually within the prescribed limits for chloride concentrations, while those situated in other areas are out the limits and have problems with often penalties, although having same final products and same values for measured chlorid concentrations. Moreover, reported results showed that all plants had significantly high values of chlorides, showing measured chloride concentrations higher from 6 to 150 times than limit values prescribed by law, depending on quantity of processed fish produced in period of monitoring analyses. Chloride concentrations showed values in range 6674.0 – 153770.0 mg/L with an average of 35614.9 mg/L (Fig. 1).

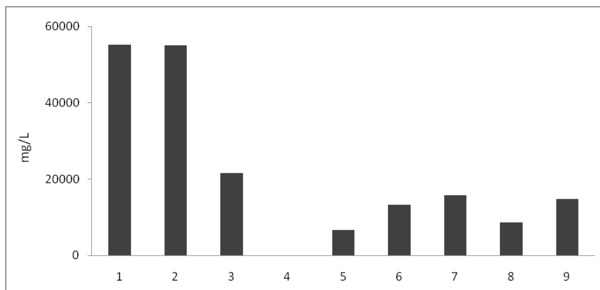

Figure 1. Chlorides concentrations measured in the wastewater from nine processing plants, showing significantly higher values than those prescribed by the law (1000 mg/L).

Monitoring of BOD5, COD and pH values is obliged for all fish processing plants and the limit values are prescribed by effective regulation (250 mg O2/L for BOD5, 700 mg O2/L for COD and 6.5-9.5 for pH). The results presented in this paper showed that effluent pH from fish processing plants is usually close to neutral, showing pH values in range from 5.7-8.3 with an average pH of 6.9 ± 0.6. Wastewater from fish processing operations usually showed high values of BOD5 and COD, while the effluent COD was higher than BOD5 (Chowdhury et al., 2010). The results from analyzed plants suggest that processing effluents were high in BOD5 and COD, confirming that COD values are higher than BOD5 values (Fig. 2). In fact, COD values show range 244.1 – 11750.0 mg O2/L with an average COD of 2405.1 mg O2/L, while BOD5 values were recorded in range 0.2 – 4600.0 mg O2/L with an average BOD5 of 990.2 mg O2/L.

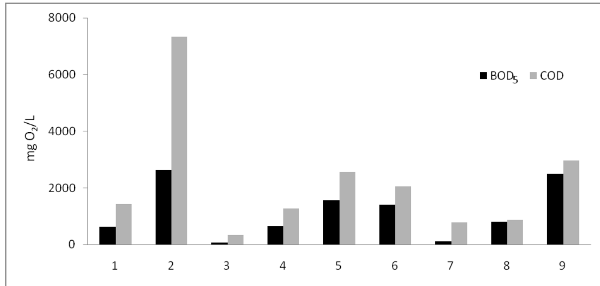

Figure 2. Parameters BOD5 and COD measured in the wastewater from nine processing plants, showing higher values than those prescribed by the law (250 mg O2/L for BOD5 and 700 mg O2/L for COD).

Literature on regional and global loads of effluents produced by the seafood processing industries and related impacts on ecosystem is hardly available (Islam et al., 2004), while the details on best practices examples, particularly those related to the reduction of chloride concentrations in fish processing wastewaters, are impossible to find. Omil et al. (1995) described the possibility of the treatment of high saline wastewater with anaerobic filter operating at loadings up to 24 kg COD/m3 day-1, while Chowdhury et al. (2010) individuated anaerobic reactor that can achieve more than 80% COD removal, operating at high salt concentration. However, no studies were reported on reduction of high chloride concentrations in wastewaters from fish processing industry. The crucial problem of Croatian processing plants in this moment is that the existing wastewater treatment systems are not designed to reduce high chloride concentrations, thus discharge of these wastewaters in collecting systems and urban wastewater treatment plants is not possible because chlorides obstruct the functioning of mechanical and/or biological treatment processes. Mechanical parts of treatment plants are destroyed by high concentrations, while anaerobic treatment of wastewater is inhibited by the presence of high chloride concentrations. The main problem of wastewater handling is present in plants situated in hinterland regions and sensitive water protected areas without possibility of wastewater treatment, while the plants situated on the coast and islands at this moment do not cause problems discharging wastewaters with high chloride concentrations directly in the sea. It is important to notice that the verification of the accuracy of the organic loads measurement systems was questionable due to high concentrations of chlorides, while the mechanical and biological wastewater treatment processes are not possible for the same reason. It is obvious that the first step in management of wastewater from fish processing plants in Croatia should be the reduction of high chloride concentrations. The reduction of chlorides in wastewaters can be obtained through technological improvements in processing operations and mechanical or chemical removal of chlorides from waters. Removal of organic pollutants from wastewaters can be resolved with biological treatments, including both anaerobic and/or aerobic processes, but these can be applied only after decrease of existing chloride concentrations. One of the possible solutions for removal of chlorides from fish processing wastewaters is an evaporation method. Considering large volumes of wastewaters with high concentrations of organic loads, a need to create large surface areas with evaporation basins (which is not possible in areas where processing plants are situated) and the problem of sludge disposal, the removal of chlorides by known evaporation methods does not seem as the ideal solution. Therefore, ulterior studies on reduction or removal methods of chloride concentrations from fish processing wastewaters are needed.

For sustainability of fish processing industry, the establishment of effective effluent treatment and monitoring facilities is needed in order to reduce waste loads and pressure on the ecosystem. It is necessary to find the sustainable solution in which all pollutants will be removed from wastewaters in order to make possible the recycling and reuse of water, reducing in this way also water consumption costs. Moreover, reliable estimate on waste loadings is needed in order to implement waste management and cleaner production systems, while the focus should be on microzonation and plant-specific studies regarding specific emission limits and possible solutions.

CONCLUSION

Impropriate organization in the waste management system can have a variety of negative effects on environmental components such as water, air, soil and human health. The lack of proper waste management is the biggest problem in Croatian environmental sector; therefore specific improvements of existing regulations are needed. Without the proper legislation, fish-processing industry is facing the lack of organized disposal sites for solid waste and adverse regulations without specified emission limits and recipients for wastewaters. The improvements in processing technologies, cleaning procedures and waste treatment systems are needed in order to reduce chlorides and other pollutants and enable the reuse of water in the plants, reducing in this way consumption of water and economical expenses. In order to determine the magnitude of waste from processing plants and the impact on the environment, the improvement of monitoring programs and existing regulations on waste management is obligatory. An important waste reduction strategy is the usage of undervalued fish parts and by-products for remarketing and additional use in other industries. Long-term sustainable solutions, primarily based on clean production principles, recycling and reuse of wastes, are necessary, while all effluents generated from different processing

phases need to be treated in order to make possible a reuse of the water in the plant. Thus, an integrated approach for water assessment in Croatian fish processing plants is essential.

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GROWTH AND SURVIVAL OF PROBIOTIC BACTERIA IN OAT-BASED SUSPENSION ENRICHED WITH GLUCOSE

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ABSTRACT

Oat is valuable food which has beneficial effects on organisms, including improving of digestive system and reduction of cholesterol in the blood. After appropriate processing, oat can be a suitable substrate for fermentation with lactic acid bacteria. This paper researches glucose impact on growth of *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-1 bacteria in oat-based suspension and their survival during 28 days of cool storage.

Oat-based suspension fermentation without (i.e. control) and with 2.5 and 5% (w/w) of glucose was done with *Lactobacillus acidophilus* La-5 or *Lactobacillus casei* Lc-1 bacteria at 37°C. Fermentation was stopped when pH 4.6 was reached. Fermented oat-based suspension samples were stored at 6°C for 28 days. pH-value, lactic acid content and viable cell count were observed during fermentation and storage.

Oat-based suspension fermentation with *Lactobacillus acidophilus* La-5 or *Lactobacillus casei* Lc-1 bacteria lasted for about 12 hours, and glucose did not influence fermentation dynamics, regardless added quantity. Better growth of *Lactobacillus acidophilus* La-5 was noticed in oat-based suspension with 2.5 and 5% (w/w) glucose (1.69 and 1.92 Δlog CFU/mL), than in control sample (1.63 Δlog CFU/mL). Determined difference in viable cell count was not statistically important (P=0.053). *Lactobacillus casei* Lc-1 shows similar dynamics of growth in oat-based suspension, and glucose did not significantly influenced its growth (P=0.140). *Lactobacillus acidophilus* La-5 does not survive well in fermented oat-based suspension, and on the 21st day, viable cell count was less than 6 log CFU/mL in all samples. Regardless added glucose *Lactobacillus casei* Lc-1 bacterium survives well in fermented oat-based suspension (about 9.62 log CFU/mL) during 28 days of cool storage.

Research results show that addition of glucose into oat-based suspension did not significantly influence growth and survival of *Lactobacillus acidophilus* La-5 or *Lactobacillus casei* Lc-1 bacteria regardless the added quantity.

KEYWORDS: *fermentation; glucose; oat-based suspension; probiotic bacteria*

INTRODUCTION

During last decades consumer demands in the field of food production have changed considerably. Consumers, more and more, believe that foods contribute directly to their health. Today foods are not intended only to satisfy hunger and to provide necessary nutrients for humans but also to prevent nutrition-related diseases and to improve physical and mental well-being of the consumers (Menrad, 2003; Siró et al., 2008). Continuous development of new functional foods is the response of science and industry to increased consumer awareness regarding health and the role of foods for improving quality of life (Angelov et al., 2006). Long known for its benefits, oats is becoming popular as a part of healthy diet and new oat products emerge at the functional food market.

Nutritional composition of oats is exceptional. Oats contain a higher percentage of protein with superior amino acid balance in comparison to other cereals. Lipids are highly unsaturated and contain substantial amounts of essential fatty acids. Oats are also a rich source of antioxidants as well as a number of essential vitamins and minerals, such as vitamin E and folic acid. Further, oats contain dietary fibres, including water-soluble β-glucans (Öste, 2008), recognized as the main functional component of cereal fibres. Studies have indicated the hypocholesterolemic effect of this compound, leading to 20-30% reduction of LDL–choles-

terol, and to an expected overall effect of reduced cardiovascular disease risk (Prado et al., 2008).

In order to obtain a health promoting non-dairy alternative that could satisfy the need of humans not being able to consume cow milk, a technology to manufacture a milk-like product from oats was developed (Lindahl et al., 1997). It is based on a unique, patented enzymatic process which involves dry milling or wet milling of rolled oats or oat flour at 60°C, followed by an enzymatic reaction using β-amylase. Maltose and β-limit dextrins are thus the main carbohydrates in the final product formed from oat starch. After the enzymatic procedures the insoluble fibres can optionally be separated using a decanting step. The final product, high or low in insoluble fibres, can be modified further by the addition of nutrients, oil and flavourings (Mårtensson et al., 2000).

Johansson et al. (1998) reported that after appropriate processing, oat is a suitable substrate for fermentation with lactic acid bacteria. Enzymatically treated oat base has been developed and applied by Mårtensson et al.(2002) as substrate for lactic acid fermentation with dairy starter cultures.

The aim of the present paper is to research glucose impact on growth of *Lactobacillus. acidophilus* La-5 and *Lactobacillus casei* Lc-1 bacteria in oat-based suspension and their survival during 28 days of cool storage.

MATERIALS AND METHODS

Fermentation media

The sterile oat-based suspension (Alnatura, Germany) was fermented with probiotic bacteria *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-1. To examine the influence on growth and survival of probiotic bacteria, three samples were prepared as it follows: (a) the first sample was without glucose added (i.e. control), and (b) glucose was added to the other two samples at the rate of 2.5 and 5% (w/w), respectively. Oat-based suspension inoculated with reactivated probiotic culture. Inoculated samples were incubated at 37°C and fermentation of all samples was stopped when pH 4.6 was reached. Fermented oat-based suspension samples were cooled and stored at 6°C for 28 days.

Used probiotic culture

Used cultures were DVS *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-1 (Chr. Hansen, Denmark). Inoculum was prepared from 100 mL sterile oat-based suspension at 37°C, in which 1 g of culture *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-1 was added. After 30 min. of activation, 2.5% (v/v) of inoculum was added in oat-based suspension.

Chemical and microbiological analyses

Acidity of oat-based suspension samples was analysed as pH and titratable acidity. pH was measured by Docu-pH+ Meter (Sartorius AG, Germany). Lactic acid, reported as titratable acidity, was estimated by titrating 10 mL of each sample with 0.1 M NaOH using phenolphthalein as indicator (Egan et al., 1981).

The viable cells count of bacteria (i.e. expressed as colony forming units (CFU/mL)) was determined by the standard method on MRS agar plates (Biolife, Italy) at 37°C for 3 days. *Lactobacillus acidophilus* La-5 or *Lactobacillus casei* Lc-1 was incubated in microaerophilic conditions, which were obtained by a layer of MRS agar over the MRS agar inoculated with culture (ISO, 2006).

The samples were analysed during fermentation (at the start of fermentation, after 3, 6, 9 hours and when pH value reached 4.6.). Survival of probiotic bacteria in fermented oat-based suspension was observed after 1, 7, 14, 21 and 28 days of cool storage at 6°C.

Statistical analysis

Each experiment was performed in three separate trials. Empirical data were analyzed by applying descriptive statistic (mean, standard deviation) and inference statistics (ANOVA). Level of significance was 0.050. SPSS 12.0 was used for data analyzing.

RESULTS AND DISCUSSION

Oat-based suspension samples were fermented with probiotic bacteria *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-1. To improve activity of probiotic bacteria, glucose in amount of 2.5 and 5% (w/w) was added. Fermentation was led up to approximate pH 4.6.

Fermentation of oat-based suspension with probiotic bacteria *Lactobacillus acidophilus* La-5 or *Lactobacillus casei* Lc-1 lasted between 11 hours and 30 min. and 12 hours, regardless to the amount of glucose added. Literature reports that fermentation of oat-based suspension with probiotic bacteria *Lactobacillus casei subsp. paracasei* and *Lactobacillus plantarium* lasts for about 8 hours or 6 hours (Angelov et al., 2005). Some authors (Mårtensson et al., 2000) report that fermentation of oat-based suspension lasted even for 18 hours, which can be caused by higher quantity of total solid content (between 16 and 18%).

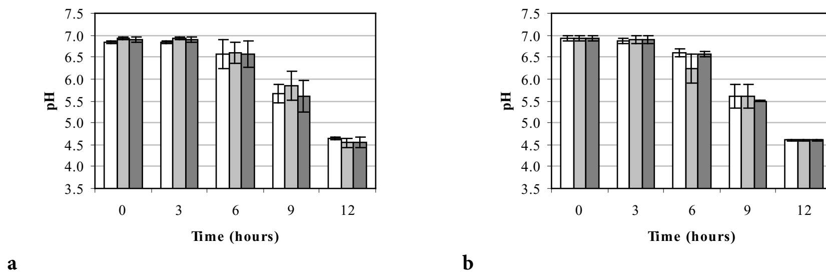

Figure 1. Changes in the pH-value of oat-based suspension without ( ), with 2.5 (▪) and 5 (▪) % (w/w) addition of glucose during fermentation with Lactobacillus acidophilus La-5 (a) and Lactobacillus casei Lc-1 (b).

Initial pH-value of all oat-based suspension samples was about 6.93 (Figure 1). Significant decrease in pH-value, in all oat-based suspension samples, was recorded after 6 hours, with both probiotic bacteria, when fermentation became more intensive. Glucose did not have statistically important effect on decrease of pH-value of oat-based suspension fermented with *Lactobacillus acidophilus* La-5 (P= 0.741) or *Lactobacillus casei* Lc-1 (P=0.644) bacteria, as it was expected (Figure 1). In literature glucose is presented as a promoter of lactobacillus growth (Božanić et al., 2008), and besides that, glucose also improves energetic value of oat-based suspension (Mårtensson et al., 2002). However, the results obtained show that glucose did not have any influence on dynamic of fermentation.

Table 1. Lactic acid content in oat-based suspension without, with 2.5 and 5% (w/w) addition of glucose after fermentation with *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-1.

Samples of oat-based suspension	Lactic acid % (w/w)
without glucose; La-5	0.21 ± 0.0086
with 2.5% (w/w) added glucose; La-5	0.24 ± 0.0258
with 5% (w/w) added glucose; La-5	0.23 ± 0.0275
without glucose; Lc-1	0.22 ± 0.0180
with 2.5% (w/w) added glucose; Lc-1	0.22 ± 0.0129
with 5% (w/w) added glucose; Lc-1	0.22 ± 0.0211

After fermentation oat-based suspension samples contained between 0.22 and 0.24% (w/w) of lactic acid (Table 1), regardless the quantity of glucose added. Similar content of lactic acid in fermented oat-based suspension is reported in literature (Mårtensson et al., 2000). Content of lactic acid is closely related to pH-value in oat-based suspension. Lactic acid bacteria make less lactic acid in oat-based suspension, but they easier reach pH-value of 4.6. Reasons for that are mainly because of lack of fermentable sugars and lower buffering capacity of oat-based suspension (Mårtensson et al., 2002).

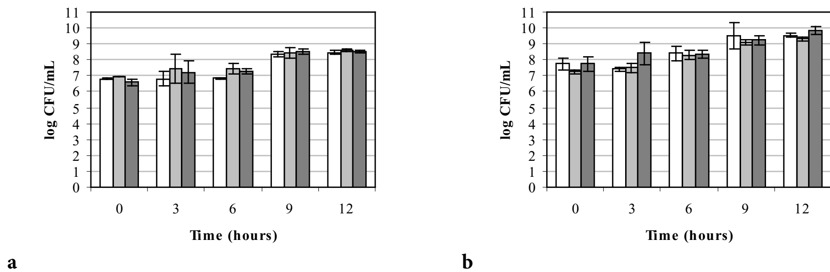

Figure 2. Changes in viable cells count of oat-based suspension without (▪), with 2.5 (▪) and 5 (▪) % (w/w) addition of glucose during fermentation with Lactobacillus acidophilus La-5 (a) and Lactobacillus casei Lc-1 (b).

Lactobacillus acidophilus La-5 grew well in all three samples of oat-based suspension (Figure 2), but in oat-based suspension with 2.5 and 5% (w/w) of glucose, the growth was better (1.69 and 1.92 Δlog CFU/mL) than in control sample (1.63 Δlog CFU/mL). At the and of fermentation, viable cell count of *Lactobacillus acidophilus* La-5 in oat-based suspension was between 8.45 and 8.62 log CFU/mL, regardless the glucose added. Differences in viable cell count during fermentation are not statistically important (P=0.053), which proves that glucose has no influence. Besides, *Lactobacillus casei* Lc-1 also grow well in oat-based suspension. At the beginning of fermentation, viable cell count of *Lactobacillus casei* Lc-01 was about 7.7 log CFU/mL (Figure 2). More significant growth of *Lactobacillus casei* Lc-01 was recorded after the 6th hour of fermentation (Figure 2). Oat-based suspension with 5% (w/w) of glucose at the end of fermentation had more *Lactobacillus casei* Lc-1 bacteria (about 9.83 log CFU/mL) than control sample (about 9.55 log CFU/mL). Regardless the difference in viable cell count of *Lactobacillus casei* Lc-01 during fermentation of oat-based suspension, statistically important influence of glucose on their growth is not recorded (P=0.140). Control sample of oat-based suspension, as a source of carbon, contains maltose. However, test samples, together with glucose which is added in oat-based suspension, also contain maltose. *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-01, besides glucose probably use maltose, because of which there is not significant influence on their activity and growth in oat-based suspension.

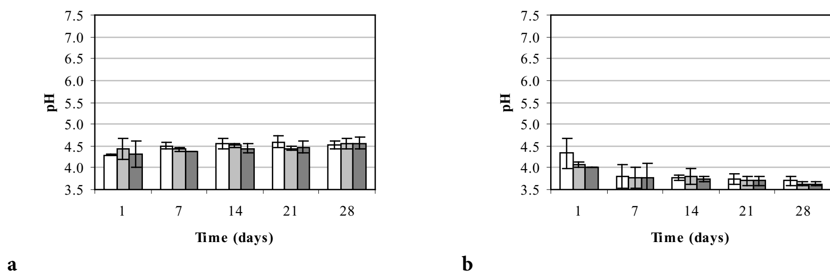

Figure 3. Changes in the pH-value of oat-based suspension without (□), with 2.5 (□) and 5 (□) % (w/w) addition of glucose and fermented with Lactobacillus acidophilus La-5 (a) and Lactobacillus casei Lc-1 (b) during 28 days of cool storage.

In the first day of storage, oat-based suspension samples fermented with *Lactobacillus acidophilus* La-5 had pH–value of about 4.3. The value increased to pH of about 4.5 during 28 days of storage. There is no statistically important difference in pH-value (P=0.503) between samples of oat-based suspension fermented with *Lactobacillus acidophilus* La-5, regardless the amount of glucose added. At the beginning of storage, control sample of fermented oat-based suspension with *Lactobacillus casei* Lc-1 had pH-value of about 4.3, and sam-

ple with 5% (w/w) of glucose about 4.0 (Figure 3). Just after the 7th day of storage, pH-value dropped below 4.0 in all samples of oat-based suspension fermented with *Lactobacillus casei* Lc-1, and at the end of storage, on the 28th day, pH-value was about 3.6 (Figure 3). Glucose did not have statistically significant influence on changes in pH-value of oat-based suspension fermented with *Lactobacillus casei* Lc-1 during 28 days of storage (P=0.269). Obtained results are in accordance with data recorded in literature (Mårtensson et al., 2000).

Table 2. Lactic acid content in oat-based suspension without, with 2.5 and 5% (w/w) addition of glucose and fermented with *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* Lc-1 after 28 days of cool storage.

Samples of oat-based suspension	**Lactic acid** % (w/w)
without glucose; La-5	0.24 ± 0.0216
with 2.5% (w/w) added glucose; La-5	0.23 ± 0.0295
with 5% (w/w) added glucose; La-5	0.23 ± 0.0288
without glucose; Lc-1	0.43 ± 0.0227
with 2.5% (w/w) added glucose; Lc-1	0.44 ± 0.0131
with 5% (w/w) added glucose; Lc-1	0.44 ± 0.0126

After 28 days of storage, oat-based suspension samples fermented with *Lactobacillus acidophilus* La-5 contain less lactic acid (about 0,24% (w/w)) than oat-based suspension samples fermented with *Lactobacillus casei* Lc-1 (about 0,44% (w/w)). Content of lactic acid in fermented oat-based suspension is closely related to pH-value, and literature also records similar values (Mårtensson et al., 2000).

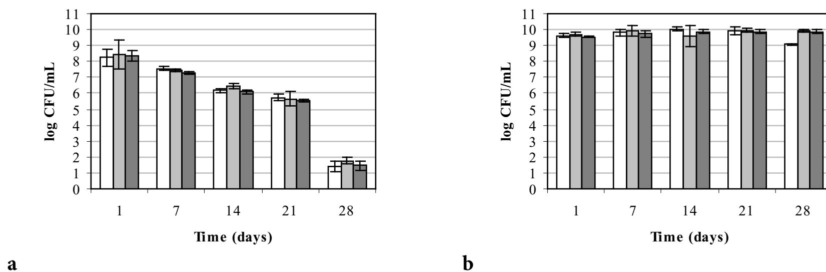

Figure 4. Viable cells count of Lactobacillus acidophilus La-5 (a) and Lactobacillus casei Lc-1 (b) in fermented oat-based suspension without (◻), with 2.5 (◻) and 5 (◻) % (w/w) addition of glucose during 28 days of cool storage.

Viable cell count of *Lactobacillus acidophilus* La-5 in all samples of fermented oat-based suspension, on the first day of storage, was about 8.47 log CFU/mL (Figure 4), regardless the amount of glucose added. After the 21st day of storage viable cell count of *Lactobacillus acidophilus* La-5 was considerably less, even below minimal therapeutic level (6 log CFU/mL). Literature also reports poor survival of this bacteria in cereal media such as oat-based suspension, and complete dying after the 21st day of storage (Helland et al., 2004). As causes of poor survival of *Lactobacillus acidophilus* La-5 in different suspensions, many authors often point out lack of micronutrients, such as peptide, amino acids, short-chain fatty acids, available carbohydrates, and presence of oxygen (Shah, 2007). Viable cell count of *Lactobacillus casei* Lc-1 on the 1st day of storage was about 9.7 CFU/mL and it was stable during the storage period (Figure 4). Glucose did not have statistically important influence on survival of *Lactobacillus acidophilus* La-5 (P=0.063) or *Lactobacillus casei* Lc-1 bacteria (P=0.363) in fermented oat-based suspension during 28 days of cool storage.

CONCLUSION

Fermentation of oat-based suspension with *Lactobacillus acidophilus* La-5 or *Lactobacillus casei* Lc-1 lasted for about 12 hours, regardless to glucose added. After fermentation of oat-based suspension viable cell count of *Lactobacillus acidophilus* La-5 bacteria was about 8.45 and log CFU/mL about 8.62. Determined difference

was not statistically important (P=0.053). *Lactobacillus casei* Lc-1 had similar dynamics of growth in oat-based suspension and viable cell count at the end of fermentation was about 9.55 and 9.83 of log CFU/mL. Glucose also did not have statistically important influence on growth of *Lactobacillus casei* Lc-1 (P=0.140) in oat-based suspension. *Lactobacillus acidophilus* La-5 bacteria poorly survive in fermented oat-based suspension, and on the 21st day viable cell count was less than 6 log CFU/mL in all samples. *Lactobacillus casei* Lc-1 survive well in fermented oat-based suspension (about 9.62 log CFU/mL) during 28 days of cool storage regardless to glucose added.

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IMPROVEMENT OF CITRIC ACID PRODUCTION FROM RAPESEED OIL BY YEAST *YARROWIA LIPOLYTICA* THROUGH INITIAL STEPS OF TRIGLYCERIDE METABOLISM AND ITS REGULATION

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ABSTRACT

The initial step of assimilation of triglycerides in the yeast *Yarrowia lipolytica* is their hydrolysis by extracellular lipases with the formation of glycerol and fatty acids, which appear in the medium in the phase of active growth. The concentrations of these metabolites change insignificantly upon further cultivation. Lipase and the key enzymes of glycerol metabolism (glycerol kinase) and the glyoxylate cycle responsible for the metabolism of fatty acids (isocitrate lyase and malate synthase) are induced just at the beginning of the growth phase and remain active in the course of further cultivation. These results, taken together, suggested that glycerol and fatty acids according in the medium do not suppress the metabolism of each other. This suggestion was confirmed by experiments on the cultivation of *Y. lipolytica* on two substrate pairs: glycerol + oleic acid and glucose + oleic acid. These experiments indicated that glycerol does not suppress the assimilation of oleic acid, whereas glucose (or its metabolites) suppresses the assimilation of oleic acid in such a manner that oleic acid begins to be consumed only after the concentration of glucose in the medium falls to about zero. The fact that glycerol and fatty acids can be consumed simultaneously is of special importance for the development of the efficient regime of oil feeding in citric acid production process by *Y. lipolytica*. *Y. lipolytica* NG40/UV7 produced citric acid from rapeseed oil with the concentration 175 g/L and the yield of 1.50 g/g.

KEYWORDS: *Yarrowia lipolytica; assimilation of triglycerides; glycerol; fatty acids; glyoxylate cycle*

INTRODUCTION

Recent years show an increasing interest to vegetable oils as substrates for the microbial production of practically important products, such as lipids, lipases and organic acids, including citric acid (CA) and *threo*-D(S)-(+)-isocitric acid (ICA).

The main component of vegetable oils and animal fats is triglycerides, which are entrained in microbial metabolism through the action of extracellular lipolytic enzymes, lipases. These enzymes hydrolyze triglycerides, forming glycerol and the respective fatty acids.

The mechanism of action of microbial lipases is not fully understood. In particular, little is known on the mechanism of consumption of the products of triglyceride hydrolisis, glycerol and fatty acids. Theoretically, glycerol and fatty acids can be consumed either simultaneously or successively (the latter mechanism of substrate consumption is known as diauxie).

The aim of this work was to study the metabolism of triacylglycerols and its regulation in the yeast *Y. lipolytica* grown on rapeseed oil in order to improve CA production.

MATERIALS AND METHODS

Strains *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7 were obtained from the All-Russian Collection of Microorganisms (VKM) and from the collection of the Laboratory of Aerobic Metabolism of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino, Russia). The strains were maintained at 4 °C on agar slants with n-alkanes as the carbon source.

In order to obtain yeast cells for the inoculation of fermentor, a 750-mL Erlenmeyer flask containing 100

mL of cultivation medium was inoculated with a yeast colony grown on the agar medium. The flask was incubated on an orbital shaker (130 rpm) at 28±1 °C for 36 h (to a cell mass density of 6.7 g/L). At regular intervals, the pH of the medium was adjusted to a value of 5.0 by adding an appropriate volume of 10 % NaOH. The basic medium had the following composition (in g/L): $(NH_4)_2SO_4$ - 3.0, KH_2PO_4 - 1.0, K_2HPO_4 - 0.1, $MgSO_4 \cdot 7H_2O$ - 0.7, $Ca(NO_3)_2$ - 0.4, NaCl - 0.5, yeast extract "Difco" - 0.5; trace elements as described by Burkholder et al. (Burkgolder et al., 1944). The thiamine concentration was 0,5 mg/L. Depending on the aim of the experiment, the medium was supplemented with one of the following growth substrate: rapeseed oil – 10 g/L, or glycerol – 20 g/L, or oleic acid -10 g/L.

For the growth experiments, *Y. lipolytica* was cultivated in a 10-litre ANKUM-2M (SKB, Pushchino, Russia) half filled (to a volume of 5 L). This medium contained (in g/L): $(NH_4)_2SO_4$ - 5.0, KH_2PO_4 - 2.0, K_2HPO_4 - 0.2, $MgSO_4 \cdot 7H_2O$ - 1.4, $Ca(NO_3)_2$ - 0.8, NaCl - 0.5, yeast extract "Difco" - 0.5; and Burkholder's trace element solution. The thiamine concentration was 0.02 mg/L. The fermentation conditions were maintained automatically at the constant level: temperature 28 °C; pH=4.5 was adjusted with 20 % NaOH; dissolved oxygen concentration (pO_2) was 60 % (air saturation); agitation was 800 rpm. At regular intervals, the culture was sampled for analyses.

CA production experiments were performed in a 10-litre ANKUM-2M with an initial working volume of 5.0 L. The medium contained (in g/L): $(NH_4)_2SO_4$ - 6.0, $MgSO_4 \cdot 7H_2O$ - 1.4, $Ca(NO_3)_2$ - 0.8, NaCl - 0.5, yeast extract "Difco" - 0.5; and Burkholder's trace element solution. The thiamine concentration was 0.02 mg/L. Rapeseed oil was added, as indicated in the text. The fermentation conditions were maintained automatically at the constant level: temperature 28 °C; pH=4.5 was adjusted with 25 % NaOH; dissolved oxygen concentration (pO2) was 60 % (air saturation); agitation was 800 rpm.

Biomass determination. From 10 to 50 ml of the culture liquid was filtered through Synpor membrane filters, and the yeast cells on the filters were washed with n-hexane to remove lipids probably adsorbed on the cells. Then membranes with the cells were dried in a vacuum desiccator at 110 °C to a constant weight. The mass of the dry cells was determined as the difference between the weights of the dried filters with cells and control dried filters without cells.

Oil assay. The filtrate was extracted twice with n-hexane. The filtrate-hexane mixture was allowed to stay and separate into two phases. The upper phase contained extracted lipids in n-hexane, while the lower aqueous phase did not contain them. The hexane extracts were pooled and dehydrated with anhydrous sodium sulphate. Then sulphate crystals were removed by passing the extract through a glass filter, and hexane from the extract was evaporated under vacuum.

Glycerol was analysed enzymatically using biochemical kit (Roche Diagnostics GmbH, Germany). The determination of glycerol was based on the measurement of NADH produced during the conversion of glycerol to L-lactate in coupled reactions; reactions were catalyzed by glycerol kinase, pyruvate kinase and L-lactate dehydrogenase.

Concentration of organic acids was determined using high-performance liquid chromatography (HPLC) with an HPLC chromatograph (Pharmacia, LKB, Uppsala, Sweden) on an Inertsil ODS-3 reversed-phase column (250x4 mm, Elsiko, Russia) at 210 nm; 20 mM phosphoric acid was used as a mobile phase with the flow rate of 1.0 mL/min; the column temperature was maintained at 35 °C. Moreover, diagnostic kits (Roche Diagnostics GmbH, Germany) were used for the assay of CA and ICA. The determination of CA was based on the measurement of the NADH produced during the conversion of CA to oxaloacetate and its decarboxylation product pyruvate, and following the conversion to L-malate and L-lactate. Reactions are catalysed by citrate lyase, malate dehydrogenase and L-lactate dehydrogenase. The determination of ICA was based on the measurement of the NADPH produced during the conversion of ICA to α-ketoglutarate, a reaction catalysed by isocitrate dehydrogenase.

Lipase assay. The sample containing culture medium and cells was filtered through a 0.20 µm filter in order to remove cells and analyse extra-cellular lipase. Lipase activity was measured by a titrimetric assay, described earlier with slight modification (Kamzolova et al., 2005). The substrate emulsion was prepared with rapeseed oil (m/V=40%) and emulsificator (2% polyvinyl alcohol). The solution was emulsified in a warring blander in order to obtain the dispersion of fat into the aqueous phase. The sample (0.2-0.5 mL) was added to the

substrate emulsion (5 мL) and 50 mM phosphate buffer (pH=8.0; 4.5 mL) and incubated for 1 h on a shaker (220 rpm) at 30 °C. Lipase activity was determined by titration of the released fatty acids with 50 mM sodium hydroxide (up to final pH=10). The amount of enzyme that catalyzed the release of 1 μmol of fatty acids per ml per min at 30 °C was taken as the units of lipase activity (U). Specific lipase activity was expressed as units per mg of cells (U/mg of cells).

Enzyme assays. Yeast cells were collected by centrifugation at 3000 g for 10 min (4 °C) and washed with an ice-cold 0.9 % NaCl solution. The cell pellet was suspended in a proportion of 1:10 in 100 mM phosphate buffer (pH 7.4) supplemented with 1 mM EDTA. Cells in the suspension were disrupted with Ballotini glass beads (d=150-250 μ) on a planetary mixer for 3 min at 1000 rpm (0 °C). The cell homogenate was centrifuged at 5000 g for 30 min (4 °C), and the supernatant was used for the assay of cytoplasmic, mitochondrial and peroxysomal enzymes: glycerol kinase (EC 2.7.1.30), citrate synthase (EC 4.1.3.7), aconitate hydratase (EC 4.2.1.3), NAD^+ - (EC 1.1.1.41) and NADP-(EC 1.1.1.42)-dependent isocitrate dehydrogenases, isocitrate lyase (EC 4.1.3.1), malate synthase (EC 4.1.3.2) and pyruvate dehydrogenase (EC 1.2.4.1). The activity of enzymes was measured by standart methods described earlier (Kamzolova et al., 2008)

The amount of enzyme catalyzing the conversion of 1 μmol of substrate per min was taken as one unit of enzyme activity (U). Specific enzyme activities were expressed as units per mg protein (U/mg of protein). The amount of protein in cell-free extracts was determined by the method of Bradford.

RESULTS AND DISCUSSION

Growth of Y. lipolytica on rapeseed oil and the dynamics of lipase activity

The wild-type strain *Y. lipolytica* VKM Y-2373 was grown in the medium with rapeseed oil as the single carbon source. For comparison, this strain was also grown on glycerol and oleic acid. The results are presented in Fig. 1, which shows the accumulation of biomass and lipase, and the consumption of the carbon sources. As evident from Fig. 1, immediately after the inoculation of the cultivation media, the yeast culture began to grow and the concentrations of the carbon sources began to decrease. After 24 h of cultivation, the growth substrates were exhausted and the cultures entered the stationary growth phase. At that time, the biomass comprised 10.0, 9.3 and 8.7 g/L in the cases of cultivation on rapeseed oil, glycerol and oleic acid, respectively. When *Y. lipolytica* was grown on rapeseed oil, the activity of extracellular lipase began to increase just from the start of yeast growth and remained at a high level (approximately 10 U/mg cells) during the whole cultivation period. Upon the yeast cultivation on glycerol, the activity of lipase began to rise only when the concentration of glycerol fell to 4.0 g/L. In this case, the activity of lipase did not exceed 3.0-3.2 U/mg cells. Upon the yeast cultivation on oleic acid, the activity of lipase slowly increase to about the same level as during the yeast cultivation on glycerol. The early induction of lipase and the maintenance of its activity at a high level during the yeast cultivation on vegetable oil suggest that the primary products of oil assimilation do not accumulate in the medium in noticeable amounts. Indeed, the concentration of glycerol in the cultivation medium varied at a very low level between 0.028 and 0.056 g/L (Fig. 1) as rapeseed oil was consumed and no accumulation of glycerol was observed. Earlier we indicated that the glycerol rapidly increased during the first 9 h of cultivation (up to 0.125 g/L) and then remained at this level in *Y. lipolytica* grown on sunflower oil (Kamzolova et al., 2008). On the basis of these results, one can suggest that the glycerol and fatty acids formed from the oil are consumed by yeast cells concurrently rather than by the type of diauxie.

Enzyme activities in Y. lipolytica cells grown on rapeseed oil, glycerol, and oleic acid

The suggestion made at the end of the previous section was confirmed by the results of enzyme assay in the *Y. lipolytica* cells grown on rapeseed oil, glycerol, and oleic acid (Table 1). As early as after 6 h of yeast cultivation on the oil, the activity of glycerol kinase in the yeast cells was notably higher that in the yeast cells grown on oleic acid, although lower by 30% than in the yeast cells grown on pure glycerol. The key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, which are involved in the metabolism of fatty acids, were also induced early during the yeast cultivation on the oil. The activities of these two enzymes in the 6 h-old yeast cells grown on

oleic acid were, respectively, 14.7 and 10 times higher than in the glycerol-grown yeast cells. In older yeast cells (12- and 24 h-old) grown on the oil, the activity of glycerol kinase was also high, probably due to the fact that the glycerol produced from the oil was actively utilized during the whole cultivation period. Similarly, the high activities of isocitrate lyase and malate synthase in the 12- and 24 h-old yeast cells grown on the oil suggest that the fatty acids produced from the oil are actively metabolized during the whole cultivation period.

The active functioning of the glyoxylate cycle upon the assimilation of rapeseed oil and oleic acid is confirmed by the high activities of citrate synthase and aconitate hydratase, which operate both in the glyoxylate cycle and in the tricarboxylic acid cycle of yeast. For comparison, NAD- and NADP-dependent isocitrate dehydrogenases, which are not involved in the glyoxylate cycle, exhibit higher activities in the yeast cells grown on glycerol than on oleic acid.

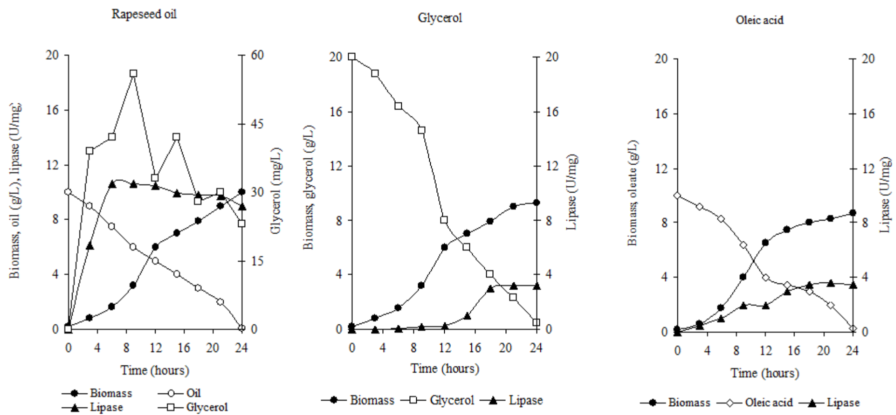

Figure1. Growth of Y. lipolytica on various carbon sources

Carbon source	Time (h)	GK	TCA cycle				Glyoxylate cycle	
			CS	AH	NAD-ID	NADP-ID	IL	MS
Rapeseed oil	6	0.254	2.04	0.92	0.063	0.058	0.089	0.054
	12	0.250	2.44	1.02	0.062	0.068	0.109	0.064
	24	0.244	1.96	0.87	0.058	0.068	0.093	0.035
Glycerol	6	0.363	0.82	0.33	0.105	0.120	0.009	0.012
	12	0.350	0.90	0.43	0.115	0.132	0.009	0.014
	24	0.312	0.87	0.40	0.117	0.152	0.011	0.010
Oleic acid	6	0.112	1.70	0.53	0.110	0.072	0.133	0.105
	12	0.112	1.90	0.59	0.112	0.068	0.134	0.131
	24	0.112	1.80	0.48	0.105	0.071	0.144	0.121

Table 1. Activities of enzymes of *Y. lipolytica*, grown on various carbon sources

Activities are expressed in U/mg protein. Abbreviations: GK, glycerol kinase; CS, citrate synthase; AH, aconitate hydratase; NAD-ID, NAD-dependent isocitrate dehydrogenase; NADP-ID, NADP-dependent isocitrate dehydrogenase; IL, isocitrate lyase; MS, malate synthase.

Citric acid production

The fact that glycerol and fatty acids can be consumed simultaneously is of special importance for the development of the efficient regime of oil feeding in CA production by *Y. lipolytica*.

The necessary condition for the synthesis of CA and its excretion into the surrounding medium is the retardation of growth of *Y. lipolytica* under the conditions of carbon excess and nitrogen deficiency in the cultivation medium. The wild strain *Y. lipolytica* VKM Y-2373 and its mutant *Y. lipolytica* NG40/UV7 were studied for CA production under optimal conditions of oil feeding under nitrogen limitation. The curves of growth of both strains, lipase activity, CA and ICA production were presented in Fig. 2.

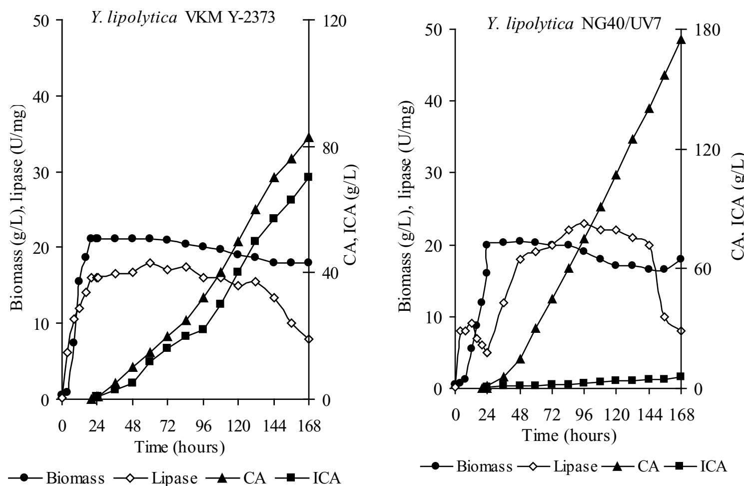

Figure 2. Time courses of growth, lipase activity, CA and ICA production by Y. lipolytica, grown on rapeseed oil

The initial concentration of rapeseed oil was 20 g/L and then the addition of oil (140 g/L) was performed as the pO_2 value increased by 5% indicating a decrease in respiratory activity of cells due to the total consumption of oil. Both strains showed the good growth on the rapeseed oil, and the biomass reached 21.2 g/L for *Y. lipolytica* VKM Y-2373 and 20.0 g/L for *Y. lipolytica* NG40/UV7 at 36 h; then the yeast culture transited to the stationary phase because of the nitrogen exhaustion in the medium. The developed regime of oil feeding provided the high lipase activity and, hence, the high rate of the oil transformation into CA during whole process.

CA and ICA were accumulated in the medium just during the stationary growth phase. At the end of cultivation (168 h) the wild strain *Y. lipolytica* VKM Y-2373 produced almost equal amounts of CA and ICA. The mutant strain *Y. lipolytica* NG40/UV7 produced 175 g/L of CA, and 5.6 g/L of ICA. This is the first time that *Y. lipolytica* grown on rapeseed oil has been shown to produce CA at high concentration with insignificant amount of by-product ICA. Earlier, we indicated that the use of rapeseed oil resulted in CA production of 135 g/L by other mutant strain *Y.lipoltica* 187/1 (Kamzolova et al., 2005). The productivity and the specific CA production rate using mutant *Y. lipolytica* NG40/UV7 reached 1.34 g/(L·h) and 0.063 g/g •h. respectively, which corresponded to the best values reported previously for citrate-producing strains. The mass CA yield of *Y. lipolytica* NG40/UV7 was 1.5 g/g of oil consumed.

CONCLUSIONS

The results of the efficient CA production from rapeseed oil by yeast *Y. lipolytica* obtained in the present work indicated that the application of yeast has a considerable promise for the industrial CA production. Traditional producer *Aspergillus niger* can produce CA from molasses with the productivity of the process of 0.8 g/(L·h) and the yeild of 0.9, while the mutant *Y. lipolytica* NG40/UV7 produced CA from rapeseed oil with the productivity of 1.34 g/(L·h) and the yield of 1.5 g/g.

The use of yeasts instead of moulds for CA production also represents a novel approach, since the traditional production of CA by using *A. niger* is associated with the accumulation of significant amounts of solid and liquid wastes. Moreover, yeasts are characterized by greater resistance to high substrate concentrations than fungi with comparable conversion rates and have greater tolerance to metal ions, which allows the use of less refined substrates.

Y. lipolytica is considered as nonpatogemic and CA production based on this organisms can be classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA).

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GAS CROMATOGRAPHIC DETERMINATION OF FREE AMINO ACIDS IN SAMPLES WITHDRAWN DURING LACTIC ACID PRODUCTION BY *LACTOBACILLUS AMYLOVORUS* DSM 20531^T

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ABSTRACT

In this work derivatization of amino- and carboxy groups of amino acids by ethyl-chloroformate in water solution was described. In this reaction N(O,S)-alkoxycarbonyl-alkyl esters have been synthesised in water phase and, subsequently, they have been extracted to organic phase - mixture of chloroform and ethyl-chloroformate. In samples prepared for analysis by optimized gas chromatographyc method, 15 out of 20 alkoxycarbonyl-alkyls derived from amino acids have been detected. This analytical method is precise, accurate, sensitive and robust and, therefore, it is appropriate for determination of concentration of amino acids in complex media which have been often used in research as well as in biotechnological production. By using the method it was proven that sterilization of water solutions of amino acids affects its concentration. In this particular case amino acids from meat extract, yeast extract and peptone - three compounds which are usually added to media as nitrogen sources, have been taken into account. Concentration of amino acids from samples whithdrawn during biotechnological batch production of lactic acid in a lab-scale strirred tank bioteactor has also been determined. Bioprocess for lactic acid production has been carried out by Lactobacillus amylovorus DSM 20531T. The lactic acid bacterium posse's proteolytic and amylolytic activity, and it is good candidate for lactic acid production by simultaneous saccharification and fermentation. Results of this work will contribute to better understanding of physiology of bacteria which may be further adapted and applied in lactic acid production in highly efficient and sustainable bioprocess.

KEYWORDS: *determination of amino acid concentration; gas chromatography; biotechnological production of lactic acid; lactic acid bacteria; Lactobacillus amylovorus*

INTRODUCTION

Simpler carbohydrates, mainly mono- and/or disaccharides (*e.g.* glucose or sucrose) have been often used as carbon sources in industrial production of lactic acid, while meat extract, yeast extract and peptone have been usually added to the growth media as nitrogen sources. Such media are rather expensive and their utilization do not strengthen concept of sustainable development (Reddy et al., 2008; Altaf et al., 2007). Economically and ecologically attractive bioprocesses for lactic acid production have been planned to be carried out in growth media consisted of different relatively cheap and accessible renewable raw materials (green biomass) and/or different by-products from food industry (John et al., 2007). Such media can met all nutritive requirements of industrial microorganisms employed in order to produce different value added products. Cost of different ingredients and raw materials have significant impact on cost of the growth media as well as on cost of final product (Altaf et al., 2005). Therefore, it is of great importance to define which amino acids are essential for the growth and optimal activity of lactic acid bacteria. Based on those results it should be possible to reduce complexity of the media for bioprocesses in which lactic acid bacteria are involved (John et al., 2009).

The aim of this work was to adapt and optimize gas chromatographic method for determination of amino acids concentration in samples withdrawn during biotechnological production of lactic acid. The acid was produced by amylolytic lactic acid bacterium *Lactobacillus amylovorus* DSM 20531^T in MRS medium.

MATERIAL & METHODS

Microorganism

Lactobacillus amylovorus DSM 20531^T (ATCC 33620, NRRL B-4540) was used in this work.

Batch cultivation

Batch fermentation was carried out in MRS medium (Biolife, Italy) (De Man et al., 1960) in a lab-scale stirred-tank bioreactor (Chemap AG, Switzerland) at 40°C without aeration and with mixing (400 rpm). The medium was inoculated with 2.5 % (vol/vol) of the overnight pre-grown bacterial culture.

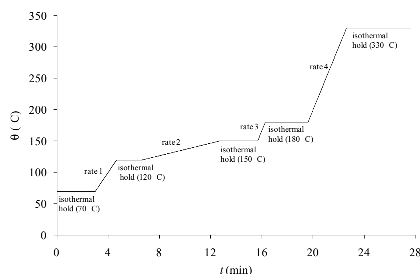
Analytical methods

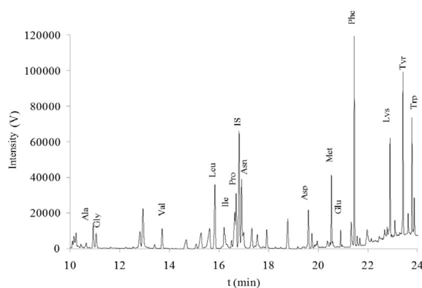
Bacterial growth was followed by determination of dry cell biomass (γ_X) and linear correlation between γ_X and optical density of withdrawn suspensions (OD_{600}) (Cary 13E Varian; Mulgrave, Australia) can be described by the equation: $\gamma_X = 0.9648OD_{600} - 0.3980$ ($R^2 = 0.9830$).

Derivatization of amino acids. Solutions of 20 amino acids (standards; Sigma-Aldrich, USA) in redistilled water were prepared (c = 2,5-25 mM) and added in different volumes, as needed, to sterile MRS medium. Then, derivatization of amino and carboxyl groups of free amino acids in the medium was performed with ethyl chloroforoformate (ECF) (Fluka, USA) and derivatives were extracted to organic phase [chlorophorm (Carlo Erba Reagents, Italy)/ECF] (Hušek, 2005). Prepared samples containing derivatized amino acids (DA) and internal standard norleucine (Sigma-Aldrich, USA) were analyzed by gas chromatography (GC)-flame ionization detection (GC-2010Plus AF system; Shimadzu, Kyoto, Japan) using low-polarity capillary column (RTX®-5 capillary column; 30 m L, 0.25 mm ID, 0.25 μm d_f; Restek, USA) (helium, hydrogen, and synthetic air; all 99,999%, Messer, Croatia). Intensity (I, V) of peaks for 15 amino acids, separated during GC analysis, were used for construction of calibration curves (Table 1.). Amino acids in supernatants of samples whitdrawn during cultivation and lactic acid production by *L. amylovorus* DSM 20531^T were derivatized and analyzed in the same way, and its concentration was determined by using calibration curves. If not otherwise stated all other chemicals were purchased from Merck (Germany).

RESULTS AND DISCUSSION

In this work procedure for derivatization of amino acids by ECF has been applied. The derivatization of: (1) 20 amino acids from their separate water solutions, (2) 20 amino acids from their mutual solution, (3) amino acids from meat extract, yeast extract, and peptone, and (4) amino acids from MRS medium have been accomplished. After extraction, 15 DA's were separated, identified and quantified by optimized GC method (Figure 1.) and calibration curves were created (Table 1.).





(A) (B)

Figure 1. Multi-ramp temperature program (A) and separation of 15 DA¢s by optimized GC method (B). Internal standard, IS.

The method was validated (data not shown) and effect of sterilization on concentration of amino acids in water solution of meat extract/yeast extract/peptone was determined (Marić and Šantek, 2009). In this solution 14 DA's were identified and quantified. Their concentration before sterilization (121°C/15 min) was in range from 0.379 mM (Glu) to 4.115 mM (Asn), and after sterilization from 0.431 mM (Glu) to 3.359 mM (Asn). Due to thermal treatment concentration of Gly, Val, Ile, Asn, and Asp was reduced while concentration of Leu, Phe, Met, Lys, and Glu was increased. Content of Ala, Tyr, Trp, and Pro was unaffected.

Table 1. Calibration curves parameters (retention time, t_R; calibration curve equation, eq; and coefficient of determination, R^2) for 15 DA's after derivatization in MRS medium.

DA	t_R (min)	eq	R^2 (-)
Ala	10.915±0.007	$I = 113112.96c + 213.57$	0.9819
Gly	11.034±0.006	$I = 66565.02c - 655.98$	0.9689
Val	13.712±0.007	$I = 45132.76c + 2764.57$	0.9109
Leu	15.848±0.017	$I = 147570.49c + 1407.44$	0.9912
Ile	16.217±0.005	$I = 29058.27c + 2324.02$	0.8518
Phe	21.473±0.004	$I = 619699.10c - 7321.31$	0.9976
Tyr	23.434±0.002	$I = 664455.47c - 31784.34$	0.9777
Trp	23.807±0.007	$I = 572816.58c - 86669.77$	0.8746
Asn	16.910±0.012	$I = 122955.15c - 4589.81$	0.9530
Met	20.555±0.005	$I = 193813.84c - 3093.45$	0.9943
Pro	16.701±0.009	$I = 173814.11c - 3382.50$	0.9551
Lys	22.908±0.001	$I = 272293.43c - 23625.03$	0.9589
Asp	19.645±0.004	$I = 25851.63c + 158.73$	0.9819
Glu	20.927±0.001	$I = 42570.56c - 60.94$	0.9849
His	21.996±0.032	$I = 57683.32c + 8090.98$	0.9795

Afterwards, concentration of amino acids in supernatants of samples withdrawn during lactic acid production by amylolytic lactic acid bacterium *Lactobacillus amylovorus* DSM 20531^T (Trontel et al., 2010; Trontel et al., 2011) has been determined (Figure 2.). During batch cultivation of *L. amylovorus* DSM 20531^T in MRS medium concentration of Glu followed trend of growth curve (Figure 2.A). This result is in agreement with previously described data by Chistensen et al. (1999). Namely, expression of *gadBC*, genes coding for glutamate decarboxylase and corresponding antiporter, was enhanced at the begging of stationary growth phase. In the presence of Glu viability of the cells and energy yielding is higher than in environment without this amino acid, as needed in stationary growth phase. Amino acids: Asn (Figure 2.C), Pro (Figure 2.D), Lys and Gly (Figure 2.E) showed similar pattern. Metabolism of Asn and Gly has not been described in many details (Chistensen et al., 1999). Lys can be catabolised in cells of lactic acid bacteria, and Pro is usually accumulated as response to osmotic shock (Tammam et al., 2000). No significant change in concentration of Leu (□ 3 mM) (Figure 2.A) and Met (□ 0.6 mM) was observed (Figure 2.B). In terms of branched chain amino acids (Leu, Ile, Val) it is generally accepted that lactic acid bacteria are auxotrophs (Chopin, 1993). Leu, Ile and Val were present in concentrations that supported growth and activity of the amylolytic bacterium and they were not depleted from the medium. Stochastic alterations of concentration of Ala (Figure 2.B), and Phe (Figure 2.F) have been obtained. It should be pointed out that during lactic acid production His was completely depleted from the medium (Figure 2.C). Usually this amino acid is involved in maintenance of pH value in cytoplasm and metabolic energy yielding (Molenaar et al., 1993). Trp was not detected.

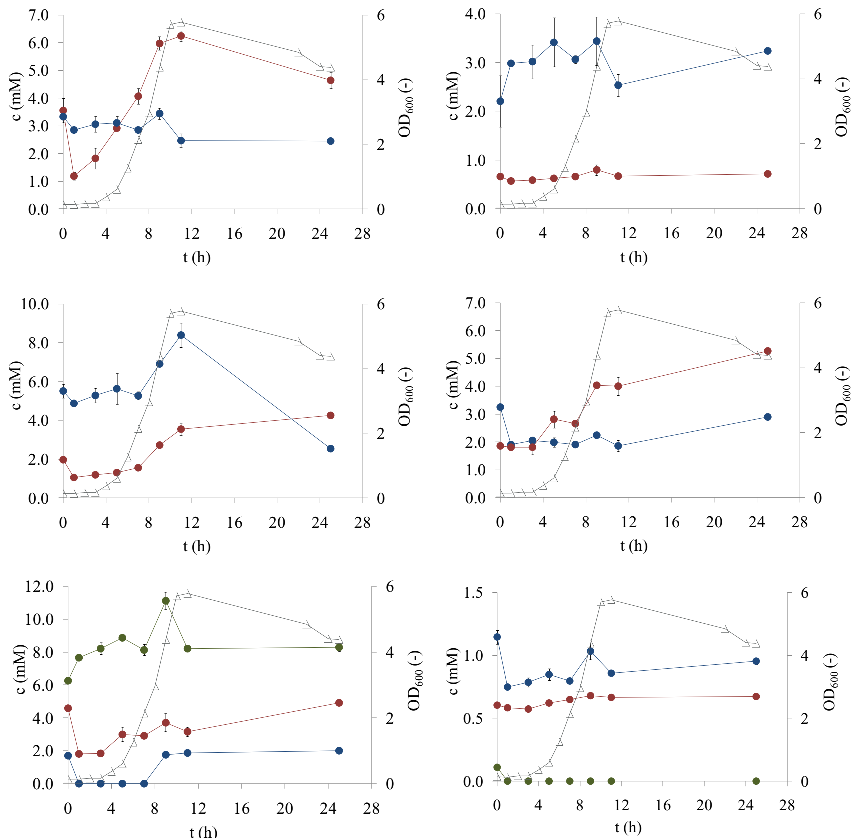


Figure 2. Changes in concentration of DA¢s (c) and bacterial biomass (OD600, D) during fermentation of glucose from MRS medium by L. amylovorus DSM 20531T: Leu (●), Glu (●) (A); Ala(●), Met (●) (B); Val (●), Asn (●) (C); Asp (●), Pro (●) (D); Lys (●), Gly (●), Ile (●) (E), Phe (●), Tyr (●), His (●) (F).

CONCLUSIONS

Derivatization of amino- and carboxy groups of amino acids by ECF in MRS medium was successfully applied. In this reaction *N*(*O,S*)-alkoxycarbonyl-alkyl ester has been synthesised in water phase and, subsequently, it has been extracted to organic phase. 14 out of 20 alkoxycarbonyl-alkyls derived from amino acids Ala, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Asn, Met, Pro, Lys, Asp and Glu have been detected. Ser, Thr, Cys, Gln i Arg cannot be detected by the method, while concentration of His was mainly below corresponding LOD value. The GC method is precise, accurate, sensitive and robust and, therefore, it is appropriate for determination of concentration of amino acids in complex media which have been often used in research as well as in biotechnological production. *Lactobacillus amylovorus* DSM 20531^{T} does posses amylolytic and proteolytic activity. Results of this work will contribute to better understanding of physiology of bacteria which may be applied in lactic acid production in highly efficient and sustainable bioprocess.

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Clinical Nutrition
Food and Consumers
Food, Health and Nutrition

CONSUMERS' OPINIONS ABOUT OLIVE OIL AND CEREALS AS FUNCTIONAL FOOD

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ABSTRACT

This study investigated perceptions related to olive oil and cereals as functional foods. It was also examined how these perceptions are influenced by gender, age and the region of Croatia. One of the aims was to identify the public acceptance of the concept of functional food. In this paper we used a convenience sample and data were collected using a self-administrated questionnaire designed to assess opinions and attitudes about consumption and opinion of olive oil and cereals as functional foods. Interviewed individuals were of both genders aged from 19 to 65 with different education levels. Stratification of the units from the sample has been made according to gender, age and educational structure as well as social-economic status of respondents in the sample (N=1122).

The study showed that over 50% of female consumers is familiar with the term functional food, more than male consumers (33.8%). Results also indicate regional differences, especially between continental part of Croatia and the coast. The continental consumers consider olive oil as functional food (51%) which is not the case in the coastal part of Croatia. Regarding cereals, the opinions are not regionally divided (p>0.05) because 50% of the consumers from the continental part of Croatia and 47.5 % from the coastal part of Croatia consider cereals as functional food.

The results have shown that generally the familiarity with functional food varies systematically as a function of age, gender and education level.

KEYWORDS: *olive oil, cereals, functional food*

INTRODUCTION

Term "functional food" was introduced in the 1980s, in Japan for food products that were fortified with special constituents with advantageous physiological effects (Hardy, 2000; Kwak & Jukes, 2001a; Stanton, Ross, Fitzgerald, & Van Sinderen, 2005). Numerous study have reaffirmed that functional foods may improve the general conditions of the body (Chen, 2011; Bhat & Bhat, 2011), decrease the risk of some diseases (Mesias et al., 2011), and could even be used for curing some illnesses (Ngo et al., 2011; Siro et al., 2008). It was recognized that there is a demand for these products in all age and gender groups (Mark-Herbert, 2004; Menrad, 2003; Side, 2006). Functional food products typically include health claims on their label touting their benefits: for example for cereals ("Cereal is a significant source of fiber. Studies have shown that an increased amount of fibre in one's diet can decrease the risk of certain types of cancer in individuals") or for olive oil as an integral ingredient of the Mediterranean diet. In the study of Dean and others (2007) is pointed out that in the UK "People with a healthy heart tend to eat more wholegrain foods as part of a healthy lifestyle" where the term "wholegrain" refers to the major cereal grains including wheat, rice, maize and oats (JHCI, 2002). Functional cereal (grain) products are grains, such as wheat, maize, rice, oats, etc. that have been modified to provide health benefits over and above basic nutrition (Elleuch et al., 2011; Dean et al., 2007). Consumption of cereals in Croatia is 94 kg per capita (MAFARD, 2009). Functionality of olive oil suggests that it may have health benefits that include reduction of risk factors of coronary heart disease, prevention of several varieties of cancers, and modification of immune and inflammatory responses (Stark et al., 2002). Olive oil appears to be an example of a functional food, which may contribute to its overall therapeutic characteristics (Bhat & Bhat, 2011). The health benefits of olive oil have been studied and documented in numerous publications. Olive oil is known for its high levels of monounsaturated fatty acids and is also a good source of phytochemi-

cals including polyphenolic compounds, squalene, alpha-tocopherol

(Martinez-Gonzales & Sanchez-Villegas, 2004; Brownlee, 2005), melatonin (Reiter,Tan, 2002) and oleocanthal (Fogliano, Sacchi, 2006). In 2004, the Food and Drug Administration (FDA) announced that "eating about 2 tablespoons (23 g) of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil" (FDA, 2004). Consumption of olive oil in Croatia is less than 2 l per capita compared with Greece (20 l) Italy (12,5 l) and Spain (10,6 l). The European Union is the greatest producer of olive oil (80% of world production) with its annual consumption of 1,8 mil l. The greatest consumers of olive oil within European countries are Greece, Italy and Spain (MAFARD, 2010). Using a survey, among the Croatian citizens, the public perceptions related to functionality of cereals and olive oil was investigated as well as how these perceptions are influenced by gender, lnationality and regionality.

MATERIALS AND METHODS

In this paper the sample frame consists of participants over 18 years of age (42.4% women, 57.6% men) from four Croatian regions (Zagreb, Zadar, Split and Rijeka). Data for the study were collected through a questionnaire elicited by face-to-face interviews. The group of participants were recruited by approaching individuals that appeared to fall within the recruitment criteria (age, gender) and inviting them to participate. The questionnaire that was used for data collection included questions about functional food knowledge, attitudes regarding functional food as well as the socio-demographic variables (Table 1). The questionnaire was distributed among respondents, self-administered and collected (N=1122). From the collected data two different matrices were developed – one for those who know the meaning of functional food and the other one for those that are not sure or are not familiar with the term.Those matrices were developed new matrices regarding the (i) olive oil consumption and (ii) the other, for cereals consumption. Consumers were divided into four categories based on the

region they live in. Data were analysed using SPSS (Statistical Package for Social Science, v.15). The ANOVA (Two- Factor without Replication) was used to determine differences between observed variables in different cities. And crosstabs from the descriptive statistics were used to observe interactions for at least 2 variables.

Table 1. Socio-demographic characteristics of the data set (N=1122)

characteristic	%
Gender	
Female	42.4
Male	57.6
Age (y)	
18-30	52.0
31-40	40.3
41-50	3.9
51-60	2.8
61+	0.9
Education level	
Elementary school	6.8
High school	58.1
University degree	35.1

RESULTS AND DISCUSSION

In the total data set, 41.3% of the consumers are familiar with the term "functional food", and 58.7% are not. One of the aims was to determine similarities and/or differences in the regional affiliation. The regional difference in global perception of functional food is confirmed ($p<0.001$) and is in accordance with Markovina et al. (2011). There are also differences in opinions if cereals and olive oil are considered as functional food ($p<0.05$) as well as on the regional level ($p<0.005$). The regional perception of cereals and olive oil as functional food is presented in Fig. 1. and as it can be seen, olive oil is considered as functional food in the continental part of Croatia (65%) followed by the region Rijeka that has included also a part of the continent (Lika and Gorski Kotar). It seems that Dalmatian consumers use olive oil traditionally without considering it as food with "special properties". In all regions consumers considered cereals as functional food (ZG=65%; ST=53.9%; ZD=55% and RI=54.7%).

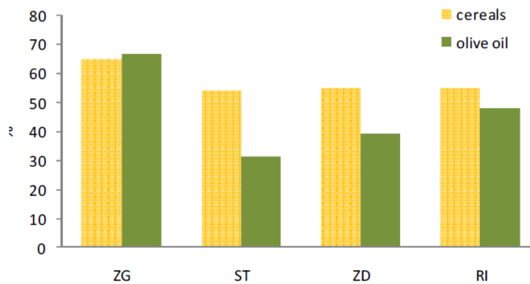

Figure 1. Consumers' opinion of cereals and olive oil as functional food according the region of living (Zagreb-ZG; Split-ST; Zadar-ZD and Rijeka-RI).

The regional samples were analyzed according the gender, age and education level (presented in tables 2 and 3).

Table 2. Consumers' opinion regarding cereals as functional food.

characteristic	%			
	Zagreb	**Split**	**Zadar**	**Rijeka**
Gender				
Female	66.9	57,7	85	18,2
Male	33.1	42,3	15	81,8
Age (y)				
18-30	25,6	57,7	90	75,8
31-40	50,4	42,3	10	24,2
41+	25	/	/	/
Education level				
Elementary school	3,4	/	/	6,0
High school	50,9	61,5	75	62
University degree	45,8	38,5	25	32

Cereals are considered as functional food in all parts of Croatia and according studies of Elleuch and his co-workers (2011) as well as Camire and co-workers (2011) the consumers show interest in foods such as breakfast cereals, and their number is growing. It has to be emphasised that the interest in different regions is depending on the education level as well as the age, whereas in only one region (Zagreb) the participants aged 31-40 years are the dominant age group consuming cereals. In other 3 regions the preponderance is on the side of those aged between 18 and 30. The functionality of the olive oil is recognized (Fig. 1) in all age and gender groups but again the education level is crucial because in all regions over 50% of participants with the highest education level have named olive oil as food that should be classified as functional food. The results of this study, regarding olive oil are in accordance with some studies (Delado et al., 2011; Santosa et

al., 2010) in which it was concluded that olive oil is a staple food for most countries in the Mediterranean region (i.e. Spain, Italy, and Greece). On the other hand, it is a relatively new product in areas outside of it, as in the continental part of Croatia. In the US in particular, however, interest in consumption of olive oil has been growing exponentially during the last 20 years (Santosa et al., 2011). Numerous studies have pointed out the health benefits associated with olive oil because is believed to help to lower cardiovascular risk (Martinez-Gonzales et al., 2004).

Table 3. Consumers' opinion regarding olive oil as functional food.

characteristic	%			
	Zagreb	**Split**	**Zadar**	**Rijeka**
Gender				
Female	68,9	50,5	82,6	8,0
Male	33.1	49,5	17,4	92,0
Age (y)				
18-30	7,8	50,0	65,2	61,2
31-40	60	50,0	34,8	38,8
41+	33,9	/	/	
Education level				
Elementary school	1,1	/	8,7	9,1
High school	36,7	43,4	39,1	39,1
University degree	62,2	56,2	52,2	51,8

CONCLUSION

Differences were found in this sample of consumer population based on four groups (consumers from Zagreb, Split, Zadar and Rijeka) that differ in their demographic characteristics and also in their pREFERENCES. Over 40% of Croatian consumers (41.3%) are familiar with the concept of functional food but the opinion of the foods that are considered as food with functional properties differ with the region of living. Majority of continental consumers choose olive oil as a food that will improve their health, and cereals are desirable in all Croatian regions. The findings of this work also imply that the impact of the gender, age and education level is not negligible. Cereals as functional food are mostly classified by female consumers that have university degree, aged between 31-40 years. On the other hand, classification of olive oil as food with functional properties fluctuates depending of the region and is mostly chosen by females with finished high school aged 31-40.

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MARKET SEGMENTATION BASED ON CONSUMERS PERCEPTION OF BOSNIAN AND HERZEGOVINIAN VINE QUALITY

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ABSTRACT

Since 2000 number of new small private vineries has been established in Bosnia and Herzegovina. To ensure its business sustainability and to become an „engine“ of rural development, they strive to develop loyal local clientele. It is not an easy task because they face competition of well-known former Yugoslav and international vineries. Therefore, aim of this paper is to provide insights into Bosnian and Herzegovinian (B&H) consumers' behaviour and to segment market on the basis of their opinion about B&H vineries' performances in comparison with foreign vineries.

Market survey had been organized in two B&H towns (Sarajevo and Ţepče) during April and May 2010. Survey sample included 300 respondents. Sample was validated by Bowman-Shelton and Crombach alpha test. Agglomeration schedule and Ward methods were used to define market clusters. Cluster validity was tested by Monte Carlo method.

Three different market clusters have been formed. First, „low involved consumers“, where price and trust in producers were the main factors driving purchase decision. Second, „prospective consumers“, consisted of young population with modest purchase power as well as habit to consume vine in cafés and restaurants. They are convinced that foreign companies offer better overall quality and quality-price ratio and pay more attention to consumers´ needs. They tend to prefer foreign vines. Third market cluster, „highly involved consumers", had higher purchase power and university degree. They believe that B&H vineries marketing services are worse, as well as their information explaining tradition and quality of domestic vine production.

Research results suggest that B&H consumers assume B&H vineries trail behind foreign vineries considering product assortment, overall quality, quality-price ratio and communication with consumers. So, B&H vineries have to change its' market image and to innovate in order to satisfy the needs of young, future customers.

KEYWORDS: *Market segmentation; targeted customers; vine consumers*

INTRODUCTION

Global and national market place, driven by the process of globalization, blurs boundaries between countries (Hallatt, 2005) and develops strong global interdependence in many economic sectors. That is why national markets, especially the food market, are becoming ultra competitive. Therefore, competitiveness as a concept is deeply connected with economic performances and sustainable development including rural development (Krom, Sagi, 2005), and this concept becomes major concern and preoccupation of national, regional and local governments, industry groups, business executives, and social stakeholders (Esterhuizen, van Rooyen, 2006; Bojnec, Ferto, 2006). Although companies' competitiveness depends on macroeconomic surroundings, it is still mostly based on it's ability to meet consumers needs better than the others (Nikolić et al., 2011). So, customer's perception and its satisfaction is a base on which companies have to build up its competitiveness and success. But, it is not an easy task to define customer's perception because it is an ABSTRACT concept of multidimensional nature, and it is connected with external and internal features of a product (Olson, Jacoby, 1972; Steenkamp, 1989; Gellynck et al., 2008; Jover et al., 2004). Besides the product itself, one must mention the importance of consumers' attitude, their willingness to spend time and money to get a better insight of the desired vine as product. That willingness is called "the level of involvement" which is defined by correlation between the consumer and the vine itself (Lockshin et al., 2001), and it affects the level of buyers loyalty to a specific product. It is accepted that there are consumers with high as well as

those with low level of involvement. Highly involved consumers are those who want to learn about a product and expect the producer to inform them and, also, they spread that knowledge forward to other potential consumers. When a consumer takes a product which requests high involvement, as vine does, he mostly wants to enjoy, to be intellectually „challenged", and not only to satisfy some of the basic needs. Consumers with low involvement do not have enough time to spend shopping and gaining knowledge about all quality characteristics, and they try to simplify their choice during shopping by assessment of price suitability (Hollebeek et al. 2007) and other quality characteristics of product which are derived from consumers trust in a producer and his brand name. It is logical, if one has in mind that a brand name, as part of external quality characteristics, is entitled to attract a buyer, inform him about the key attributes of a product as well as to persuade the consumer to repeat shopping, enlarge his loyalty (Lau, Lee, 2000), and eventually, to pay extra price for the product (Myers, 2003). Consumers with low involvement in public (e.g. restaurant), are much more often subjects of society and environment influence (Howlett et al., 2002; Hollebeek, 2007). Although brand means more for consumers with low involvement, it also affects the perception of highly involved consumers. All aforesaid signifies the importance of brand performance and it's sophisticated appearance on the market, which are some of the key factors in developing brand competitiveness (Atilgan et al., 2005), because it allows companies to dictate prices, evaluate market power and gain unexpected profit (Gupta et al., 2008). In other words, a successful vine producer has to take care about nutritive, microbiological and sensory attributes of a product, as well as „additional services", meaning attributes of business process which provide the buyer additional services such as: saving time on shopping and usage, reflection of society status etc. Relative importance of different quality dimensions are connected to consumers' level of involvement (Charters, Pettigrew, 2007). Thus, it indicates that sensory/tasting attributes are the most important for highly involved consumers. Consumers with low involvement, which are mainly „new consumers", pay more attention to extrinsic characteristics, meaning their decision to purchase is based on previous experience with product and on product price. Accordingly, cheaper vines, accompanied with adequate and catching package, may become a hit on the market. That was the strategy many countries of "New vine world" implemented. In addition, while consumers with low involvement respect mostly straightforward intrinsic quality characteristics as appearance, taste, smoothness; consumers with high involvement recognize complexity and potential for aging, while balance and alcohol concentration are important for all of them. It should be underlined that studies so far have shown that one values vine as much as he think it is expensive (Brochet, 2001; Plassmann et al., 2008). In literature this effect is called "placebo effect".

Current situation where import is almost six times bigger than export (in 2009 import was above 30 millions of BAM and export was above 5 millions of BAM1[1]) suggests that B&H vine producers have problems in building their own competitive performances. Hence it suggests that strong foreign competition is able to offer better quality-price ratio and to make customers more satisfied in comparison to B&H vine producers. So, it is possible that "new consumers" or "future consumers" become accustomed to consume trendy vines whose characteristics (especially taste, aroma, sweetness and colour) are significantly different from traditional B&H vines like Țilavka and Blatina, which are the basic product of each B&H vineries. It can decrease B&H vine consumers capability to recognise uniqueness of B&H traditional vines, making them less able to make informed decision about quality-price ratio of B&H vines. It can discourage local demand of B&H vines and affect negatively on future prospects of the whole sector. This is not a unique situation. Studies of vine consumers perception in South Africa, where vine consumption is neither usual nor traditional (like in B&H), and where majority of consumers are „new consumers" concluded that vine producers have to make an effort to educate these consumers in order to make them change their opinion on a vine (Leah et al., 2009). It is important to research the market in order to enable vineries to develop brands connected to consumers, and to reach targeted groups, especially women and those who just begin to consume vine, because they are the most willing to become highly involved consumers.

This paper aims at assessing consumers' estimation of B&H vineries related to foreign (regional) and to try to define market segments to group consumers, in order to enable B&H vineries to choose a targeted group of consumers as well as to create a marketing strategy which is going to satisfy its needs and create loyal local clients.

1 1 € = 1,9558 BAM

MATERIALS AND METHODS

The basic postulate of this study is that B&H consumers are mainly in the group of „new customers“ who are just starting to consume vine, because they are most likely to become highly involved consumers. That is why it is important to research their attitude regarding B&H vineries compared to foreign ones and their perception on quality of B&H vine. Consumers' perception should be the base for defining different market segments.

Method of survey is used. Questionnaire was consisted of 17 questions sorted in several groups: level of vine recognition, consuming habits (e.g. what occasions, frequency of consuming), attributes important in vine selection, position of B&H vineries compared to region producers, basic socio-demographic information of examinee (age, level of education, monthly income, social status). Most of questions were to answer on scale between 1 and 5 (so-called Likert scale).

The survey comprised 300 subjects by non-random selection, by forming sample that is, according to Newbold et al. (2010) „regularly used by public opinion testing organization“, and is recommended to identify attitude and perception of examinees. According to Geraghty, Torres (2009) such sampling method beside them was used by Hall (2004) and Bruwer et al. (2004). The first question about vine drinking habit was used to select target population, more frequent vine consumers. There were 250 examinees from Sarajevo, and 50 from Țepče. In Sarajevo, survey has been done in front of main supermarket chains, while in Țepče it took place in front of the Cultural Centre ("Dom kulture") during April and May 2010.

Sample validity was checked using Bowman Shelton (split – half) test, which, in our case is 0.461, and it is a little bit lower value than recommended for this coefficient for exploratory type of research (Markovina, 2009). Reliability of results for total sample is 0.671 (Crombarch alpha coefficient), which represents acceptable level of measurement reliability (Markovina, 2009, Karpati, Szakal, 2008).

Cluster analysis is used to define market segments. This analysis is done for 17 factors (two for involvement, 10 for motives/life style and five for way of purchase). Squared Euclid distance is used to measure distance (differences) between clusters, Ward method used to connect objects into clusters, optimal number of clusters was defined on "Agglomeration schedule", and validity of defined clusters was verified by Monte Carlo method.

RESULTS AND DISCUSSION

In this survey there were 168 (56%) male examinees and 132 (44%) female examinees, which is satisfactory due to the fact that women are lesser consumers of alcohol beverages. Out of total number of examinees, 121 (40%) were younger than 25, none had more than 60 years, and out of reminder 60%, 86 (29%) belongs to age group 25-39, and 93 (31%) to age group between 40 and 60. Such age structure supports the focus of study to „new consumers“.

Major part of examinees indicated possessing slight knowledge about vines, while only two of them stated that they are expert for vines. This is in line with identified customer's ability to connect company name and brand name of six of the most popular vines listed (45% of examinees were not able to connect any company with brand name). Income, according to results, is the most significant socio-demographic characteristic of examinees. The most important vine attributes for high income consumers is trust to producers and geographical indications, while others matter the most to price, colour, grape variety and vintage year. Additionally, survey showed that participants, while purchasing vine, paid main attention to the previous experience and recommendation by their friends. All mentioned suggest that B&H vine consumers need significant amount of reassurance. This finding is in line with research done by Rithcie (2009), Resnick (2008) and Kolyesnikova et al. (2008).

According to our results, majority of participants think that B&H producers are worse in marketing strategies than foreign producers in the region, worse in packaging of vines, and that they are neither aware of consumers' taste, nor promote adequately tradition of the product, vineyards and/or cultivar. Major part of participants (81%) consider that B&H producers do not have high enough level of services and do not care what consumers need. Therefore, it is not surprising that the major proportion of participants, asked

to quote two of the most consumed vines, specified Ždrepčeva krv (SRB) and Vranac (MNG), the imported vines. The main variables shaping customer behaviour are grouped into three identified and validate clusters. Statistically, structure of clusters according to level of cognition of vines doesn't differ.

Table 1. Vine attributes gruped into the identified clusters

	Cluster 1	Cluster 2	Cluster 3
Variables	Confidence in producer – important attribute	BiH vine producers are worse in packaging of vines	BiH vine producers are worse in marketing strategies
	Vine price– important attribute in purchasing decision	BiH vine producers has low quality-price ratio	BiH vine producers do not promote adequately tradition
	Proces proizvodnje – irrelevant attribute in purchasing decision	BiH vine producers has not enough high quality of vines	BiH vine producers do not promote vineyards and cultivar
	Vinification year – irrelevant attribute in purchasing decision	BiH vine producers are not aware of consumers' taste	

Cluster 1 – "low involved and price conscious" are the consumers that, when purchasing vine, pay the most attention to price, and main reason is their confidence in producer. These are low involved consumers. This type of consumers wants to consume proper vines on proper prices, which means average vines affordable to wider population, with no demand of too high level of knowledge about intrinsic characteristics of vine quality. They are older than 25, mostly men with lower level of income (less than 750 KM) and lower education (secondary education).

Cluster 2 "prospective consumers" are consumers whose opinion is that B&H producers are not competitive to producers in region and that total quality of B&H vine production is not satisfactory. Also, neither package nor ratio between price and quality of B&H vines is satisfactory. This should be an alert indicator for B&H vine producers, because consumers are turning to imported vines. Especially if we know that they are young (>25 years) and some of them are still students. So, they have potential to become loyal local consumers and to provide "home basis" for B&H vineries supporting development of its competitiveness.

Cluster 3 "highly involved consumers" who always need something new. These consumers believe that current marketing strategies do not meet buyers' needs and cannot attract consumers. Also, they need producers to accent tradition of vine production in their regions, as well typical sorts for that area. There were mainly participants with monthly income of 750 KM and higher. Also, there were predominantly beer, and not vine consumers. If vineries want to focus on this segment, they have to think how to deal with "beer competition".

Clusters do not differ neither by way of consuming of vine nor purchasing it as gift. Significant difference occurs when consuming vine in restaurants, during lunch/dinner or other circumstances. The "prospective consumers" prefer to consume vine in restaurants, while all examinees stated that they like to consume vine in good company and to bring vines as a present.

CONCLUSION

B&H vine industry faces strong competition of imported vines. According to examinees opinion B&H vineries trail behind regional competitors in their capacity to create favourable product perception, which is, according to Martinez et al. (2006), key to market success. So, B&H vineries have to improve their marketing efforts significantly. Additionally, research findings suggest that B&H consumer's quality perception is shaped by extrinsic quality cues such as price, brand etc. It makes them more open to "the power of suggestion", meaning that their brain literally tastes price and region, before it even begins to consider merits of vine itself. According to Priilaid (2006) this "placebo effect" is a usual situation, especially with less involved and less knowledgeable vine consumers.

The research findings indicate higher level of B&H consumers' recognition of imported regional vines. According Wilcox et al. (2008) brand awareness has strong positive relationship with brand survival. This is

an early warning for B&H vineries to change their market strategies and to use markets segmentation to provide better focus on customer needs (Geraghty, Torres, 2009). According to identified market clusters, B&H vineries should consider the following:

- To create product (decent quality and fair price) attractive for price conscious consumers for everyday consumption. This vine should be sold at supermarkets with clear in store information (such as point of sale display, labels or expert opinion) to provide needed reassurance. All such product attributes have to convey a message "these products bring together good friends and make life enjoyable". Companies can make new, fresh brand connected with two authentic grape varieties. Promotion activities have to focus on developing trust between producers and consumers either trough activities of building individual brands or generic, common "B&H vine" brand .
- To improve "value package" of B&H vines to satisfy the needs of prospective consumers, including strong promotional campaign in order to educate consumers and make information about uniqueness of B&H traditional vines more accessible. This should include different types of culinary shows, strong tasting campaigns, and different types of story tellers connecting local identity history, culture, life-style and vine. The promotion program has to focus on restaurants and other amusement facilities where young people prefer spending their time. The result of such program has to be increased capability of hospitality service/waiters to recommend vine, to tell catching stories about them and to increase sale of local vines. Such improved vines have to be offered in different package sizes and easy to handle, storage or to drink in restaurants (as house vine or vine per glass).
- To develop strong brand of high quality vine, based on two autochthonic grape varieties and geographic denomination. Such vine has to ensure that the possibility of consumers reaching full and total enjoyment is maximized. It has to be served properly with full explanation and with an adequate food selection. All its extrinsic quality cues have to convey message of uniqueness, style, cultural sophistication and local identity. This is crucial for success because most people think about the impression which a bottle of vine will give about them. Such vine should to be offered and consumed at special occasions. The main distributional channels should be restaurants, vine roads and special/souvenir shops.

The B&H vineries should also aim to pursue some generic and joint programs:

Generic promotion to strengthen recognition and knowledge about B&H traditional vines Ţilavka and Blatina and to make their geographical indications and connections with local culture, life-style and history visible. This way B&H vineries will be prepared to satisfy future vine consumers needs which are, according to Bruwer, Jonson (2010), the needs for authentic and traceable products.

Program to create B&H souvenir or "B&H gift". Such program will strengthen recognition of B&H traditional vines and satisfy B&H vine consumer's needs to "give a present" and to convey right impression about consumers personality, sophistication and life-stile.

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FUZZY LOGIC BASED MENU PLANNING IN PREVENTION AND TREATMENT OF HYPERTENSION

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ABSTRACT

Fuzzy sets are used to represent the inherent imprecision and fuzziness of food quantities and nutrient values as well as to model the gradual boundaries of the daily recommended values associated with each nutrient. Knowing that cardiovascular diseases are considered as one of the leading causes of today mortality, especially for men aged 31-50 years, who conducted low active way of life with a diet that is mainly consumption of ready to eat or fast food, and that small interventions in the nourishment can achieve significant positive impact, fuzzy approach was used to plan daily menus. DASH diet as a lifestyle was proven as a good treatment in the prevention of height blood pressure for the high-risk population, correcting inadequate nutrition. The principles of fuzzy logic were used in the analysis and optimization process, based on developed membership functions for nutrients and food groups, for the target population of men aged 31-50 years, according to guidelines that are outlined in the treatment of hypertension (DASH guidelines). The algorithm used in the analysis and optimization is written in the programming system *W.R. Mathematica v.6*. The final result of the optimisation process is a set of daily menus, 65% of which are nutritional acceptable. The acceptable daily combinations can be used in healthy meal planning and in prevention and treatment of hypertension, changing nutritional habits according DASH guidelines. This would also help to avoid consumption of unacceptable daily offers (almost 35%) from the set. The results indicated that use of fuzzy logic is well suited to deal with the inherent imprecision of data associated with food quantities and their nutrient values, and to propagate it through computations in a mathematical way with a great applicability in diet planning as well as in the demanding cases as DASH diet planning.

KEYWORDS: *Optimisation; Fuzzy logic; menu planning, DASH*

INTRODUCTION

Studies have shown the Dietary Approaches to Stop Hypertension (DASH) diet to be as effective for lowering blood pressure (BP) levels as the daily consumption of one prescription medication (Appel et al., 1997; Svetkey et al., 1999; Kwiterowich et al., 1997). Uncontrolled hypertension can cause arteriosclerosis, cerebrovascular accidents, myocardial infarction, and end-stage renal disease (Alderman, 1999) and because physically devastating is called the "silent killer" (Burt et al., 1995; Hajjar, Kotchen, 2003; Kumanyika, 1997). For a person with hypertension new eating style is a shock, and the following shock is a radical change in the costume eating habits (Cook et al., 1995). Guidelines given by the DASH diet are valuable for those who know their daily intake, but the less troublesome step from the guidelines could be the reduction of sodium intake as well increase of fruit and vegetable consumption. Sometimes the first step is searching of readymade recipes based on the DASH diet guidelines (AHA, 2004). This is the reason way we have analysed such offers (internet recipes), what was the first goal in this work. The second goal was to propose – computer based menus that will help in the further menu planning. Having crisp values for daily needs it is possible to use them in computer-based planning of optimal menus with respect to agreed evidence-based dietary recommendations and guidelines. Recommendations are usually expressed as specific quantities (crisp values). In meal planning, crisp values are to rigorous limitations, such as restrictions on nutrient intake like, for instance, the limits for sodium in the DASH diet – 2300 mg/day. Crisp values define the exclusive affiliation in the set, yes or not (Boolean logic). If the daily sodium recommendations are defined as 2300 mg, the daily offer that contains for example, 2350 mg is completely unacceptable. The overload of 50 mg of sodium per day could

be an acceptable overload, but this offer with 2.2% more sodium than recommended will be excluded if the crisp value is overdrawn (Rumora et al., 2009). Fuzzy logic is used to describe unreliable (imprecise) data and knowledge, using linguistic variables, such as slightly deficiency or surplus, much more or less of some nutrient, etc. (Gajdoš Kljusurić, Kurtanjek, 2003; Čerić, 2007).

In order to visualize fuzzy concept, on previous example, excessive intake of sodium (50 mg), will be estimated with a value between 0 (not belonging to the set) and 1 (completely belongs). This value of belonging to a set is defining the acceptability or unacceptability grade (AHA, 2004). Bernd Wirsam (1995) was the first that has applied fuzzy logic in defining nutrient intakes using membership functions. For each nutrient was determination a fuzzy set, μ (xi). The tendency is to achieve maximal value (value 1) in the membership function μ, for each observed ntrient, which would mean that the input of the optimal nutrient (Hahn et al., 1995). The goal, using fuzzy sets in nutrition is to optimize the diet so that the requirements for all observed nutrients are met. For example if there is too little dietary fibre and too much energy in the diet, adding wholemeal bread gives more dietary fibre but also more energy – so there is a conflict (Wirsam, Uthus, 1996; Wirsam, Hahn, 1999; Wirsam et al., 1999; McBride, 1997). To solve this conflict and to represent the logical "and" (because it is desirable to optimize both dietary fibre and energy), compromises are made (Wirsam, Hahn, 1999; Wirsam et al., 1999). Wirsam suggested the application of the product of the minimal membership vale and the harmonic mean to the fuzzy sets of the rest of observed nutrients, gives the name to the crisp value that defuzifies the optimisation – the Prerow value (PV):

$$\boldsymbol{PV} = \min\left[\boldsymbol{\mu}(\boldsymbol{x}_i)\right]\cdot(\boldsymbol{n}-1)\cdot\left(\sum_{i\neq i_{\min}}\frac{1}{\boldsymbol{\mu}(\boldsymbol{x}_i)}\right)^{-1} \qquad (1)$$

μ(xi) are the fuzzy sets for *i* nutrients that are observed. The Prerow value so defined is now a measure for how close one comes on average to the recommendations - or how healthy our food is. With the Prerow value one can decide whether a certain nutrition situation is better or worse than another. Wirsam has graded the PV values (between 0 and 1) of a nutritive offer concerning their impact on health (Wirsam, Hahn, 1999; Wirsam et al., 1999). In accordance with the health impact, the preferred PV values are PV>07, and an optimal meal or menu offer, energy and nutrient balanced, will have PV >0.9, if it is possible PV ≈ 1, what would be an indication for optimal offer of all observed nutrients. The Prerow value so defined is a measure of how close meals come on average to the recommendations; in other words it means an index of measure how healthy is a given menue . The aim of this paper was to use fuzzy logic in the (a) menu analysis and (b) menu planning. In the analysis and planning the primary idea was to balance the daily energy and nutrient needs significant in DASH diet (Wirsam, Uthus, 1996; Wirsam et al., 1997; Gajdos, Kurtanjek, 2003; Wirsam, 1995; Rumora et al, 2007).

MATERIALS AND METHODS

Long-term adherence to dietary modification is difficult for most people (Little et al., 2004) and, necessary are interventions that will help people adhere to dietary modifications. DASH diet guidelines like: total fats (27 % of calories), saturated fats (6 % of calories), proteins (18 % of calories), carbohydrates (55 % of calories), cholesterol (150 mg), sodium (1,500 mg), potassium (4,700 mg), calcium (1,250 mg), magnesium (500 mg) and fibbers (30 g) are crisp values that should be achieved for a daily intake based on 2,100 kcal eating plan (USDA, 1995). A weak plan diet according DASH diet guidelines were used (AHA, 2004). Each day offer consisted of one breakfast, lunch, snack and dinner. Daily offers for a weak were on-line (i) analysed in order to evaluate the eligibility with DASH diet guidelines, and (ii) using the optimisation tool, the dish offers were combined in new daily menus and (iii) new menu offers were evaluated with the PV value. During the analysis the goal is to determine the adequacy of mutual combining of individual dishes of daily meals, and to determine the number of daily combinations that are nutritionally acceptable (PV > 0.7 connotes energy and nutrient intake of all observed nutrients in the acceptable range). Basic emphasis was on nutrients whose intake, in the DASH diet, is general important in the treatment measures: energy, fat, saturated fat, cholesterol, magnesium, potassium, calcium, sodium, dietary fibre, proteins and carbohydrates (Table 1, column "observed parameters"). DASH diet guidelines emphasize the quality of fat, or fat consisting mostly

n-3 and n-6 unsaturated fatty acids. Their membership functions were modelled according to their share in the total daily fat share. So, 11 membership functions were modelled according the DASH recommendations (table 1), as presented on Fig. 1. The algorithm used in the analysis and optimization is written in the programming system *W.R. Mathematica v.6.*

RESULTS AND DISCUSSION

Membership function for each of the observed parameter (11 of them) was modelled and used in the menu analysis and planning. Membership functions were modelled for the male population group aged 31-50, with low physical activity (PA=1), and potentially suffering from hypertension.

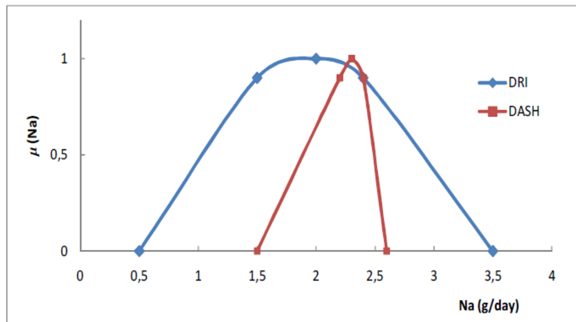

Figure 1. Membership function, of (i) sodium as recommended by DRI and (ii) sodium according DASH guidelines

Using fuzzy logic, during the analysis of present DASH menu offers (AHA, 2004), it was possible to give the total acceptance grade using the PV value as well as to identify insufficient nutrients in the offer. The analysis of DASH meal offers given by NHLBI (2010) is presented in Table 1.

Table 1. Energy and nutrient content of daily offers found on the internet, regarding DASH diet guideline (*AHA, 2004)

Observed parameters	DASH* Recommendations	Different daily offers in one week						
		D1	D2	D3	D4	D5	D6	D7
Ed, kcal	2100	2062	1997	2086	1801	2039	1939	1993
Total fat , % Ed	27	27,5	31,1	29,8	13,0	19,9	26,9	28,9
Saturated fat , % Ed	6	5,7	4,5	3,9	3,0	5,3	5,6	5,9
Cholesterol , mg	150	155	76	61	88	264	171	71
Magnesium, mg	500	594	n.d.	n.d.	n.d.	n.d.	548	537
Potassium, mg	4700	4909	3384	3249	3196	344	4710	4693
Calcium, mg	1250	1220	1035	1132	819	1201	1210	1616
Sodium, mg	2300	2101	2014	1786	1395	1335	1671	2069
Dietary fibre, g	30	37	34	37	24	23	36	32
Protein, % Ed	18	27,9	18,6	17,4	21,6	9,6	21,7	18,7
Carbohydrate, % Ed	55	55,1	54,9	51,8	67,8	67,7	55,3	56,8

n.d. – not detected; D1 – D7 are daily offers where each offer included one breakfast, supper, dinner and lunch

The menu offer of the day 4 (D4) is unacceptable regarding the insufficient daily offer (total fat, saturated fat, cholesterol what can be sighted positive, but the insufficient intake of calcium and dietary fibre is unsatisfactory. The offer of the 5th day has significant insufficient share of dietary fibres as well as proteins. Next step was to include optimisation in new menu offers, based on dish offers included in the week offer provided by the offer on internet (NHLBI, 2010)**.** Optimisation provided assistance in the selection of foods and meals as well as their combination with each other. The above weakly offer (DASH menus) consists of 7 daily offers that included breakfast, lunch, brunch and dinner. Combining the dishes, it would be possible to gain 2401 different menus (7Bx7Dx7Sx7L) but are all possible offers really acceptable regarding the DASH recommendations?

The self-developed software that analyses and plans meal offer based on membership function was used. The input variables (nutritive share of a menu or a dish) were estimated using membership functions of 11 parameters in order to get an output set of meal combinations. The software included a feature that allowed decoding fuzziness, in order to understand and compare the results. Fuzziness was decoded with the assistance of Prerow features (PV value, Eq.1). As acceptable offer was considered a daily offer whit the PV value within the range of 0.7 to 1 what is according to Wirsam and co-workers (1997) a nutritionally acceptable offer. If the final PV value is closer to 1 there are more nutrients whose daily intake is optimal or close to it.

The final result of the optimisation process is a set of daily menus, where 65% of them are nutritional acceptable. The acceptable daily combinations can be used in healthy meal planning and in prevention and treatment of hypertension, changing nutritional habits according DASH guidelines. Using optimisation tool based on fuzzy sets would also help to avoid consumption of unacceptable daily offers (almost 35%) from the set. Result of 35% of unacceptable daily offers is an effect of inappropriate combining of dishes that has as an outcome insufficient intake of one or more nutrients per day. This is not a problem if such intakes are rare, but with the enlargement of such offers, can result with negative impacts on human health (Little et al., 2004; McCarron, 1998; MacMahon, Rogers, 1993).

CONCLUSION

The results indicated that use of fuzzy logic is well suited to deal with the inherent imprecision of data associated with food quantities and their nutrient values, and to propagate it through computations in a mathematical way with a great applicability in diet analysis and planning as well as in the demanding cases as DASH diet planning. The use of fuzzy logic has also highlighted the possibility of insufficient energy and nutrient intake when dishes from different daily offers are combined in an inaptitude way. Using optimisation in meal planning show that in the optimised meals the energy amount is in proportion with DASH recommendations with no fear of the influence on health as Wirsam and Hahn (1999) pointed out.

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A FERMENTED WHEY PRODUCT BENEFITS MALE PATIENTS WITH LOWER URINARY TRACT SYMPTOMS

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ABSTRACT

After the age of 50, the prostate begins to increase in size. This is known as benign prostatic hypertrophy (BPH). Compression of urethra by enlarged prostate causes dribbling reduced force of the urinary stream, pain and occasional bleeding or infection. This causes difficulty in urinating and requires many men to get up several times during the night to urinate.

The aim of our study was to evaluate the efficiency of consuming new fermented whey based product (FWP) to several biochemical parameters and lower urinary tract symptoms (LUTS).

Patients from the outpatient department of Andrology Center were randomized. The patients had minor to moderate LUTS, International Prostate Symptoms Score (IPSS) range 3-19 but not prostatitis (NIH-Chronic Prostatitis Symptom Index (NIH-CPSI) <4 and 2-glass test negative for prostate inflammation and infection).

During consumption of the FWP, the IPSS score decreased in patients with moderate LUTS/BPH ($p<0.001$) whereas irritative and obstructive symptoms changed in parallel. LUTS symptoms changed in correlation of blood high-sensitive C-reactive protein (hsCRP), glycated haemoglobin (HbA1c), oxidized low density lipoprotein (oxLDL), interleukine-10 (IL-10) and 8-isoprostanes in urine. Statistically significant changes in mentioned parameters occurred in study group but not in control group. Compression of urethra by enlarged prostate explains LUTS in BPH patients. Due to elevated oxidative stress (OxS) the peroxidation of cell membrane phospholipids occurs. This generates 8-isoprostanes, the prostaglandin-like compounds that can exaggerate LUTS. 8-isoprostanes may cause constriction of bladder and urethra in nanomolar concentrations.

Consuming FWP may improve LUTS as well as OxS and diminish LUTS-related inflammatory response.

KEYWORDS: *lower urinary tract symptoms (LUTS), benign prostatic hypertrophy (BPH), prostate, fermented whey product (FWP), oxidative stress*

INTRODUCTION

Lower urinary tract symptoms (LUTS) due to benign prostatic hypertrophy (BPH) affect approximately 25% of men worldwide after the age of 40. When prostate enlarges, urethra becomes compressed. This causes difficulty in urinating and requires many men to get up several times during night to urinate including dribbling, reduced force of urinary stream, pain and occasional bleeding or infection. The prevalence of BPH reaches as high as 70% in the older men, although it is debatable whether such prevalence is reached in the 60-69 or 80-89 years old age group (Wei et al., 2005; Parsons et al., 2008). Thus, the prevalence is age-dependent. As reviewed by Parsons, 2010 the epidemiologic correlates of BPH are serum dihydrotestosterone, obesity, elevated fasting glucose, diabetes, fat intake and inflammation that increase the risk while vegetables, regular alcohol consumption, exercise, and non-steroidal anti-inflammatory drugs (NSAIDs) decrease the risk. The prostate enlargement ordinary risk factors are age, decreased sexuality and the level of androgens. Besides that, LUTS associates with oxidative stress (OxS). During the OxS, the peroxidation of cell membrane phospholipids generates of 8-isoprostanes (8-EPI), the prosta-glandin-like compounds that can cause

LUTS. It has been demonstrated that antioxidative treatment reduced LUTS in BPH patients (Matsumoto et al., 2010). It can be partially explained by reducing 8-EPI level in the bladder as it has been shown that 8-EPI can constrict bladder and urethra at nanomolar levels (Tarcan et al., 2000).

Fermentation of whey isolate by specific lactic acid bacteria (LAB) strains could be an interesting possibility to produce dairy functional food with high nutritional content (whey proteins have high bio quality) and perhaps a therapeutical effect to LUTS/BPH *via* reduction of OxS. The aim of our study was to evaluate the efficiency of original FWP in the light of some biochemical parameters and IPSS (International Prostate Symptom Score), a questionnaire to evaluate LUTS.

MATERIALS AND METHODS

Patients were selected from outpatient department of Andrology Center Tartu University Hospital. The patients gave written informed consent and the Tartu University Ethics Committee approved the study. All subjects enrolled met the inclusion and exclusion criteria and fulfilled International Prostate Symptom Score (IPSS) questionnaire. 58 patients of light and moderate LUTS/BPH were recruited into randomized double blind control group included clinical study. The inclusion/exclusion criteria were: 55±5 years of age, prostate specific antigen (PSA) <4 ng/ml, light to moderate LUTS (IPSS score 3-19), excluded prostatitis (NIH-Chronic Prostatitis Symptom Index (NIH-CPSI) <4 and 2-glass test negative for prostate inflammation and infection. All patients were randomized into two groups, control group (n=19) consumed apple juice 50g/day and study group (n=39) consumed 50g/day of yellow fermented whey product during 2 weeks (7 patients consumed FWP, fulfilled IPSS but could not give blood samples). Whey from semi-hard cheese production (cheese dust and fat removed) was heated to fermentation temperature and the strain *L. plantarum* MCC1 and the strain *L. gasseri* MCC2 were added. After addition, whey was fermented 20 hours and then pasteurized. The product was flavored with sea buckthorn jam, cooled, bottled and stored at 2-6oC. Chemical/physical parameters of whey and end product are presented in Table 1 (Estonian patent application EE201100012 (2011, unpublished); Kullisaar et al., 2011). Blood and urine samples were collected before and after treatment. Routine blood indices were assayed: low-density lipoprotein-cholesterol (LDL-Chol), high-density lipoprotein-cholesterol (HDL-Chol), glucose, HbA1c and Immunoglobulin E (IgE) using a certified Hitachi 912 automated analyzer in the local clinical laboratory (Roche Diagnostics LDL-Chol and HDL-Chol). Assayed blood OxS- and inflammation-related indices were as follows: oxLDL, IL-10, hsCR and urine indices 8-EPI. OxLDL level was measured by using the Mercodia Oxidized LDL Enzyme-Linked Immunosorbent Assay (ELISA) kit, manufactured by Mercodia AB, Sweden. Plasma level of IL-10 was measured by an ELISA using a commercially available kit (Human IL-10 Immunoassay, catalogue number D1000B, R&D Systems Inc.®, Minneapolis, USA). To characterize systemic OxS the content of 8-EPI was assayed in urine. This assay is a competitive enzyme-linked immunoassay for determining levels of 8-EPI in biological samples (BIOXYTECH 8-Isoprostane Assay, Cat. No. 21019; Oxis International, Inc., Portland, OR, USA). The hsCRP was determined by a latex particle-enhanced immunoturbidimetric assay (Roche Diagnostics GmPh, Germany) with the automated analyzer Hitachi 912 (Pihl et al., 2003).

RESULTS AND DISCUSSION

The parameters of whey and end product are presented in Table 1. Changes in nutritional parameters (fat, protein, carbohydrates and dry matter content) caused by addition of sea buckthorn jam. The drop of pH is the result of biological activity of used strains and also addition of sea buckthorn jam.

Table 1. Chemical parameters of whey and end product

Ingredient/parameter	Whey	Product
Fat (%)	< 0.1	0.72±0.03
Protein (%)	0.66±0.10	0.98±0.04
Carbohydrates (%)	4.07±0.59	9.9±1.2
Dry matter (%)	5.37±0.27	11.99±0.12
pH	6.14±0.41	3.99±0.14

The inflammatory prostatitis and prostate cancer were excluded on the basis of investigation of prostate secretion and NIH-CPSI questionnaire. IPSS score (consists of 3 questions that measure type of irritation and 4 questions that measure obstruction), decreased in patients of moderate LUTS/BPH from 13.0±3.2 to 8.6±3.5 ($p<0.001$) after the consumption of the FWP during two weeks. In the control group as well as in light LUTS/BPH group separately, the reduction of LUTS did not reach statistical significance (Table 2).

Table 2. International Prostate Symptoms Score (IPSS) in LUTS/BPH patients

	Control group of patients (n=19)		Study group of patients (n=39)	
	Before	**After**	**Before**	**After**
Light LUTS/BPH IPSS (3-7 point)	n=9 5.0±1.1	3.5±1.6	n=13 5.0±1.3	4.2±1.4
Medium LUTS/BPH IPSS (8-19 point)	n=10 13.7±3.8	11.6±3.5	n=26 13.0±3.2	8.6±3.5*

*Significant difference compared before and after treatment value $p<0.001$

The level of 8-EPI (the gold standard marker of systemic lipid peroxidation as well as OxS) in urine decreased in patients after consumption of FWP in comparison with control (Table 3). The next marker of OxS – oxLDL (approved recently also by EFSA panel of NDA) as well as the new marker of inflammation – hsCRP, decreased (Table 3) and had statistically significant correlation between each other (the Spearman correlation coefficient $r=0.4$, $p=0.017$). In addition, the level of anti-inflammatory cytokine IL-10 increased and the percent of Hb glycation decreased (Khaw et al., 2001) (Table 3). Hb glycation is just a marker of the formation of advanced glyca-tion/lipoxidation end products (AGE-s/ALE-s) that may be both causes and amplifiers of OxS that are linked to physiology of aging and, especially, to patophysiology of diabetes (reviewed by Rahbar, 2005). While hsCRP, glycation of Hb, reduced glutathione (GSH), 8-EPI and oxLDL can be considered a cluster of markers very closely related to OxS, the increase of cytokine IL-10 is more difficult to interpret. BPH is associated with IL-10 polymorphisms (Yoo et al., 2011), as well as with level of IL-10 in seminal fluid (Penna et al., 2007). It is known that enzymatically hydrolyzed whey protein isolate could increase intracellular reduced glutathione concentrations and protect against oxidant-induced cell death in a human prostate epithelial cell line (Kent et al., 2003).

LUTS may have several causes. BHP is characterized by hyperplasia of prostatic stromal and epithelial cells, resulting in the formation of large, fairly discrete nodules in the periurethral region of prostate. When sufficiently large, the nodules compress the urethral canal and cause a partial, or sometimes a virtually complete obstruction of the urethra, which interferes with the normal flow of urine. LUTS associates also with OxS. Reactive oxygen species (ROS) can be produced by the cells other than leucocytes and OxS level is not always a marker of leucocytes *per se*, but rather a marker of tissue injury (Pontari, 2002). Motor activity of lower urinary tract can induce a release of ROS into the urine (Tarcan et al., 2000). The iatrogenic bladder obstruction of rabbits resulted in a dramatic elevation of urinary 8-hydroxy-deoxyguanine (8-OHdG), an oxidation product of DNA, concentration that could be reversed by 70% with antioxidant treatment (Matsumoto et al., 2010). Obstructed tissues might generate ROS and release 8-EPI into urine via circulation or direct diffusion through urinary epithelium. Prostatic cells by themselves are able to secrete inflammatory mediators and finally to stimulate their own growth (Robert et al., 2010). The 8-EPI can affect lower urinary tract of rabbits in even nanomolar concentrations (Tarcan et al., 2000). This suggests that 8-isoprostanes could contribute to LUTS by contracting bladder and urethra. In addition, the clinical arm of the same study demonstrated that antioxidant treatment reduced LUTS in BPH patients (Matsumoto et al., 2010).

Table 3. Clinical /biochemical parameters of LUTS/BPH patients before and after consumption of special fermented whey product

	Control group of patients (n=19)		Study group of patients (n=32)	
	Before	**After**	**Before**	**After**
hsCRP mg/L	1.58±0.98	1.92±1.38	1.92±1,24	1.55±0,98*
HbA1c % of total Hb	5.88±0.27	5.86±0.32	5.87±0,35	5.79±0,36**
oxLDL U/L	60.3±11.1	61.0±9.2	72±16	70±16*
IL-10 pg/mL	12.6±7.2	11.4±5.7	8.6±2.2	10.0±2.8*
8-isoprostanes ng/mmol creatinine	47.7±8.4	44.6±10.2	47.8±15.9	37.2±10.1***

* $p<0.05$, ** $p<0.01$, ***$p<0.001$

There were no statistically significant differences between the patients groups in baseline values.

In our previous study, we observed an increase of urinary 8-EPI that correlated with urinary 8-OHdG (both of them in use as OxS markers) in inflammatory prostatitis patients (Kullisaar et al., 2008). In this study, we observed a decrease of 8-EPI and a strong correlation between the former and hsCRP in LUTS patients after consumption of FWP (Table 3). Increased value of hsCRP associated with LUTS/BPH may refer to tissue damage associated with higher level of OxS. Thus, antioxidative treatment may reduce BPH symptoms caused by obstruction, especially those mediated by 8-EPI. The consumption of phytoterapeutic agent – Eviprostat decreased IPSS and lowered the level of OxS marker 8-OHdG (Matsumoto et al., 2010). Possibly, an antioxidant supplementation might keep the obstruction or consequent LUTS subclinical.

It is interesting that the patients with BPH and LUTS have a considerably higher prevalence of cardiovascular diseases than the general population (Karatas et al., 2010). It might be at least partially explained by increased level of oxLDL, decreased level of anti-inflammatory IL-10, effect of 8-EPI on the thromboxanes receptors (Parsons, 2010), and increased level of systemic OxS in their organism that poses risk for development of these diseases. The causal relationships between BPH, its comorbid diseases and the analyzed markers (IL-10, hsCRP, 8-EPI, etc) are not yet established.

The benefit of FWP is in part explainable by high bioquality (biovalue) of whey protein that is superior to other proteins (Smithers et al., 2008). The whey proteins are valuable because of its high content of branched-chain essential amino acids isoleucine, leucine and valine (Pescuma et al., 2010) and sulfur-containing amino acid cystine, the rate-limiting precursor of glutathione synthesis (Griffith, 1999; Zilmer et al., 2005). It is possible that the elevation of GSH in the system and tissues is the FWP's mechanism of action due to the decreased level of redox ratio (GSSG/GSH) and this can prevent OxS and consequent release of 8-EPI. Another explanation is that the whey protein promotes the growth of the beneficial bacteria, as was apparently the case in a study of a whey-based infant formula (Hascoët et al., 2011).

Finally, it is possible that the two lactobacilli strains used for fermentation contributed to benefit because the lactobacilli strains used for fermentation do have moderate antioxidative properties (Estonian patent application EE201100012 (2011, unpublished); Kullisaar et al., 2011). Other studies showed that certain probiotic strains of lactic acid bacteria (LAB) and bifidobacteria may induce oral tolerance to β-lactoglobulin, restore aberrant protein transport and have specific effect on protein degradation in the intestinal mucosa (Prioult, Fliss, 2003). It is also possible that the lactobacilli contributed to the benefit by modulating the gut microbiota. According to the perhaps yet preliminary evidence, INTRODUCTION of new microorganisms into the gut may either decrease or increase the microbial diversity (Uronis et al., 2011). Composition of gut microbiota, and modulation thereof seems to have a profound ability to influence widely diverse facets of the hosts' physiology and metabolism (Larsen et al., 2010).

CONCLUSION

We can conclude that consuming FWP may improve LUTS as well as OxS and diminish LUTS-associated inflammatory response.

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DO ZADAR COUNTY NURSERIES FOLLOW THE MEDITERRANEAN DIET?

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ABSTRACT

Mediterranean diet is probably one of the healthiest dietary models. It is rich in vitamins and minerals sourced from local fresh fruits and vegetables, cereals, olive oil, and fish. Nutrition affects health throughout life, and pre-school age is significant for adopting healthy eating habits. The aim of this study was to assess daily dietary energy value, macronutrient content, and presence of typical food groups according to the principles of the Mediterranean diet in food served at nurseries to pre-school children from Zadar, which is a typical Mediterranean town. The central nursery kitchen prepares meals for eight nurseries that are attended by about 1200 children from the city of Zadar and its suburb area. In collaboration with nursery staff, we collected duplicate portions of meals, four a day, which were served at the nurseries for 15 consecutive days over the winter, spring, and autumn in 2007/2008.

Daily energy and macronutrients content was evaluated and compared with the recommended national daily values. Daily average dietary energy values for the total sample were 1053 kcal in the winter, 1222 kcal in the spring, and 1169 kcal in the autumn. The menus did not sufficiently include typical Mediterranean food such as fresh fruit and vegetables, wholegrain cereals, olive oil, and fish. The obtained results were lower than the recommended values for all three studied seasons. To conclude, the menus indicate that Zadar nurseries do not follow strict Mediterranean model of diet.

KEYWORDS: *pre-school age; Mediterranean diet; meals and/or menus; dietary energy value; recommended daily values*

INTRODUCTION

Children are vulnerable population group for a large scale of ill effects due to various environmental exposures. Three major groups of factors affect children growth and health: genetic and epigenetic inheritance, mental factors, and food consumption (Davaney et al., 2005; Dubois et al., 2007 Mikkila et al., 2007). During pre-school age children develop their behaviours and attitudes towards surrounding environment, including their nutritional habits. Eating patterns can have a significant positive effect on healthy growth and development during childhood and adolescence (Loewen & Pliner, 1999; Kroke et al., 2004) and on health issues later in life (Garemo, et al., 2007; Neumark-Sztainer, et al., 2003). Balanced nutrition has an important role in children's development and helps to avoid the occurrence of a number of health disorders both in adolescence and adulthood. It also ensures physical growth of the child to its full potential (Bruening, et al., 1999; Sepp et al., 2002; Jennings et al., 2011).

Nowadays pre-school children spend most of the day time (8-10 h) in nurseries where they get about 80 % or more of their daily dietary needs through four regular meals (Vranešić Bender, 2006; NN 2007; Vučemilović, Vujić Šisler 2007).

Mediterranean diet (MD) model is considered among the healthiest dietary models because it is well balanced in protein, lipid and carbohydrate content, and, at the same time, rich in vitamins and minerals sourced from local fresh fruits and vegetables, cereals, olive oil and fish (Keys, 1995; Serra-Majem et al., 2003; Trichopoulou 2004). MD warrants attention, in adolescence and/or adulthood because it has been repeatedly associated with protection against several chronic degenerative diseases and disorders. Although

it is not yet clear which components of the diet provide the greatest health benefits, it is likely that certain components, eaten together, provide a dietary pattern that is highly protective. Several possible explanations and biological mechanisms have been proposed for these foods, against the pathogenesis of chronic disease (Brill, 2009).

The main challenge for further research in this area is the lack of representative data on essential elements and other nutrient intake in children's meals. Especially insufficient are information on

different nutrient impact on children's overweight and obesity that is becoming an increasing global public health concern and a matter of national and international research projects (Royo-Bardonada et al., 2003; Lazarou et al., 2009; Farajian et al., 2011; Jennings et al., 2011).

The aim of our study was to assess daily dietary energy value, macronutrient content, and presence of typical food groups according to the principles of the Mediterranean diet in food served at nurseries to pre-school children from Zadar, which is a typical Mediterranean town. Major significance of such study is the assessment of quality of nutrition and daily dietary intake of nutrients in vulnerable pre-school population group that bears multiple health and social benefits as important part of measures for prevention of metabolic health disorders in childhood and adulthood and reduction of significant health costs. All these have been widely recognized as an important issue of the strategy in European research area (ERA) and research topics have been set-up accordingly over the last years.

MATERIALS AND METHODS

The kitchen of public nurseries of Zadar County involved in this research prepares meals for about 1200 children (4 to 6 years of age) attending eight public nurseries. The research was based on evaluation of collected meals served to the children who were attending nurseries over three seasonal periods during year 2007/2008 in order to assess daily energy values and macronutrients content. The food records (menus) about the presence of food groups were taken accordingly.

Daily and seasonal differences of macronutrients in meals were examined by standard methodological approaches. In collaboration with staff in Zadar nurseries, we collected four duplicate portions of meals served a day for 15 consecutive work days over the winter, spring and autumn in 2007/2008. Daily samples were homogenized and analyzed for major nutrient content in accordance to Bruening et al., 1999 and AOAC, 1997. We calculated energy value (Atwater factors) in pre-treated duplicate portion samples and determined macronutrients content by direct analyses of protein (by Kjeldahl method), lipid (by Soxhlet method), carbohydrate (calculated), water (by gravimetric method), and ash content (by gravimetric method).

Table 1. Food consumption according to basic food groups offered in daily meals in public nurseries of Zadar County - winter 2007, spring 2008, and autumn 2008.

Food groups	**Winter** average week consump.	**Spring** average week consump.	**Autumn** average week consump.	**Winter** food serving per day	**Spring** food serving per day	**Autumn** food serving per day
Milk and dairy products	9	6.3	6.3	1.67	1.40	1.27
Meat, poultry, and egg products	8.3	9.3	7.7	1.67	1.73	1.47
Fish	1.5	1	1.3	0.20	0.2	0.27
Fats	3	2.3	2	0.60	0.4	0.4
Olive oil and oil products	1.7	1.3	1.7	0.20	0.27	0.33
Legumes	0.7	0	0.3	0.13	-	0.07
Sugar, honey, and sweets	4.3	6.7	4.3	0.80	1.33	0.87
Vegetables and vegetable products	4.3	5.7	4.7	0.87	1.13	0.80
Fruit and fruit products	4.3	4	7.7	0.87	0.80	1.53
Grains, bread, rice, and cereals	16.3	14.7	12	3.73	3.80	2.87
Potato	2.3	2.3	1.7	0.5	0.5	0.3
Drinks	4	4.7	4.7	0.80	0.93	0.93
Tea	6.3	6	3.3	1.27	1.20	0.67
Water	0.3	0.3	0.3	0.07	0.07	0.07

Served food was distributed to 13 main food groups (Table 1). We determined serving seasonal and daily frequency for each food group and compared the results against the list of typical Mediterranean food groups to verify the compliance.

RESULTS AND DISCUSSION

Energy value in duplicate portions collected during winter, spring and autumn periods of 15 consecutive work days should be in the range between 1239 and 1319 kcal. The range was calculated as 80 % of daily dietary intake recommended by the National programme (NN 127/07), because most of the children attending public nurseries spend there 8 to 10 hours a day. During that time children are offered for meals meaning 80% of total daily energy intake, since it is assumed that the remaining 20 % refers to dinner at home. The National programme limits toleration for deviation of recommended energy values for between each meal to 10%. We found that the winter, spring and autumn periods average energy values were lower than recommended lower limit for 16 %, 1 % and 6 %, respectively. The largest deviation of the daily recommendation was observed for winter period (Table 2, Figure 1).

Table 2. Energy values of meals in winter, spring and autumn

	Distribution Percentiles (kcal)					
	10 th	**25 th**	**Median**	**Mean**	**75 th**	**90 th**
Winter	954	1023	1058	1052	1106	1148
Spring	1045	1136	1193	1222	1326	1390
Autumn	946	1039	1190	1169	1309	1388

Lower limit (LL) – 1239 k cal; Upper limit (UL) –1319kcal
Values are calculated as 80 % of recommended daily energy intake for normal nourished and moderately active child in accordance with the National programme (Narodne novine NN, 121/07).

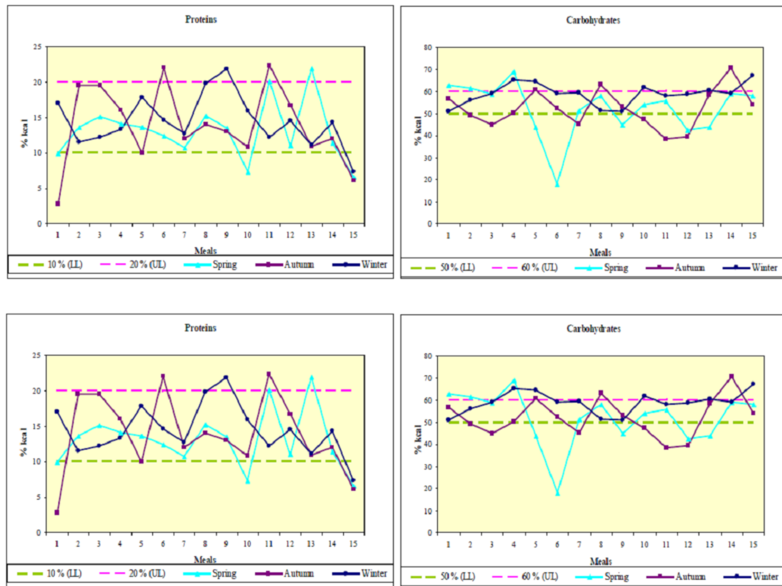

Figure 1. Energy value and % of macronutrient content (proteins, lipids and carbohydrates) in complete duplicate portions in public nurseries of Zadar County during winter, spring and autumn periods in 2007/2008

Target ranges for the macronutrients expressed as percentage of the total daily energy intake were: 30-35% kcal for lipids, 10-20% kcal for proteins and 50-60% kcal for carbohydrates. Average values for all the examined macronutrients during three monitored seasons corresponded the recommended range (Tables 3 and 4, Figure 1), except lipids over the winter period. Nevertheless, large oscillations of the lipid content from day-to-day were observed (Table 5, Figure 1).

Table 3. Protein intake in winter, spring and autumn

	Protein intake (%kcal)					
	10 th	**25 th**	**Median**	**Mean**	**75 th**	**90 th**
Winter	11	12	14	15	16	21
Spring	9	11	13	13	14	19
Autumn	9	11	13	14	18	22

Adequate intake limits for protein content in daily meals offered in nursery:
Lower limit (LL)= 10%; Upper limit (UL)=20%

Table 4. Carbohydrates intake in winter, spring and autumn

	Carbohydrates intake (%kcal)					
	10 th	**25 th**	**Median**	**Mean**	**75 th**	**90 th**
Winter	52	57	59	59	61	65
Spring	44	46	55	54	59	63
Autumn	43	47	52	52	57	63

Adequate intake limits for carbohydrate content in daily meals offered in nursery:
Lower limit (LL)=50%; Upper limit (UL)=60%

Table 5. Lipids intake in winter, spring and autumn

	Lipids intake (%kcal)					
	10 th	**25 th**	**Median**	**Mean**	**75 th**	**90 th**
Winter	22	26	28	28	29	31
Spring	24	27	34	33	39	43
Autumn	27	30	35	35	42	45

Adequate intake limits for lipid content in daily meals offered in nursery:
Lower limit (LL)=30%; Upper limt (UL)=35%

General agreement on Mediterranean diet (MD) as one of the healthiest diet models is based upon evidence on its protective role in metabolic syndrome and diabetes as well as various chronic diseases such as coronary heart disease, several types of cancers, age-related cognitive decline and other. According to current knowledge improper diet just in period of intensive growth and development is responsible for the majority of these chronic diseases. A working definition of the traditional MD is that it is characterised by (cited) 'an abundance of plant foods such as bread, pasta, vegetables, salad, legumes, pulses, fruit, nuts; olive oil as the principal source of fat; low to moderate amounts of fish, poultry, dairy products (yogurt and cheese, primarily derived from goats and sheep, milk consumption less than current intakes) and eggs (a few times per week); sporadic red meat intake, only a few times per month, with greater consumption of lamb, poultry, rabbit or fish; in combination with an active lifestyle, complete the characteristic MD model' (Trichopoulou, 2004; Serra-Majem et al., 2004, Willett, 2006; Jaklin Kekez, 2007).

Zadar County, including the city of Zadar and its suburbs fits geographically to a typical Mediterranean region. However, according to the monitored public nurseries menus, typical Mediterranean food groups such as fresh fish, vegetables and fruits, legumes, olive oil, and water were not presented or not sufficiently represented. Dairy products yogurt and cheese were primarily derived from cow not from goats and sheep milk and were consumed more often then in typical MD, in spite of the evidence that it is desirable in menus for this age group (Jaklin Kekez, 2007). In addition, it should be pointed out that there are certain differences between the traditional MDs when one compares local dietary habits in different countries. In Croatia, cow milk and milk products, except cheese, are more represented in local diet than goat and sheep milk. Similarly applied for the meat; Croats mostly consume poultry, pork, beef, and lamb meat. In general, red meat and all types of meat are common at Croat menus (Table 2) then in MD. Lamb, poultry, and rabbit meats preferred at MD were not at all represented on the menu in public nursery of Zadar County. Although there is a great choice of fresh fish from the Adriatic Sea offered on the local

market, public nurseries tend to supply their kitchen with mostly imported frozen fish and fish products. Legumes are represented rarely, despite of their availability and low price. Sugar, honey, sweets, and various beverages were consumed more often. There were no differences in food consumption frequency with the respect to the season. The limitation of the study is that the adherence to the Mediterranean diet was evaluated just according to frequency of occurrence in offered meals therefore, further research on this topic is necessary.

CONCLUSION

Children are vulnerable population group for adverse effects of xenobiotics and dietary imbalances. in order to ensure healthy growth and development in the young, it is necessary to evaluate and balance their overall diet quality habits and local dietary habits. Our results showed that average values for all the examined major nutrients during three monitored seasons corresponded with the recommended range, except lipids over the winter period due to the lipid content oscillations. The collected food records indicate that Zadar nurseries do not follow strictly so-called Mediterranean diet (MD) model of diet, as it was traditionally represented during the last century which is in accordance with the results of some other researches on MD issue (Naska et al., 2006).

The results yielded by this study are of public health relevance and they may help in setting-up food guidelines in Zadar County nurseries. The results may bear potential to initiate implementing measures to preserve the traditional characteristics of typical local Mediterranean diet, through the promotion of a healthier diet, such as reducing red meat consumption, and increasing wholegrain cereal intake, fresh fish, fresh fruits and vegetables and water instead of various beverages.

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LIGHT OR NOT? - AN OVERVIEW ON STUDENTS\' DIETARY HABITS

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ABSTRACT

Accepting the trend of healthy eating and strengthened care for own health and the desire to enjoy food led to the development of products with lower fat, sugar and sodium (\"light\"). Today, there are a number of different products labeled \"light\" - milk, cheese, yogurt, margarine, beverage and others available on the market. The aim of this study was to investigate the attitudes of students from different faculties towards \"light\" products, and to determine the difference in dietary habits by type of study.

The survey was conducted during June 2010 at Faculty of Economics and Faculty of Medicine, University of Mostar (Bosnia and Herzegovina) and the Polytechnic of Karlovac (Croatia). The sample consisted of 269 students. The original questionnaire, with questions about the knowledge of \"light\" products, motives, attitudes towards them and the frequency of consumption, was used. Data were analyzed by descriptive and inferential statistics methods.

Almost all respondents believe that they know what \"light\" products are (app 98.5%), and describe them as products with less fat and sugar, or with lower energy values (app 35%). Most respondents consumed \"light\" products, while a smaller part of them just tried it (15%) or did not consum at all (6%). Only about 18.6% of the respondents believe that the label \"light\" on the product is trustworthy. The results show that there is no statistically significant difference in dietary habits and attitudes of students of economics, medicine and food technology to light products, but differ in the understanding of light products ($p<0.050$) and the skepticism toward these products ($p=0.007$).

Most of the students consume \"light\" products, but not on every day basis. They do not know the full meaning of the label \"light\". Selection of studies did not affect the dietary habits of students.

KEYWORDS: \"light\" products, students, dietary habits

INTRODUCTION

Today's way of living is, unfortunately, characterized by irregular diet, frequent consumption of fast food and development of new and modification of existing diseases. In order to reduce these bad trends, experts from fields of medicine, food technology and nutrition introduce different innovations in the form of new products and food regimes. All of this led to the development of so called 'light' products (products with reduced amount of fat, sugar and sodium) and functional food (line of new products whose consumption is leading to aimed improvement of physiological functions in the body).

Presented purpose of both of these types of food is to influence the health of the consumers by reducing the risk of illness (by different chronic diseases) and by helping the treatment of already diagnosed diseases. The results of different researches focused on consumption of products with reduced amount of fat/sugar ('light' products) available in current literature are following:

- 'Light' products that are most often consumed are dairy products (Mabić, Matijević, 2010).
- Examinees' attitudes about their own health and diet, and attitudes about 'light' products directly influence the consumption prevalence of 'light' products (Cerjak et al., 2007).

- Girls consume light products more often (Fulkerson et al., 2004). 'Light' products are less tasty compared to conventional products and consumers are more sceptic to them (Hamilton et al., 2000). Persons who believe in connection between nutrition and health consume 'light' products more often (Nayga, 1998).
- Most common reasons for consuming light products are control (regulation) of body weight and nutrition improvement (Viaene, 1997).

Acceptance of 'light' product as a potential "source" of health and the prevalence of its consumption in everyday diet can vary and it is mostly dependent of sex, age, level of education, scepticism, diet habits etc.

The researches have shown that people with higher level of education bear more positive attitude towards 'light' products and also consume them more often. In further consideration of education as an item that influences the acceptance of innovations in nutrition, the authors have put their focus on professional orientation of examinees, that is: on their field of studying. During their study, students of

medical orientation learn about different diseases, causes of their occurrence, treatment and possibilities of their prevention. On the other hand, students of food technology learn about food, its composition, ingredients, value and importance for human growth and development throughout their study. Students of economic orientation do not have much contact with either of these previously mentioned fields, but learn about ways of presentation, advertising and selling products. Guided by mentioned differences in chosen field of studying, the authors wanted to investigate if there is a statistically significant difference in comprehension and consumption of 'light' products between students of different study orientation, economic, medical and food technology specifically.

MATERIALS AND METHODS

The survey was conducted during June 2010 at Faculty of Economics and Faculty of Medicine, University of Mostar (Bosnia and Herzegovina) and the Polytechnic of Karlovac (Croatia). The random sample comprised 29Q students in every year of study having 48.7% students from economic orientation (Economy students), 17% from orientation in food technology (Food technology student) and 35% in medical orientation (Medical students). Distribution by age was as following: 37% men and 63 %female. Age range of subjects included in the survey was 19 to 25 years. After technical control of collected questionnaires, 269 of them were accepted for further analysis. The original questionnaire, with questions about the knowledge of 'light' products, motives, attitudes towards them and the frequency of consumption, was used. Questions were formed as closed type questions (answered by choosing one or more offered answers) and list of statements (Likert scale of five degrees was used for measuring agreement). Examinees took about 10 minutes in average to answer entire questionnaire. Data were analyzed in SPSS 16.0. by descriptive (relative frequency, mean, standard deviation) and inferential (t test, Chi-square test, ANOVA with post-hoc tests) statistics methods. Level of significance was $p<0.05$.

RESULTS AND DISCUSSION

The results of the research for the sample in total are as follows:

- 99% of the examinees claims that they know what 'light' products are, and only 2% of them (4 examinees) have no knowledge of this kind of products,
- Around 35% of the examinees define mentioned products as products with reduced fat and sugar, that is products with lower energetic value, 16% of them consider them to be products with reduced amount of fat, 5% with reduced sugar amount and 9% of the examinees have different way of defining them without stating correct dermition,
- 80% of the examinees consume 'light' products, 15% have tried them at some point, but are not regular consumers and the rest (6%) never tried them, although 19% of these have the will to try them,
- From those examinees who are also regular consumers, only 4% consumes 'light' products on daily bases, 26% do it several times in a week and 70% do it rarer,

- Somewhat more than half (53%) of the examinees are sceptic to mentioned products,
- Only 19% of the examinees trust the label ‘light’, 40% consider it to be deception for the consumer, a mere marketing stunt; the others have no formed opinion,
- Equal number of the examinees (27%) consumes ‘light’ products for the purpose of controlling body weight and for the purpose of the improvement and correction of diet habits, 18% consume it in order to improve their health, 5% because of the recommendation by their physician and the other didn’t state their motives.

The results of the research, when it comes to knowing ‘light’ products and having confident at them according to the selected field of the education (orientation), are shown at Table 1:

Table 1. Habits of consuming and student's attitudes about the ‘light’ products

‘Light’ products	**Economy students (%)**	**Food technology student (%)**	**Medical students (%)**	**p***
Familiar with them	99	98	99	0.871
Not familiar with them	2	2	1	
Have less percentage of fat	23	7	12	**<0.05**
Have less percentage of sugar	8	4	0	
Have less percentage of fat, sugar and sodium	36	49	27	
Have less energetic value	2	38	54	
Other	12	2	8	
Trust the label	18	31	13	0.103
Do not trust the label	37	36	45	
Have no opinion	45	33	42	
Sceptic	44	56	65	**<0.05**
Not sceptic	57	44	36	
Never tried it	7	9	3	0.316
Tried it, not consuming it	16	7	17	
Consuming it	77	84	80	

* Chi-square test

As visible from the table 1, more than 95% of the examinees, in all of the observed groups, are familiar with the term ‘light’ products. This was expected to be the result, but it was surprisingly to find that some of the young people never heard the term ‘light’ product. This is quite a surprise considering the fact that due to the strong influence of the media to everyday life of the individual and frequent and aggressive advertising for the healthy way of eating, caring for one’s health and innovations in the area of food technology, it is almost impossible to get left out. It is even more peculiar that some of the students from the food technology orientation never heard of ‘light’ products, considering that the main theme of their study is food itself.

Although there is almost 100% positive answer when it comes to the results about knowing the term ‘light’ product (Table 1), the true meaning of the label ‘light’ in food industry is very variable. Somewhat over one third of the examinees from economic field have the correct interpretation of the term ‘light’ in nutrition, the same goes for almost half of the examinees from the food technology filed and this is the most present answer in these groups. In the group of the medical field, somewhat over one quarter of the examinees shares this opinion, while most of them consider ‘light’ products to be products of reduced energetic value. Obtained results demonstrate the existence of statistically significant difference when it comes to understanding the term ‘light’ product between observed groups ($x2=5.392$; $p<0.05$).

When it comes to having confidence and believing in the label ‘light’, the results show that most of the examinees from the food technology field believe in it (31%), while in the case of economic and medical field,

the percentage is much lower (Table 1). There was no statistically significant difference between the fields of the studying when it comes to believing in the label 'light'. It was interesting to find that quite the number of the examinees have no opinion of this issue so it was desirable to conduct an analysis without these collected questionnaires. The label 'light' is trustworthy mostly to the examinees from the field of food technology (47%), followed by those from economic field (33%) and then from medical field (22%). There is no statistically significant difference between these groups when it comes to believing in the term 'light' ($p<0.05$).

More than 90% of the examinees have tried the 'light' product at some point in time (Table 1). From these, part of them didn't continue to consume it, but from those who did 77% are from the economic field, 84% from the field of food technology and 80% from medical field.

The reasons for trying 'light' products are observed at all examinees who had at least tried it once. Most of the examinees, without some big difference concerning their orientation, tried 'light' products out of the curiosity (Economy students: 53%; Food technology student: 63%; Medical students: 49%). Second biggest reason, in all three groups, is the care for their own health (Economy students: 14%; Food technology student: 15%; Medical students: 20%). There is no statisticallr significant difference between these groups when it comes to the reasons for trying 'light' products (X2=5.727; p=0.678).

Further analysis comprised only those examinees who have continued to consume 'light' products. From the group of the economic field, only 4% of the examinees consume these products on daily bases, 21% do it several times a week and others rarer than this (75%). In the group of the food technology orientation none of the examinees consume 'light' products on daily bases, 32% do it several times a week and 68% do it rarer than this. From the group of medical field, 5% consume it on daily bases, 30% do it several times a week and 65% do it rarer. There is no statistically significant difference between these groups when it comes to the frequency of the 'light' food consumption (i=4.570; p=0.334).

Relying on the researches from the literature, for the most common reasons for consuming 'light' products, control of body weight, improvement of nutrition, improvement of health and physician's recommendation were listed There was a possibility of adding some other personal motives. The results by the groups were as follows:

- Economy students: improvement of nutrition (27%), body weight control (23%), improvement of health (20%), physician's recommendation (5%),
- Food technology student: improvement of nutrition (29%), body weight control (24%), improvement of health (24%), physician's recommendation (11%),
- Medical students: body weight control (32%), improvement of nutrition (24%), improvement of health (14%), physician's recommendation (3%). The examinees from different groups have no statistically significant difference when it comes to motives for 'light' products consumption (i=9.562; p=0.48).

The prevalence of certain type of products available in 'light' version according to field of studying is demonstrated in Table 2.

Table 2. The prevalence of 'light' products in student diet according to field of studying

	Economy students (%)	Food technology student (%)	Medical Students (%)
dairy products	81	71	81
candy	2	5	4
salty snacks	0	5	0
juices	9	5	7
mayonnaise	3	11	7
other	5	3	1

As visible from Table 2, examinees have stated that, when it comes to different types of 'light' products, they mostly consume dairy 'light' products such as milk, yoghurt, cheese, etc. Dairy products are consumed more than 80% of examinees from economic and medical field and somewhat more than 70% of the examinees

from food technology field There is no statistically significant difference between these groups concerning the type of 'light' product they consume (i=24.739; p=0.212). Table 3 is demonstrating some of the 'light' products consumers attitudes towards different statements related to 'light' products.

Table 3. Assessment of attitudes about the 'light' products

Statement	Mean±Standard Deviation (of scores 1 to 5)			p*
	Economy students	**Food technology student**	**Medical students**	
I am very careful about the quality of the products I consume	3.4±1.0	3.5±1.1	3.4±1.1	0.847
Healthy diet is very important to me	4.1±0.9	3.9±1.1	4.0±1.0	0.513
Consuming food with less calories is very important to me	2.9±1.3	3.1±1.2	3.2±1.2	0.266
It is very important to know the ingredients of the food I consume	3.4±1.2	3.8±1.1	3.5±1.2	0.235
I like to try new products in my diet	3.8±1.2	3.9±1.3	3.7±1.2	0.804
Consumption of 'light' products has positive effect on health	3.2±1.1	3.3±1.1	3.1±0.8	0.612
Consumption of 'light' products helps in maintaining body weight	2.9±1.2	2.8±1.1	2.7±1.0	0.343
'Light' products are healthier than conventional products	3.0±1.1	2.7±1.0	2.5±1.0	**<0.05**
'Light' products taste better than conventional products	2.2±1.0	2.2±1.1	2.1±1.0	0.578
I'll buy more 'light' products in the future	2.6±1.1	2.3±1.1	2.5±1.1	0.554
I'll eat a certain product no matter the taste it has if it is healthy (good for my health)	3.4±1.4	3.3±1.3	3.1±1.3	0.216
The taste of 'light' food is something one can get use to	3.9±1.0	3.7±0.8	3.6±1.1	0.171
'Light' products are only for sick people (people with health problems)	1.7±0.9	1.5±0.9	1.7±1.0	0.641
'Light' products are only for people who want to reduce/maintain their body weight	2.0±1.1	1.9±1.2	1.8±1.0	0.511
The price of 'light' products is affordable to everyone	3.1±1.0	3.2±1.1	2.9±1.1	0.375
'Light' products are cheaper than conventional products	2.6±0.9	2.6±1.0	2.4±1.2	0.584
The food I consume will have effect on my health in later age	3.9±1.2	4.1±1.1	3.9±1.0	0.663
'Light' products provide same amount of energy as a conventional products	3.0±1.0	2.6±0.9	2.6±1.0	**<0.05**

*ANOVA (post hoc: Dunnett's C)

The examinees of economic and medical field have the greatest agreement with the statement

Healthy nutrition is very important to me and the examinees from the food technology field agree with the statement: *The food I consume will have effect on my health in later age* the most. All three groups agree with the following statement: *'Light' products are only for sick people (people with health problems).* Although the obtained results are pretty close, it is interesting to highlight some of the statements (Table 3):

- *Healthy diet is very important to me*- given statement is fairly graded in every group (mean around 4) which is pointing to a fact that the younger people are developing sense for healthy diet although
- It is very common to think that younger people are prone to consumption of fast food). *It is very important to know the ingredients of the food I consume* - the ingredients of the food are most important to the examinees from the field of food technology which was an expected result given their area of interest and study, but this statement is also almost as important to the examinees from other fields.
- *'Light' products are only for sick people (people with health problems)* - this statement is least agreeable to all of the examinees which is demonstrating that 'light' products have been perceived as products for entire population with the aim of positive effect on the health

- *I 'II buy more 'light' products in the future* - although all of the examinees have shown the interest for knowing ingredients of the food and they have shown care for healthy diet and perception of 'light' products as products for entire population, mean given for this statement is disputing this. They are not too interested in buying 'light' products in the future.

Statistically significant difference is determined at statements: *'Light' products are healthier than conventional products* ($p<0.05$) and *'Light' products provide same amount of energy as a conventional products* ($p<0.05$). Former statement was best graded by examinees from economic field, and worst by those from medical field. Post hoc analysis demonstrated significant difference between examinees from economic and medical orientation ($p<0.05$). Almost identical results were obtained for latter statement. The statement is most agreeable to the economic oriented examinees, while other two groups have the same mean. The difference in this case is present between examinees from economic and medical orientation ($p<0.05$).

CONCLUSION

Students are closely monitoring food trends, and the type of education is certainly one of the factors that influence the development of awareness of new food products. 'Light' products are familiar to about 99% of the students, but about 35% of them define them in a proper manner which is associated with the field of studying ($p<0.05$). A very high percentage of students consume 'light' products (79%), but a smaller number of respondents do it on a daily bases (4%), which is probably due to lack of confidence in the meaning of the label 'light'. Motives for consumption are mostly improvement of nutrition and weight control. When it comes to types of 'light' products, the diet of students consists of dairy 'light' products regardless of the field of studying (economy, food technology, medicine). However, students of food technology consume other 'light' products which is present in smaller extent in other groups. The results show that there is no statistically significant difference in dietary habits and attitudes of students of economics, food technology and medicine to 'light' products, but differ in the understanding of 'light' products and the scepticism tbward these products. At the end, it is important to emphasis that if the product is "light", that does not imply that its consumption should be strongly encouraged. Healthy diet primarily refers to adequate food choices and achievement of energy balance through portion size control.

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RESEARCH ON THE DIETARY HABITS OF POPULATION OF DIFFERENT AGES IN THE REGION OF VELES - MACEDONIA

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ABSTRACT

A questionnaire on consumption of food and physical activities in the region of Veles – Macedonia was done by 200 subjects. The results obtained shown that the most consumed were white bread and semi-white bread by 78% of participants, while wholegrain bread ate 17% of the population. The youngest respondents (children and adolescents) frequently consumed fruits and vegetables. Chicken and pork were the most consumed meat, fish and beef less and lamb very little. With aging the percentage of persons over 50 years, who consume chicken and beef decline, while growing the percentage of those who consume fish. Respondents most consumed boiled and fried food, not far behind fresh and roasted food. Only 11% of the population didn't consume milk or yogurt. Half of the participants fluid intake from 1 liter to 2 liters daily, 27% less than 1 liter. Female respondents prefer walking as physical activity, while males prefer sports such as basketball, handball, football etc. Over 50% were not informed by the media concerning the relationship between food and health, an indicator that in the future need to consider health education to promote health through proper nutrition.

KEYWORDS: *physical activity; nutrition information; children; adolescents; adults*

INTRODUCTION

Dietary habits are the habitual decisions a person or culture makes when choosing what foods to eat, Wansink, Sobal, 2007. Many cultures hold some food preferences and some food taboos.

In Alaska, meat and fish are the centre pieces of Eskimo diets and constitute 90 percent of locally harvested foods, Jones, 1983. Cereals constitute the bases of the Middle Eastern diet, historically and today. Wheat and rice are the major and preferred sources of staple foods. Barley is common in the region and is an ingredient in cheaper bread, and millet and sorghum are used in a few places to make porridge and gruel. Lamb and mutton have always been the favoured meats of the region, with veal as a subsidiary choice in some instances, and, in other places, goat, Mallos, 1979, Roden, 1985.

Dietary choices can also define cultures and play a role in religion. For example, pork, prohibited in the religions of Islam—though there are accounts of wild boar being hunted and eaten by some Bedouins—and Judaism, was also largely avoided by the Christians of the region, in Hinduism beef is restricted, Roden, 1985, Simoons, 1994. In addition, the dietary choices of different countries or regions have different characteristics. This is highly related to a culture's cuisine.

Dietary habits play a significant role in the health and mortality of all humans. Imbalances between the consumed fuels and expended energy results in either starvation or excessive reserves of adipose tissue, known as body fat, Nicklas, 2002. Poor intake of various vitamins and minerals can lead to diseases that can have far-reaching effects on health.

According to statistics published in World Health Report WHO, 2002 entitled "Reducing risk, improving healthy living" and the base of the Global Strategy on diet, physical Activity and Health of WHO, 2004 which is already implemented worldwide e confirmed that the health of the population both in developed and in developing countries depends on the diet and level of physical activity.

Faculty of Technology and Technical Sciences – Vales, Republic of Macedonia is a relatively new institution of higher education within the University St. Clement of Ohrid – Bitola. It first stepped into a range of accredited and high quality program for the first cycle of studies in nutrition in relation to all higher education institutions in the country and the region. In the frame of project activities, questionnaire on consumption of food and physical activities in Macedonia was done by 800 subjects, in region of Veles, Bitola, Prilep, Kicevo and Demir Hisar.

The aim of this study is to investigate frequency of eating: bread, vegetables, fruits, meat as well as drinking of water, milk, yogurt on population in the region of Veles, Republic of Macedonia with different age and gender. Also, we want to know what kinds of physical activities prefer participants: walking, running, cycling or sports (basketball, football, handball, etc.) and how they were informed about the relationship of food with health by the media.

MATERIALS & METHODS

In this survey 200 respondents participated. They had to answer 20 questions about their eating habits and physical activities. What type of bread they consume, how often they drink milk or yogurt. It was necessary to answer the frequency of eating fresh fruits and vegetables. Because the quality of entered food ingredients depends on how it is prepared, the respondents answered the way of preparing food. Water is essential for the proper functioning of the organism, thus they had to say what volume of fluid intake through the day. Respondents answered whether practicing walking, running, cycling or sports (basketball, football, handball, etc.). The data from surveys performed were classified by age and gender. The results were processed with the Excel program.

RESULTS & DISCUSSION

The results of the completed survey were analyzed individually or by connecting two or more parameters. For specific needs individually analysis only answers, for other purposes by gender or age. Often is pursued a combination of multiple responses both by gender and by age (Table 1). The most results are shown in percentages (%) referring to the percentage of subjects.

Table 1. Demographic properties and number of subjects according to age and gender.

place/gender	less than 18 years	19-49 years	over 50 years
Veles/male	23	36	9
Veles/female	17	27	12
surrounding villages/ male	10	15	11
surrounding villages/ female	12	17	11

Results for consumption of bread and milk/yogurt are processed first. In Fig. 1 was shown results obtained for consumption of bread, traditional food in Macedonia. The most consumed were white bread and semi-white bread by 78% means that ¾ (three quarters) of respondents' use, while only ¼ (one quarter) consume wholegrain bread.

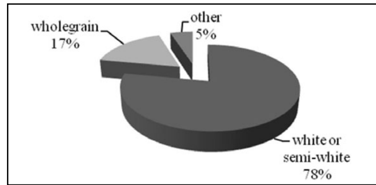

Figure 1. The type of bread that is consumed. (*the percentages refer to % subjects)

Consumption of milk and dairy products in the diet is beneficial to health – people who have diets rich in dairy products may reduce the risk of reduced bone mass throughout the life cycle. Milk and dairy products provide nutrients that are vital for health and maintenance of the body, such as calcium, potassium, vitamin D and protein. Diet rich in dairy products helps in creating and maintaining bone mass throughout life. It can reduce the risk of osteoporosis. Intake of dairy products is of particular importance to bone health during childhood and adolescence, when bone mass is created, Heaney, 2009. A diet that includes dairy products usually has higher overall nutritional quality. About 60% of all respondents of different ages consumed milk or yogurt 2-4 times per week. 22% of children and adolescents consumed milk or yogurt every day and 64% 2-4 times per week. Only 11% of the population don't consume milk or yogurt (Fig. 2).

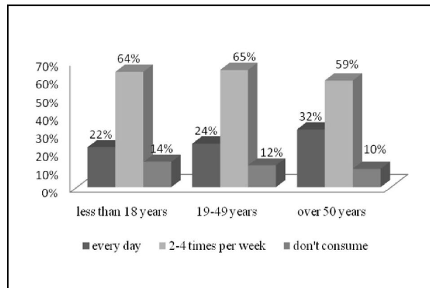

Figure 2. Drinking of milk or yogurt.

The use of milk or yogurt is positive, especially to be used yogurts with probiotics and lower % of fat (0.9%, 1.6%). Selecting dairy food group with a high percentage of saturated fat and cholesterol can have harmful effects on health. Diet containing a high percentage of saturated fat can raise levels of LDL (low-density lipoprotein) cholesterol in the blood. High levels of that, cholesterol increases the risk of coronary heart disease, Skeaff, Miller, 2009, WHO, 2010.

Table 2. Frequency of consumption of fruits and vegetables.

fruits	**less than 18 years**	**19-49 years**	**over 50 years**
every day	53%	24%	29%
2-4 times per week	27%	42%	41%
1 time per week	17%	29%	27%
don't consume	3%	5%	3%
vegetables	**less than 18 years**	**19-49 years**	**over 50 years**
every day	56%	47%	41%
2-4 times per week	22%	36%	34%
1 time per week	22%	15%	22%
don't consume	0%	2%	3%

Fruits and vegetables were consumed every day, by 53% and 56% of the respondents less than 18 years, respectively (table 2). About 26% of adults ate fruits every day, 41% consumed 2-4 times per week. Vegetables were consumed every day by 44% of participants over 19 years, while 35% of the same population ate 2-4 times per week. People, who eat more fruits and vegetables as part of a healthy diet, reduce the risk of some chronic diseases. Vegetables and fruits provide nutrients vital for health and maintenance of our body, Deneo-Pellegrini et al, 1996, WHO, 2004.

The next analysis was done to type of meat that was eaten. Our results show that chicken and pork were the most consumed meat, fish and beef less and lamb very little, by the participants from the region of Veles. 32% of men and 40% of women consume chicken. Pork is eaten about 30% and fish about 20% of all participants. Only 3% of subjects consume lamb in their nutrition (Fig. 3).

With aging the percentage of persons over 50 years, who consume chicken and beef decline, while growing the percentage of those who consume fish.

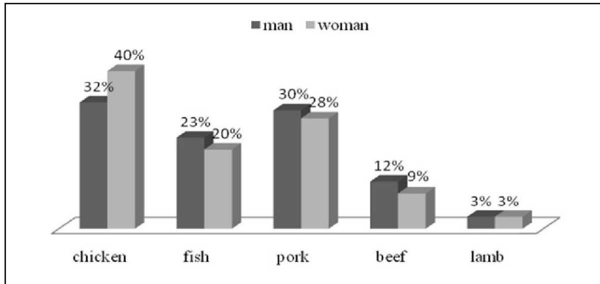

Figure 3. Eating meat by gender.

The quality of imported food ingredients depends on how it is prepared. Respondents most consumed boiled and fried food, not far behind fresh and roasted food (Fig. 4). The young population and participants aged less than 18 years, and from 19 to 49 years prefers the use of fried foods that include "fast food", while adults usually eat cooked food that is normal with age.

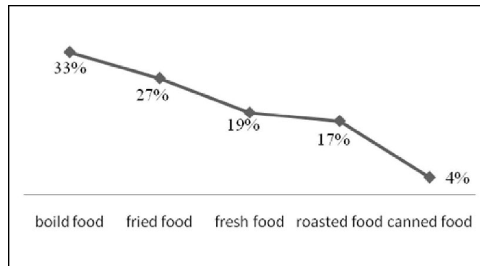

Figure 4. Way of preparing food.

Half of the participants fluid intake from 1 liter to 2 liters daily, 27% less than 1 liter. The human body consists mostly of water, but this water is lost daily. The body loses about 2.8L of fluid through urine, sweat, faeces and through breathing. Although there's quite a lot of water in solid foods (most people get about 30% of their minimum daily fluid needs from solid foods) that isn't nearly enough to replace the losses without a considerable intake from beverages. So it's important to try and get 6-10 glasses of fluid into your body everyday, Mann, Truswell, 2002, D Anci et al, 2006.

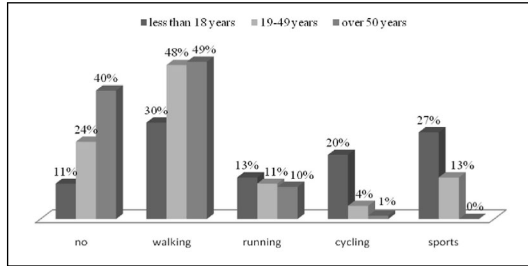

Figure 5. Physical activity.

Regular physical activity is important for overall health and vitality. It also helps control weight, balancing the calories you enter as food calories they consume daily, Bauman et al, 2009. The results obtained shows that elderly prefer walking as physical activity, while the youngest surveyed population prefers sports basketball, handball, football (Fig. 5).

To be welcomed is not only to eat healthier but to be physically active. The participants in the region of Veles, were asked also about the enough information about food and health by the media.

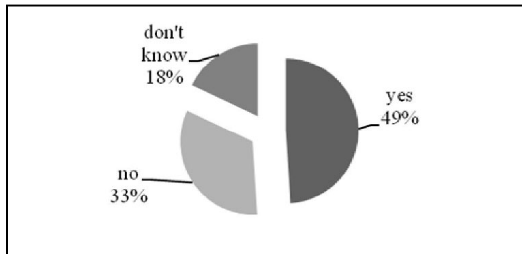

Figure 6. The media provides enough information about nutrition and health.

Over 50% were not informed by the media concerning the relationship between food and health, an indicator that in the future need to consider health education to promote health through proper nutrition (Fig. 6). The media shouldn't be the primary and the most trusted source of nutrition information, because journalist didn't interpret nutrition information on the scientific aspect. Only high educated staff could give the real nutrition information.

CONCLUSION

From this research significant results have been obtained. High rate of consumed white bread and semi-white bread by the participants, maybe indicate spoor financial strength, because it is cheaper compared to the wholegrain bread, but not healthier. The use of milk or yogurt is positive, especially to be used yogurts with probiotics and lower % of fat (0.9%, 1.6%). The youngest respondents (less than 18 years) frequently consumed fruits and vegetables, while older less.

Convenient to town people are advised to use fruit and vegetable are closer to 90% of population.

Chicken and pork were the most consumed meat, fish and beef less and lamb very little. The fried foods that include "fast food" were the most consumed by the young population and participants aged less than 18 years, while cooked food by adults that was normal with age. Veles is a city that is geographically spread on hills, so not surprising that most of the participants prefer walking as physical activity. The further goal in our activities is to educate the population about proper diet, what to consume, how to prepare food, why it is important physical activity etc. This could be achieved by certain classes by professionals, organizing ebates, TV shows.

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THE SENSORY PROPERTIES OF SPECIAL BRANDY WITH GANODERMA LUCIDUM

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ABSTRACT

Ganoderma lucidum has been used for more than 2000 years in traditional Chinese medicine. It is not edible mushroom since its fruiting bodies are tough and corky, but the extract of this mushroom can be used as additives for food or drinks. The reason for great attention of this mushroom is due to the wide variety of its bioactive compounds, such as triterpenes, polysaccharides (β-glucans), proteins, steroids etc. In the production of beverages, very important fact is that the Ganoderma is a rich source of bitter triterpenoids and bioactive polysaccharides. Brandy is an alcoholic beverage with a very long tradition in Serbia and it is produced by distillation of fermented fruits. Brandy can be mixed with different medicinal herbs, and according to Serbian tradition, these products are treated as forms of folk medicine. In this study, we investigated the sensory properties of special type of grape brandy and wine distillate, which were obtained by maceration of various amounts (0.5-2.5%) of fruit bodies of *Ganoderma lucidum*. The origin of strain of *G. lucidum* was from wild Bojčinska forest near Belgrade. Sensory characteristics of the brandies were determined using modified Buxbaum model of positive ranking (20 score max.). The samples of brandies were subject for sensory evaluation by a panel comprising 5 qualified testers. The results of the evaluation showed that total sensory quality of samples were between 16.7 and 17.85, which we consider as very good score. The best rated was the sample of grape brandy with 2.5% *G. lucidum.* The obtained results suggest that sensory properties of these special brandies were completely acceptable for the tasters. Supplementation of this mushroom with different kind of brandy can improve the sensory characteristics and health properties of spirit drinks.

KEYWORDS: *Ganoderma lucidum; grape brandy; wine distillate; sensory characteristic.*

INTRODUCTION

Ganoderma lucidum (Lingzhi) has been used for more than 2000 years in traditional Chinese medicine. The earliest mention of these fungi was in the era of the first emperor of China, Shih-huang of the Ch'in Dynasty (221-207 B.C.) (Stamets, 1993). In Chinese folklore the fruit body of *Ganoderma*) has been used for treatment of many types of diseases (hepatopathy, chronic hepatitis, nephritis, hypertension, hyperlipemia, arthritis, neurasthenia, insomnia, bronchitis, asthma, gastric ulcer, arteriosclerosis, leukopenia, diabetes, anorexia, mushroom poisoning etc.) (Jong, Birmingham, 1992). Chemical and medical properties of *Ganoderma* are still one of the most important subject of modern research, especially in Chine. The reason for great attention of this mushroom is due to the wide variety of its bioactive compounds, such as triterpenes, polysaccharides (β-glucans), proteins, steroids, etc. The most important of these compounds are triterpenoids and polysaccharides. In the production of spirits very important is the fact that red-colored *Ganoderma* is a rich source of bitter triterpenoids. Depending on the color of *Ganoderma lucidum*, this mushroom is divided into six different types (red, blue, yellow, white, purple and black). Red-colored type has bitter taste and generally has been regarded as the most potent and medical (Wasser, 2010).

Fruit brandies are alcoholic beverages with a very long tradition not only in Serbia, but also in many countries worldwide. They are produced by distillation of fermented fruits. The most important brandies in Serbia are made from plum juice and grape. After distillation these spirits can aged in wooden barrels, which colored it and adds special and distinctive aromas and flavours. In case of production of colorless beverages, raw distillate aged in inert casks (usually glass or inox). Wine distillate is made through heating wine, distilling over the alcohol and volatiles. Raw distillate aged in wooden barrel, minimum three years. Brandy can sometimes mixed with different medicinal herbs, and according to Serbian tradition, these products are

treated as forms of folk medicine. Lingzhi is not edible mushroom since its fruiting bodies are tough and corky, but the extract of this mushroom can be used as additives for food or drinks. Extraction of fruit bodies of *G.lucidum* in alcohol can be more effective than extraction in water, because in some cases more bioactive compounds can be dissolved (Nikšić et al., 2001). Extract have changed color, which is very similar with color of spirit aged in wooden barrel. In this study we investigated the possibility of production special type of brandy with *G.lucidum* produced of grape brandy and wine distillate as alcohol base. We investigate the acceptably of these products with sensory experts with large experience in evaluating of alcoholic beverages.

MATERIALS AND METHODS

In this study about the sensory properties of special brandy with *G. lucidum,* red-colored *G. lucidum* were collected in Bojčinska forest near Belgrade, Serbia. This mushroom was found on the roots and stubs of oak near the soil surface. Grape brandy (Lozovača) and wine distillate were obtained from Faculty of Agriculture, University of Belgrade, Serbia. Air-dried fruiting bodies of fungi *G.lucidum* were cut on small pieces (about 1cm) and added in 40 % alcohol medium (grape brandy and wine distillate) in three different concentrations: 0.5, 1.5 and 2.5% w/v. The extraction was carried out in shaker in dark place for 15 days at room temperature. The mixture was filtered with 70 g/m3 filter paper. The samples of brandies were stored in glass bottles in dark place at room temperature. Sensory characteristics of the brandies enriched with mushroom were determined using modified Buxbaum model of positive ranking (Tešević et al., 2005). The common quality parameters were evaluated: clearness – parameter, which contributes to the overall visual liking of drink; it's depend on the purity and hue of color (max 1 points); color - visual impression created as a result of irritation of the retina of the eye with light rays of different wavelengths (max 1 points); distinction – parameter, which clearly define specific and distinctive characteristic synonymous for certain categories of strong alcoholic beverages (max 2 points); odor - a sensory attribute resulting from stimulation of the olfactory receptors in the nasal cavity by certain volatile substances (max 6 points); taste - a sensory attribute resulting from stimulation of the gustatory receptors in the oral cavity by certain soluble substances (max 10 points) (Nikićević, 2005). In this evaluation a brandy sample may have a maximal score of 20 points. Sensory tests were carried out using a panel of five qualified testers (experts). All samples were presented in special glass cup, tulip-like shape. Sample (50 ml) were served and assessed at controlled testing room (air temperature about 20°C, adequate daylight, peace and quietness) (Nikićević, Tešević, 2008).

Physico-chemical parameters of grape brandy and wine distillate were determined using the methods prescribed by the law of spirit drinks of Republic of Serbia (Official gazette of SFRJ, 1987).

RESULTS AND DISCUSSION

The chemical characteristics of the grape brandy and wine distillate are shown in Table 1. Ethanol content is very important for the mouth feel and flavour of alcoholic beverages (Tešević et al., 2005). Therefore, the ethanol content in both alcohol mediums was adjusted on 40 % v/v. The balance of various compounds in alcoholic drinks is very important for sensory profile and overall quality. The concentration of all analyzed compounds is in according with legislation (Official gazette of SCG, 2004). In grape brandy, values for all analyzed physico-chemical parameters were higher in comparison with wine distillate, but less than the prescribed maximum values.

Table 1. Physico-chemicalproperties of grape brandz and wine distillate

Parameter	Grape brandy	Wine destillate
Ethanol (%v/v)	40.0	40.0
Methanol (%v/v)	2.64	1.32
Higher alcohol (mg/l a.a *)	1580	1340
Total acid (mg/l a.a)	563	136
Aldehydes (mg/l a.a)	97.76	78.45
Esters (mg/l a.a)	1140	862
Furfural (mg/l a.a)	4.50	3.75
Total dry extract (mg/l a.a)	0.46	0.36
Total content SO_2 (mg/l)	5.12	2.56

*- milligram per liter of absolute alcohol

The primary goal of this study was to exmine the possibility of improving the sensorial characteristics of grape brandy and wine distillate by adding *Ganoderma lucidum.* In addition, this mushroom is a rich source of many bioactive compounds, such as triterpenoids, polysaccharides, proteins, amino acids, nucleosides, alkaloids, steroids, lactones, fatty acids etc. We were investigated the possibility of producing beverages with improved sensory characteristics and health properties. Extraction of *G.lucidum* in alcohol mediums was completed after 15 days, and the obtained samples were subjected to sensory evaluation by a panel. In tables 2 and 3 are shown the results of the sensory evaluation of samples made of grape brandy (GP1, GP2, GP3) and wine distillate (WD1, WD2, WD3), respectively.

Table 2. The results of sensory evaluation grape brandy with additionof G. lucidum

Parameter	GP1	GP2	GP3
clearness	1	1	1
color	1	1	1
distinction	2	2	2
odor	5.25	5.5	5.45
taste	8.0	8.35	8.25
Total score	17.25	17.85	17.7

GP1, GP2, GP3 – grape brandy with 5, 15 and 25g/l of *G.lucidum*, respectively.

Table 3. The results of sensory evaluation wine distillate with adition of G. lucidum

Parameter	WD1	WD2	WD3
clearness	1	1	1
color	1	1	1
distinction	2	2	2
odor	5.2	5.0	5.0
taste	8.1	8.0	7.7
Total score	17.3	17.0	16.7

WD1, WD2, WD3 – wine distillate with 5, 15 and 25g/l *G.lucidum*, respectively.

The results of this evaluation show that total sensory quality of samples were between 16.7 and 17.85, which is a very good score. Mean values of the sensorial grades for samples GP1, GP2 and GP3 were higher than

those for WD1, WD2 and WD3. Hence, more favourable medium for the addition of *Ganoderma* was grape brandy. Samples WD1, WD2 and WD3 showed that addition of the large quantity of mushroom adversely effect the taste. Bitterness of samples was increased with extraction of higher quantity of bitter triterpenoids from *G. lucidum*. The best rated was the sample of grape brandy with 2.5% of *G. lucidum,* which taste were characterized with a well-rounded and balanced, tasty bitterness. The color of the samples was the most similar to brandy aged in wooden barrel. The results of this study suggested that extraction of this mushroom in these alcohol mediums can give a product with more than satisfactory sensory properties.

CONCLUSION

Obtained results suggest that *Ganoderma lucidum* could be very interesting raw material for the production of various kinds of spirit drinks. High concentration of alcohol can contributes to a better extraction of bioactive compounds of this mushroom, which affects the increase in functionality. These new products with novel and specific sensory profile and with potential physiological effects may be of potential for various kinds of customers.

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FOLATE RELATED KNOWLEDGE, DIETARY INTAKE AND SUPPLEMENTATION PATTERNS IN CROATIAN PREGNANT WOMEN

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ABSTRACT

Neural tube defects (NTD; exp. spina bifida and anencephaly) affect more than 4500 pregnancies in the European Union. Periconceptional folic acid supplementation has shown to decrease both the occurrence and recurrence of NTD. The aim of this work was to establish the knowledge of folate supplementation in periconceptuale and pregnancy period and to determine dietary folate intake among Croatian pregnant women.

Subjects were 101 pregnant women, from 3 Zagreb's health centres, interviewed during regular visit to gynaecologist. Dietary folate intake was assessed by validated food frequency questionnaire (FFQ) while specially designed questionnaire was used to establish knowledge of folate and usage of folic acid supplements before and during pregnancy.

62% of pregnant women knew that folate deficit could result in neural tube defects in newborns. However, despite of 73.3% of planned pregnancies only 17% of subjects start taking folate supplements before pregnancy. 54.5% knew that folate supplementation is recommended in the period of 4 weeks before planned conception until the 12th week of pregnancy, however only 5.9% of subjects used supplements in that period. In the first trimester of pregnancy (n=30) median intake of dietary folate was 250.1 μg per day while only 36% achieved DRI recommendation of 600 μg per day by using supplements.

These results may support need for dietary counselling in women of childbearing ages to increase intake of folate-rich foods and to use folate supplements. Despite awareness of folate importance before and during pregnancy majority of subjects did not meet recommendation, therefore further education would provide required knowledge of folate supplements selection, duration and dosages.

KEYWORDS: *dietary folate intake; folate supplementation; knowledge; pregnancy*

INTRODUCTION

The periconceptional use of folic acid reduces the risk of neural tube defects (NTD) like spina bifida and anencephaly (Smithells et al., 1980, Czeizel and Dundas, 1992). Therefore it was established that women in periconceptional period and pregnancy should consume the recommended daily intake (RDI) for folate of 400 μg and 600 μg dietary folate equivalents (DFE), respectively (Institute of Medicine, 2006). DFE include folates that are present in food as well as folic acid that is the form of folate used in vitamin supplements (Suitor and Bailey, 2000). Also to increase folate intake in childbearing ages many countries have made mandatory folate fortification of flours or grain products (Williams et al., 2002) that resulted by reduction of NTD (Lopez- Camelo et al., 2005).

Official national recommendation of folic acid intake for women planning pregnancy suggest that extra 400 μg should be taken from food supplement or fortified food (NN, 2004). Folate fortification is still not the practice in Croatia. Therefore, women in childbearing ages are on their own in deciding what supplement to use, how long and what dosages to take.

Despite worldwide public-health campaigns recommending periconceptional daily supplementation of folic acid to reduce the risk of NTD, many women are not following these recommendations. At the same time, in most European countries no decline in defects has been recorded in recent years (Eichholzer et al., 2006).

Therefore, the aim of this study was to establish the knowledge of folate, supplementation in periconceptuale and pregnancy period and to determine dietary folate intake among Croatian pregnant women.

MATERIALS AND METHODS

Subject were pregnant women (n=101) recruited during regular visit to gynaecologist in three Zagreb's health centres. All subject provided written informed consent. During visit they have completed questionnaire that included folate related knowledge and supplements usage. Dietary folate intake was assessed by validated quantitative food frequency questionnaire developed by Colić Barić et al. 2009. This FFQ consists of questions about 39 folate rich food items. For each item, subjects were asked to indicate their intake over the past month, frequencies of consumption such as never, once per month, 2-3 times per month, once per week, 2-3 times per week, 4-6 times per week and every day and quantity of consumption using food photographs. Folate intake was calculated as dietary folate equivalents (DFE), taking into account all forms of folate including synthetic folic acid from fortified food and dietary supplements, using food composition tables (Kaić-Rak and Antonić, 1990). Folate content from supplements and fortified food was taken from the nutrition information on the product labels or from manufacturers. We also took into account that folic acid taken when a person is fasting is 2 times more bioavailable than food folate, and folic acid taken with food (which includes folic acid added to food during fortification) is 1.7 times more bioavailable than naturally occurring folate (West Suitor and Bailey, 2000).

The data was analysed using SPSS version 15 for Windows (SPSS Inc. Chichago, USA). Quantitative variables were expressed as mean ± SD or %. The distribution of folate intakes was right-skewed therefore expressed as median and range.

RESULTS AND DISCUSSION

Average maternal age was 30.05 ± 4.98 years (range 18-42 years). The majority of subject (46.6 %) had university education while 40.6 % have medium and 12.9% low education level. Sociodemographic characteristics are presented in Table 1. Most subjects had planned pregnancy (73.3 %) which is in accordance with previously reported studies conducted among Croatian women reported by Gjergja et al., 2006. and Vitale et al., 2009.

A majority of the subjects were nonsmokers (70%), while 30% smoked and 19% of them stated that they wouldn't stop smoking during pregnancy.

Tabla 1. Socio-demographic caracteristics of pregnant women (n=101)

Characteristics	n	%
Age (years)		
<19	2	2.0
20-29	45	44.6
30-39	53	52.5
>40	1	1.0
Education		
Elementary school	13	12.9
Secondary school	41	40.6
University	47	46.6
Trimester of pregnancy		
I	30	29.7
II	38	37.6
III	33	32.7
Smoking status		
Yes	30	29.7
No	71	70.3

This study demonstrates subject's limited knowledge about folate. Only 32 % knew that folate was a vitamin, 10 % thought that it was a mineral supplement, 6 % thought it was essential fatty acid, 16 % thought it was essential amino acid and 37 % stated that they did not know what folate was. The level of knowledge is considerably lower than the rate found in a national survey conducted by Vitale et al. in 2009, where 52.4 % of pregnant women had heard of folic acid. High rate of subjects (81%) had heard of folate importance during pregnancy and their sources of information included the physician (43%), friends (12%), media/internet/magazines (17%) and own knowledge through education (27 %). Many had heard from more than one source. However, only 40 % of subjects gain that knowledge before becoming pregnant. These results are in accordance with study reported by Gjergja et al. 2006 indicating that still the importance of folate intake before pregnancy isn't reaching women in periconceptional period.

55 % of subject correctly knew that it was most appropriate to take folic acid just before and soon after conception while 23 % thought it should be taken throughout all pregnancy and 22 % didn't know. However, only 5.9 % of subject used folic acid supplements in recommended period. Previous researches shows that 50 % reduced odds of using folic acid periconceptionally is usually associated with lower level of education, younger maternal age and unplanned pregnancy (Ray, 2004), which is not the case in this study.

62 % of subjects knew that folic acid could prevent NTDs, but 37% did not know the use of folic acid and 2% thought that it prevents heart defects or diabetes. Awareness of folic acid in the current study was higher than that reported by two other studies (French et al., 2003; Vollset and Lande, 2000). In contrast, Sen et al., 2001 surveyed 300 pregnant women and found that 91% were well informed about folic acid's role in preventing neural tube defects.

All subjects had completed validated food frequency questionnaire used to determine folate intake expressed as dietary folate equivalent (DFE). Median dietary folate intake from food among pregnant women was similar for all trimester shown in Figure 1 ($p>0.05$). In first trimester was 250.1 μg DFE, second 243.62 μg DFE and third trimester 286.90 μg DFE which was far below DRI recommendation of 600 μg. Pucarin-Cvetković et al 2006. examined mean folate intake of women in childbearing age in Croatia and found that 85% of population consume less than 400 μg of folates indicating that it is very difficult to reach recommended value.

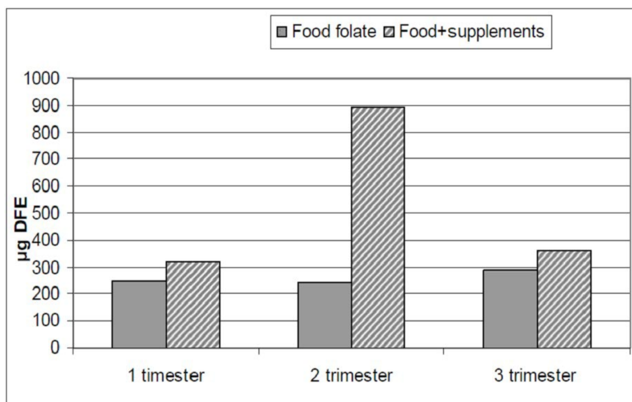

Figure 1. Median daily intake of folate from food and supplements in nutrition of pregnant women (n=101)

The major difference in folate intake among trimesters was shown when taking folate supplement into account (Figure 1). The highest folate intake was during second trimester. Overall, 71.3 % of subject was taking folic acid supplement, however only 17 % of them start taking folate supplements before pregnancy while only 5.9% of subjects used supplements in appropriate period. Appropriate folic acid intake is considered in the period of 4 weeks before pregnancy and during first 12 weeks of pregnancy.

As reported by Bailey (2000), recommendation should be done by trimester of pregnancy because of gestational difference. Folate supplement was taken in different dosage (range 200 μg folic acid to 5000 μg folic acid) and duration. Subjects consume 22 different folic acid supplements that are available on Croatian market. Very concerning was information that 10% of subjects exceed the Tolerable Upper Intake Level (UL) (1000 μg folic acid/day) by taking 5000 μg folic acid per day on their own and without consulting a physician. These higher dosages of folate are recommended but only for couples already having a child with neural tube defect (Gjergja et al., 2006). Folic acid intakes in excess of the UL, which was not based on toxicity criteria, may mask the symptoms associated with a vitamin B12 deficiency and allow for the progression of irreversible neurological damage (Rampersaud et al., 2003).

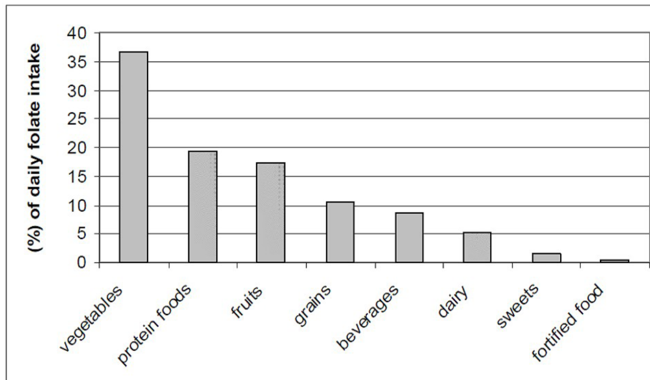

Figure 2. Average contribution of selected food groups to dietary folate intake in nutrition of pregnant women (n=101)

Humans are entirely dependent on dietary sources or dietary supplements for their folate supply (Scholl and Johnson, 2000). The major dietary source of folate among pregnant women in our research was vegetables (42%), followed by grains (20%) and fruits (18%) (Figure 2). The lower contribution of folate was from fortified food which can be explained by the fact that only few products on Croatian market are fortified with folic acid. 15% of subject drunk vitamin drinks on the daily base which may significantly increase folate intake as reported by Pucerin-Cvetković et al., 2006. Subjects were asked to identify good food sources of folate. Many had chosen more than one source. Green leafy vegetables, liver and beans as good source of folate were identified by 60%, 21 % and 13 % of subjects, respectively. It is important to educate pregnant women as well as women in childbearing ages about folate rich food. Very good source of folate are black-eyed peas and lentils (boiled, 1 cup, 358 μg), beans (white, boiled, 1 cup, 263 μg), spinach (cooked, ½ cup, 131 μg) and broccoli (cooked, 1 cup, 168 μg).

CONCLUSION

In the first trimester of pregnancy, period that folate are most important, pregnant women had median folate intake from food and supplements of 318.7 μg DFE per day which was far below recommended 600 μg DFE. These results may support need for dietary counselling in women of childbearing ages to increase intake of folate-rich foods and to use folate supplements. Despite awareness of folate importance before and during pregnancy majority of subjects did not meet recommendations, therefore further education is needed to obtain required knowledge of folate supplements selection, duration and dosages.

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Food Chemistry

STUDIES ON FURAN FORMATION IN FOODS PREPARED IN DOMESTIC PRESSURE COOKER

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ABSTRACT

The formation of furan, a possible human carcinogen, has been reported in canned and jarred foods at oncentrations up to 173 μ g/kg. Although furan is a highly volatile molecule, the processing of foods in hermetically sealed containers permits its accumulation in the products since the contaminant can not be lost by volatilization. Considering that a pressure cooker is a container with a partial hermetic seal, it is very important to evaluate the levels of furan in home processed foods prepared under such conditions. For that, several foods have been cooked in a domestic pressure cooker, including beans, whole rice, soy beans, beef, pork, potato and cassava. Furan content was determined by using an in- house validated method based on gas chromatography coupled to mass spectrometry preceded by headspace solid phase microextraction (HS-SPME-GC/MS). The results showed that furan was not found above the limit of quantification in the cooked samples. It is possible that furan, whether formed under such conditions, might be lost with the steam that is released by the valve when a determined pressure is reached. Therefore, these results indicated that the cooking in domestic pressure cooker may not represent a concern in relation to the occurrence of furan in foods. No furan has either been found in quantifiable amounts in re-heated samples after the pressure cooking and after 24 hours under cold storage.

KEYWORDS: *furan; pressure cooking; GC-MS; processing contaminant*

INTRODUCTION

Furan (C_4H_4O) is a colorless flammable liquid with an ethereal odor, having a low molecular weight of 68 and a high volatility with the boiling point of 31°C (NTP, 1993). Furan is classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer in view of its hepatotoxicity, cytotoxicity and carcinogenicity verified in experimental animals (IARC, 1995). In 2004, American researchers showed that furan can be formed during thermal treatment of several foods, especially canned and jarred products. Levels up to 112 and 173 μ g/kg were found in jarred baby food and canned gravies, respectively (US FDA, 2004). The exact mechanism of furan formation is not completely understood, but experiments conducted by using model systems have shown that ascorbic acid, sugars, amino acids and poly-unsaturated fatty acids could act as potential precursors (Locas, Yaylayan, 2004; Becalski, Seaman, 2005; Märk et al., 2006). In a recent risk assessment, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that margins of exposure (MOEs) obtained for average and high consumers (960 and 480, respectively) were low for a carcinogenic compound that might act via a DNA-reactive genotoxic metabolite, indicating a human health concern (FAO/WHO, 2010). Although several studies have been published on furan levels in commercial products, few data is available for home cooked foods. Fromberg et al. (2009) evaluated the formation of furan during domestic cooking such as grilling, roasting, baking, frying and cooking in saucepan and microwave. The foods rich in carbohydrates were most likely to form furan, probably due to the Maillard browning reaction. By the other side, meat dishes prepared by using ingredients containing furan (such as soy sauce and tinned tomato), did not show quantifiable levels of the contaminant in the final cooked product. However, the presence of furan in home cooked foods prepared in domestic pressure cooker has not been investigated so far. The occurrence of furan in canned and jarred products suggests that the processing of foods in hermetically sealed containers permits its accumulation in the products since furan can not be lost by volatilization (Hasnip et al., 2006). Considering that a pressure cooker is a hermetic container until the release of the steam, the objective of this study was to evaluate the occurrence of furan in

home processed foods prepared under such conditions. Additionally, the stability of furan during re-heating after cooking was also investigated.

MATERIALS AND METHODS

Standards and chemicals

Furan and [$^2H_4$] furan (furan-d_4) were obtained from Sigma-Aldrich at purity higher than 98%. Methanol was of HPLC-grade and water was purified by reverse osmosis. Individual stock solutions of both standards at *ca* 2 mg/ml were prepared by dissolving in methanol. Intermediate and work solutions at *ca* 20 μ g/ml and 0.2 μ g/ml, respectively, were prepared in water.

Samples

Several foods were considered in the present study, including beans, whole rice, soy beans, beef, pork, potato and cassava. The samples were obtained from the local market. With exception of beans, whole rice and soy beans, the samples were cut in cubs of 2 x 2 cm approximately prior cooking. Potato and cassava were previously peeled.

Pressure cooking

A domestic aluminium pressure cooker of 4.5 litres was used in the experiments. The cooking was performed with 400 g of fresh sample and 1.5 litre of water during 20 min after the first release of steam from the pressure valve. Before opening, the pressure cooker was cooled under tap water in order to eliminate the pressure.

Re-heating experiments

After opening, the samples were kept in the pressure cooker and were cooked for additional 10 minutes without the lid. Then, the samples were stored under refrigeration during 24 hours and re- heated in an open saucepan during 5 minutes after boiling.

Determination of furan

All samples were stored at 4°C for at least 4 hours before homogenization in order to avoid furan loss by volatilization. Furan content was determined in the fresh, cooked (before and after additional 10 minutes without the lid), refrigerated and re-heated samples by using an in-house validated method based on HS-SPME-GC/MS (Arisseto et al., 2010). A portion of 1 g of homogeneous sample was weighed in a chilled 40 ml screw-cap glass vial fitted with silicone-PTFE septum containing a 15 mm x 5 mm PTFE-coated stir bar. Aliquots of 125 μ l of furan-d_4 working standard solution 0.2 μ g/ml and 875 μ l of water were added and the vial was immediately closed. The SPME was carried out in a 75 μ m carboxen-polydimethylsiloxane (CAR-PDMS) fiber (Supelco) at 25ºC during 30 min, under a constant magnetic agitation rate of 1200 rpm, approximately.

GC-MS analysis

The analyses were performed into a HP 6890 gas chromatography equipped with a MSD 5973 mass spectrometer (Agilent Technologies). Helium was used as the carrier gas at a constant flow rate of 0.7 ml/min. The Programmable Temperature Vaporizing (PTV) injector was operated in the splitless mode under the following temperature program: 40ºC (held for 0.1 min), 700ºC/min up to 230ºC (held for 23 min). The split valve remained open for 0.7 min. The separation was performed on a 60 m x 0.25 mm, d_f 0.25 μ m HP-INNOWAX capillary column (Agilent Technologies) and the oven temperature program was: 30ºC (held for 0.1 min), 2ºC/min up to 40ºC (held for 3 min), 12ºC/min up to 200ºC (held for 2 min). The mass spectrometer was operated in positive electron impact ionization mode (+EI) with 70 eV of electron energy. The quadrupole and the ionization source were maintained at 150 and 230ºC, respectively. Selected ion monitoring (SIM) was used for the detection of furan and furan-d_4, using *m/z* 68*/39/69 for furan and *m/z* 72*/42 for furan-d_4 (*quantifier ions). A dwell time of 100 ms was used for all the ions.

Identification and quantification

The relative retention time (RRT) and the presence of diagnostic ions were considered for identification of furan in the samples. For confirmatory purposes, an acceptable deviation of 0.5% for RRT, 10% for ionic relative abundance considering *m/z* 39/68, and 50% for ionic relative abundance considering *m/z* 69/68 were used by comparing the sample with a standard solution (EC, 2002). The quantification of furan was carried out by extrapolation from a linear analytical curve, using the quantifier ions *m/z* 68 (furan) and *m/z* 72 (furan-d_4, internal standard).

RESULTS AND DISCUSSION

Analytical method

Due to its high volatility, the analysis of furan in foods is performed by gas chromatography coupled to mass spectrometry (GC-MS), preceded by headspace sampling (HS) or headspace solid phase microextraction (HS-SPME). Both HS and HS-SPME approaches are very simple and convenient for volatiles analyses, demand no expensive equipment for sample extraction and give satisfactory and comparable results. HS-SPME seems to be more advantageous since allows sample concentration and affords higher sensitivity. Care must be taken during handling of samples in order to avoid furan loss by volatilization (Wenzl, 2008). In this study, the analysis of furan were carried out by using a HS-SPME-GC/MS method previously validated according to the guidelines laid down by the Brazilian Institute of Metrology, Standardization and Industrial Quality (INMETRO, 2007). This method showed good linearity within the range 0-100 μg/kg (r^2 = 0.998). A non-significant matrix effect ($F_{calc} = 1.04 < F_{tab} = 5.82$; $t_{calc} = 0.23 < t_{tab} = 1.78$) was verified by comparison between curves set on standard solutions and on matrix by applying the F-test and *t*-test. Limits of detection (LOD) and quantification (LOQ) were calculated as 0.7 and 2.4 μg/kg, respectively. Recovery, repeatability and within-laboratory reproducibility were in satisfactory ranges (Arisseto et al., 2010). A typical ion chromatogram of a sample of cooked pork containing < 2.4 μg/kg is illustrated in Figure 1.

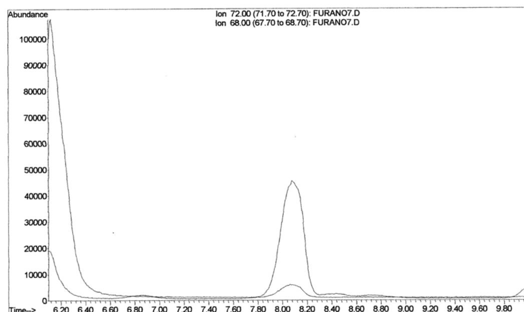

Figure 1. Ion chromatogram of a sample of cooked pork containing < 2.4 μ g/kg (m/z 68 and 72: quantifier ions; carrier gas: helium; flow rate: 0.7 ml/min; Programmable Temperature Vaporizing (PTV) injector: 40°C (held for 0.1 min), 700°C/min up to 230°C (held for 23 min); mode: splitless; column: 60 m x 0.25 mm, df 0.25 μ m HP-INNOWAX; oven: 30°C (held for 0.1 min), 2°C/min up to 40°C (held for 3 min), 12°C/min up to 200°C (held for 2 min); mass spectrometer: positive electron impact ionization (70 eV); quadrupole temperature: 150°C; ionization source temperature: 230°C; dwell time: 100 ms).

Furan levels in foods prepared in a pressure cooker

Table 1 shows the results obtained for the experiments carried out in the pressure cooker. Furan was not detected in the fresh samples. Although the pressure cooker is a container with a hermetic seal until the release of the steam, furan was not found above the limit of quantification in the cooked samples.

Table 1. Furan levels in foods cooked in a pressure cooker

Food	Furan (µg/kg)	
	Raw	Cooked
Beans	nd	<2.4
Whole rice	nd	<2.4
Soy beans	nd	<2.4
Beef	nd	nd
Pork	nd	<2.4
Potato	nd	nd
Cassava	nd	nd

Experiments simulating the conditions of a pressure cooking were performed by some authors by using aqueous model systems and retail foods heated in sealed vials (Hasnip et al., 2006; Limacher et al., 2007; Limacher et al., 2008). In both cases, an increase in furan levels was observed, demonstrating that furan can be formed under such conditions of pressure, temperature and time (83 kPa, 117/121°C, 20/25 min) when sealed vials are used. Taking into account these observations and the results obtained in the present study, it could be suggested that furan was possibly formed during the pressure cooking, but it must have been lost with the steam that is released by the valve when a determined pressure is reached. In this case, although furan was not found in the cooked foods, it should be considered that the contaminant could be released to the air kitchen and contribute for an occupational exposure. This should be further investigated since it has already been demonstrated that furan was found in the air kitchen as a result of some procedures, such as the addition of water to a cafetiere, the frying of chipped potatoes in an open chip pan, and baking some foods in an oven (Crews, 2009).

Stability of furan during re-heating after cooking

The levels of furan obtained in the experiments of re-heating after pressure cooking are illustrated in Figure 2. It can be seen that no furan has also been found in quantifiable amounts in the re-heated samples.

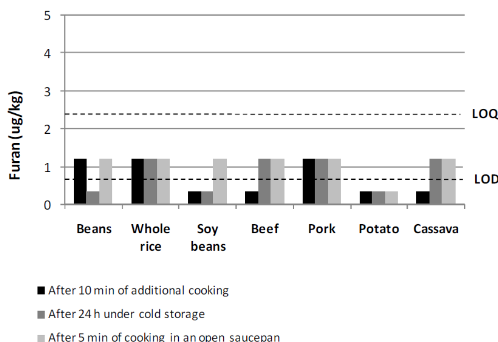

Figure 2. Furan levels obtained after re-heating (for illustration purposes, levels below limit of detection (LOD) were considered as ½ LOD (0.35 µ g/kg) and levels below limit of quantification (LOQ) were considered as ½ LOQ (1.2 µ g/kg)).

Studies on the stability of furan in foods available in the literature by applying normal warming procedures have shown conflicting results. Some authors reported furan losses of 29-85% whereas others have found that furan persists during normal heating practices (Goldmann et al., 2005; Hasnip et al., 2006; Zoller et al., 2007). Due to the non significant levels of furan found after re-heating in the present study, no conclusive results could be obtained in relation to its stability. It has also been demonstrated that furan can be even formed at levels up to 29.2 μ g/kg during the re- heating of baby-foods containing potatoes after cold storage (Lachenmeier et al., 2009). However, no significant furan formation was observed in the samples investigated here.

CONCLUSION

The data obtained in the present study indicated that the cooking in domestic pressure cooker may not represent a concern in relation to the occurrence of furan in foods.

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COMPARATIVE ANALYSIS OF DIFFERENT ALGINATE-BASED IMMOBILIZATION SYSTEMS FOR ENCAPSULATION OF POLYPHENOLIC ANTIOXIDANTS FROM RED RASPBERRY LEAVES (*RUBUS IDAEUS* L.) BY ELECTROSTATIC EXTRUSION

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ABSTRACT

Traditionally used in folk medicine for its health benefits, leaves of red raspberry (*Rubus idaeus* L.) present a rich source of bioactive compunds and contributes to an increase of the overall intake of bioactive compounds in the daily diet, so an effective supplementation form containing red raspberry leaves bioactives should be established. Up to date spray drying has been mainly used as a technique for the entrappment of polyphenolic compounds from various plant substrates. Since spray-drying requires complex equipment and high energy inputs, a need arises for development of an effective immobilization system applying a simple technique, such as electrostatic extrusion. The aim of this study was to encapsulate polyphenolic compounds from red raspberry leaves extract in different alginate-based matrices using electrostatic extrusion. For that purpose plain alginate, as well as caseine and chitosan reinforced alginate microbeads were evaluated in terms of encapsulation efficiency and release profiles of polyphenols in water. The release kinetics was determined by following total phenol content and antioxidant capacity during time period of 24h, where antioxidant capacity was measured by ABTS radical scavenging assay. The obtained results revealed the same difussion controlled release patterns for all samples. The majority of polyphenolic compounds are released during 10-15 min, while the encapsulation efficiency varied between 80.5 % for alginate and 84.4 % for chitosan-alginate microbeads. In addition to alginate-based microbeads described above, Ca-alginate microbeads containing dextrose as a filler substance were prepared by absorption of raspberry leaves extract compounds in blank Ca-alginate microbeads. In this case, somewhat lower encapsulation efficiency (77%) was determined, while diffussion rate of polyphenolic compounds was nearly the same. The results suggest the potential of alginate-based microbeads prepared by electrostatic extrusion to be used for delivery of bioactive compounds, and contribute to the development of novel functional food products, as well as antioxidant-containing supplements for pharmaceutical and cosmetic aplications.

KEYWORDS: *alginate; chitosan; electrostatic extrusion; encapsulation; polyphenols*

INTRODUCTION

Based on numerous scientific arguments, supplementation of diet with bioactive compounds derived from medicinal plants should be recommended among individual consumers, both for its healing properties and nutritive value. Since the preparation of infusions of medicinal plants and herbs is the most common way of their consumption, which is rarely able to acomplish in the modern daylife, an efficient supplementation form of the bioactive compounds from medicinal plants should be established. In this way, an effective delivery system of medicinal plants bioactives would enable their implementation in various food products and creating novel functional foods with beneficial health activities. Microencapsulation technology has faced a growing interest of both scientific community and food industry, due to the ability of immobilizing bioactive compounds and enabeling their further use in food purposes. Adding bioactive ingredients to functional foods presents many challenges, particularly with respect to the stability of bioactive compounds during

processing and storage and the need to prevent undesirable interactions with the carrier food matrix (Champagne et al., 2005). The encapsulation of polyphenolic compounds by electrostatic extrusion is a relatively novel technique, so additional investigation is required to enable the best characteristics and controlled delivery of microencapsuled bioactive compounds. Sodium alginate is one of the most widely used coating materials for the encapsulation of various substances, and its properties have been well studied (Ostberg et al., 2003; Rajaonarivony et al., 1993). Alginate gel microspheres have been conventionally prepared using extrusion by dropping an sodium-alginate solution through a needle into a $CaCl_2$ solution (external gelation), but this approach has several inconveniences, such as the limitation in reducing microsphere diameter, the teardrop shape of the microparticles produced, and the difficulty in industrial scale-up (Gombotz, Wee, 1998). Since aqueous solutions of alginate exhibit already very high viscosities even at low concentrations, maximum dry matters of approximately 2–4 % e.g. in the case of Ca-alginate are attainable for the matrix material (Chandramouli et al., 2004). As a consequence, a pronounced barrier effect is often not given, due to the low density of the resulting gel network (Crittenden et al., 2006). Therefore, there is a strong interest for the development of new ways to create small water-insoluble microcapsules, with a reduced porosity and retarded release of entrapped substances. To reduce the porosity and increase stability, alginate microparticles can be combined with proteins, chitosan or some synthetic polymers. Nontoxicity and biodegradability of these two naturally occurring biopolymers make them suitable for encapsulation of a wide variety of biologically active agents. The relatively mild cross-linking conditions required for obtaining chitosan- coated alginate microparticles also contribute to the attractiveness of this concept. In this paper the use of casein, chitosan and dextrose for coating/filling alginate microbeads will be examined in order to modify the bulk structure and alter diffusion of encapsulated polyphenolic compounds in an aqueous medium. Therefore, the red raspberry aqeous extract was encapsulated in plain alginate, alginate- caseine, alginate-chitosan, and alginate-dextran microbeads, and release of polyphenolic compounds was quantified in terms of total polyphenol content and antioxidant capacity.

MATERIALS AND METHODS

Preparation of raspberry leaves extract

For the encapsulation of raspberry leaves polyphenols, extraction was carried out by pouring 200 mL of boiling distilled water over 10 g of ground raspberry leaves at ambient temperature. The extraction was carried out for 30min with stirring, whereafter the obtained extract was filtered through a tea strainer.

Preparation of alginate, alginate-caseine and alginate-chitosan microcapsules

In order to compare the release kinetics of polyphenols entrapped in alginate microbeads, plain alginate, alginate-caseine and alginate-chitosan microcapsules were obtained and evaluated. Alginate solution (1,5% w/v) was prepared by dissolving a low viscosity sodium alginate Protanal LF 20/40 (purchased from FMC Biopolymer) in the previously obtained raspberry leaves extract. The mixture was well homogenized and drawn into a 5 ml syringe attached to 23-gauge metal needle with a blunt tip. The syringe was placed in a syringe pump ((Razel, Scientific Instruments, Stamford, CT)) and a high voltage dc unit (Model 30R, Bertan Associates, Inc., New York) was applied to control the potential difference. The solution was extruded at a voltage of 6.3 kV and flow rate of 25.2 cm^3/h to a receiving beaker containing 50 ml of 2% (w/v) calcium chloride solution prepared in raspberry leaves extract. The microparticles were allowed to stirr gently in the collection solution and then stored in that way until the analysis. For the preparation of alginate-caseine microbeads, alginate solution was prepared as described above, while caseine solution was prepared (3%, w/v) by dissolving caseine hydrolizate in raspberry leaves extract. The alginate and caseine solutions were mixed together (3:2 v/v) and processed by electrostatic-extrusion according to the above described procedure. Alginate-chitosan beads were obtained by preparing plain alginate microbeads and leaving the beads in a coating solution composed of 2% $CaCl_2$, 0.5% (w/v) chitosan and 0.5% (w/v) acetic acid in the raspberry leaves extract. The beads were left in the solution for 24h and then washed seve ral times with the extract.

Preparation of hydrogel microbeads absorbing raspberry leaves extract

Blank Ca-alginate beads were first prepared by using the electrostatic extrusion method as previously described. The microbeads were then filtered under a low vacuum and immersed into a glass containing raspberry leaves extract (one gram of microbeads in 15 mL of water), prepared as previously described, for 24h. During that time, the kinetics of absorption of polyphenols by Ca- alginate microbeads was measured by evaluating the polyphenolic content and antioxidant capacity of 1mL excerpt aliquots at defined time intervals.

Determination of total phenol content (TPC) of raspberry leaves extract

Total phenol content was determined spectrophotometrically according to a modified method of Lachman et al. (1998). Gallic acid was used as the standard and the results are expressed as mg of gallic acid equivalents (GAE)/g of dry weight of sample.

Determination of antioxidant capacity

The Trolox equivalent antioxidant capacity (TEAC) was estimated by the ABTS radical cation decolorization assay (Re et al., 1999). The results, obtained from triplicate analyses, were expressed as Trolox equivalents, and derived from a calibration curve determined for this standard (100-1000 μM).

Encapsulation efficiency

The amount of raspberry leaves polyphenols entrapped in the microparticles was estimated by dissolving a known amount of capsules in sodium citrate (2% w/v) during 60 min by vigorous shaking on a Vortex mixer (Tehnica, Železniki, Slovenia) at room temperature. The content of raspberry leaves extract loaded in the beads was determined by the previously desribed Folin-Ciocalteau method. A The percentage of loading efficiency was calculated according to the following equation:

$$\text{Loading efficiency (\%)} = \frac{A}{A_0} \times 100$$

where A is the TPC determined after destabilization of the microparticles with the sodium citrate solution and A_0 is the TPC of the initial extract.

Release kinetics of polyphenols in water

The release of the polyphenols from raspberry leaves extract in water was followed in terms of both, TPC measured by Folin-Ciocalteau method and antioxidant capacity determined by ABTS assay; both are described in previous sections. For the analysis a known amount of particles (~ 3g) was suspended in 50 mL of distilled water. The samples were submitted to continuous agitation on an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ) operating at 100 rpm. At certain time intervals, analiquot of the supernatant was taken for analysis and replaced by the same amount of fresh distilled water. The experiments were performed in triplicate.

RESULTS AND DISCUSSION

As can be seen on Figure 1., there is no significant difference beween release profiles of plain alginate, alginate-caseine and alginate-chitosan microbeads. The majority of polyphenolic compounds is released during first 10-15 minutes, which is in accordance with our recently reported results, where the release profile of polyphenolics from plain alginate and alginate-chitosan cross-linked particles were evaluated (Belščak-Cvitanović et al., 2010). The encapsulation efficiency measurements revealed that alginate-chitosan particles entrapped the most polyphenolic antioxidants (84.4%), followed by alginate-caseine microparticles (82.1%), while the lowest encapsulation efficiency (80.5%) was determined for plain alginate particles. Despite the fact that in all three cases high loads of polyphenolic compunds were achieved, rapid release of those copounds in an aqueous medium indicates that proposed alginate-based microbeads are not sufficiently efficient when prolonged release of polyphenolics is desired. Therefore, for the purpose of controlled delivery of bioactives in a hydrophilic ambient, another soultion should be looked for.

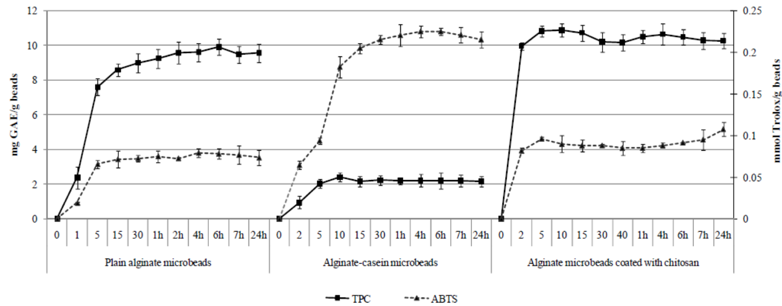

Figure 1. The release profile of polyphenolic compounds form microparticles encapsulating raspberry extract in water determined by the Folin-Ciocalteu and ABTS assays

The differences inTPC and antioxidant capacity of plain, alginate-casein and alginate-chitosan beads are attributed to the chemical interactions of caseine with polyphenolic compounds or storage duration of alginate-chitosan microbeads. The content of total polyphenols in alginate-casein is in average about 5 times lower compared to TPC of the other two samples. According to the literature, polyphenols exhibit strong inclination to form complex compounds with proteins, polysaccharides and alkaloids, which enhances the complexity of polyphenol-containing products (Hagerman, Butler, 1981). Therefore, we assume that caseine may have formed complexes with polyphenolic compounds which disabled the reaction of Folin-Ciocalteu reagent with free polyphenolic compounds; this resulted with an underestimation of polyphenols released from alginate-caseine microbeads. Similarly, the presence of caseine may have contributed to the reaction with ABTS radical during the radical scavenging capacity determination and, as a cosequence, antioxidant capacity appeared dramatically higher in comparison with values for ABTS of the other two alginat-based systems.As can be seen, the TPC of alginate-chitosan coated particles is slightly higher than in the plain alginate-particles. One possible explnation is that during coating process which lasted for 24 hours polymerization of some polyphenolic compounds might have occured. Namely, it is well established that during storage polyphenolic compounds undergo polymerization reactions which can result in the formation of oligomeric compounds; those often exhibit higher antioxidant capacity than monomeric compounds. Such polymerized compounds may also react with the Folin-Ciocalteu reagent, due to the lack of selectivity of this assay, and as a result, a slight increase in the TPC of alginate-chitosan particles is detected. In order to produce raspberry polyphenols-entrapping microparticles, another alternative approach was examined. Herein, blank alginate and alginate-dextrose microbeads were obtained and immersed in raspberry leaves extract to absorb its compounds. The diffusion of polyphenols during immersion period was followed, afterwhich such obtained particles were desintegrated and the amount of remaining polyphenolic compounds in the particles was determined.

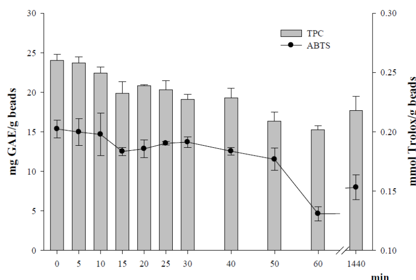

Figure 2. The absorption rate of polyphenolic compounds from raspberry leaves extract by blank alginate particles determined with the Folin-Ciocalteu and ABTS assays

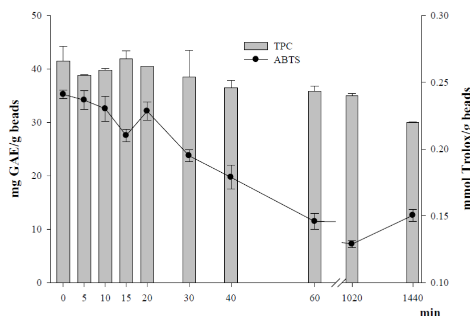

Figure 3. The absorption rate of polyphenolic compounds from raspberry leaves extract by alginate-dextrose particles determined with the Folin-Ciocalteu and ABTS assays

As can be seen on Figures 2&3, the TPC is gradually decreasing during prolonged immersion of alginate beads in raspberry leaves extracts. According to the TPC profile, it was not possible to determine at which point the concentration balance is reached. After the 24-hour absorption process is finished, the microbeads were mechanically and chemically desintegrated in order to determine encapsulation efficiency. The results revealed relatively high and equal values for encapsulation efficiency of both, blank alginate microbeads (77.2%) and alginate-dextrose microbeads (77.1%).

CONCLUSIONS

In all evaluated cases high loads of polyphenolic compunds were achieved, while rapid release of those copounds in an aqueous medium indicates that proposed alginate-based microbeads are not sufficiently efficient when prolonged release of polyphenolics is desired. The obtained results contribute to the development of encapsulation technology suitable for production of polyphenols dosage forms. With an appropriate immobilization system, the intake of antioxidants from herbs, such as raspberry could be maintained on a regular, daily basis by consuming functional food products enriched in encapsulates.

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THE MARMALADES OF SWEET ROWANBERRIES AS AN EXAMPLE OF A FUNCTIONAL FOOD

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ABSTRACT

The rowanberries (*Sorbus aucuparia*) are small orange-red "fruits" of a rowan tree. These berries have been described as an important source of flavonoids, and their antioxidant activity affects reactive oxygen species and lipid peroxidation; therefore they are suitable for production of health-food products. The ripe wild rowanberries are eatable, but very tart in flavour. Sweeter and less astringent than wild rowanberries are different cultivars of sweet rowanberries and hybrids with other species. The aim of this experiment was to prepare new product – the marmalades of sweet rowanberries with apples in different proportions, and to determine chemical and physical properties of experimental samples. The berries of cultivars „Moravica" and „Rosina" chosen for the production of marmalades are characterized by the high content of ascorbic acid and total carotenoids. The marmalades are made from puree of rowanberries and purees' mixtures with apples. The contents of organic acids, ascorbic acid, and total carotenoids of marmalades, and the colour and firmness of the experimental products were analysed. For determination of the organic acids and ascorbic acid content high performance liquid chromatography was used, and the content of total carotenoids was determined by spectrophotometric method. The colour (L*a*b*) of samples of marmalades was measured by colorimeter ColorTec-PCM and firmness - by texture analyser TA.XT.plus. The results showed that sweet rowanberries are good raw material for preparation of marmalades. The analysis of firmness showed that the marmalade from mixture of rowanberries and apples (30 % : 70 %) had a fairly hard texture. It was observed the reducing of the ascorbic acid content and total carotenoids after preparation of experimental samples of marmalades.

KEYWORDS: *Marmalade; sweet rowanberries; chemical and physical parameters*

INTRODUCTION

The rowanberries (*Sorbus aucuparia*) are small orange-red "fruits" of a rowan tree. These berries have been described as an important source of flavonoids, and their antioxidant activity affects reactive oxygen species and lipid peroxidation; therefore they are suitable for production of health-food products. The ripe wild rowanberries are eatable, but very tart in flavour. Sweeter and less astringent than wild rowanberries are different cultivars of sweet rowanberries and hybrids with other species. The berries consist mostly of water, and the main components in the dry matter are carbohydrates, primarily sugars, and non-volatile organic. Organic acids are important intermediate products of metabolism (Viljakainen et al., 2001). According to food composition and nutrition tables, sweet rowanberry *S. aucuparia* L. var. edulis contains 1600-2420 mg of organic acids per 100 g of edible portion, among others 10 mg of parasorbic acid, 98 mg of vitamin C per 100 g and 2.5 mg of total carotenoids per 100 g (Souci et al., 2008). There are reported that the vitamin C content of rowanberries genotypes, Sladkoplodá Moravská' is 22.84 mg 100 g^{-1} and antiradical activity are evaluated as 76.84 % (Paulovicsová at al., 2009). Therefore the rowanberries have characteristic patterns relating to their chemical parameters which were influenced by variety, season or geographic origin. Historically, jams and jellies may have originated as an early effort to preserve fruit for consumption in the off-season. Processing of different fruit kinds into juice, marmalade or jam is important for insuring of fruits during all year. Jellies, jams and marmalades are primary distinguished by the form from which their fruit is incorporated. Marmalades are basically jellies with fruit purée and sugar-acid-pectin gel or low- methoxyl pectin-calcium gels. Pectin is traditionally used in a wide range of fruit-based products in which it acts as a gelling agent (Grujić et al., 2010; Baker et al., 1996). Today foods are not intended to only satisfy hunger and to provide necessary nutrients for humans but alsoto prevent nutrition-related diseases and improve physical and men-

tal well-being of the consumers. In this context Functional Food play a specific role (Menrad, 2003). The term "functional food" originated in Japan in the 1980s, when it was used by the industry to describe foods fortified with specific ingredients imparting certain health benefits. There are many possible definitions for the term functional food; however, functional food is usually described as food that promotes health beyond the provision of basic nutrition (Rogelj, 2000). The aim of this experiment was to prepare new product – the marmalades of sweet rowanberries with apples in different proportions, and to determine chemical and physical properties of experimental samples.

MATERIALS AND METHODS

The experiments were done in the Faculty of Food technology of Latvia University of Agriculture, and the Customs laboratory of National Customs Board, State Revenue Service. The rowanberries of cultivars „Moravica" and „Rosina" were used for preparation of marmalades, and picked in the Pure Horticultural Research centre (HRC) collection of genetic resources in September 2010, and then were packed in plastic bags, frozen, and kept at -20±2 °C along 6 months. The sweet rowanberries purees were made from frozen and thawed berries. Thawing process of berries was made overnight in refrigerator at + 4 °C. The sweet rowanberries were homogenised by a manual blender and then were scrubbed through sieve, and the apple puree was made from boiled pieces of apples, which were scrubbed through sieve, too. Three different proportions of sweet rowanberries and apple purees were used - 100:0, 50:50 and 30:70, respectively. Then the mass of purees was mixed with sugar (sucrose 24 % from the total amount of product) and were heated till 80-85 °C, and then pectin (Gen pectin LM-101-AS powder 4 %) was added. After that all samples were put in the polypropylene boxes, cooled and dried at room temperature for 24 h. All variants of the marmalade samples of sweet rowanberries are shown in Table 1.

Table 1. The variants of prepared the marmalades of sweet rowanberries and apples.

The sample number	The cultivar of rowanberries	Proportions (rowanberries:apples)
1M	'Moravica'	100:0
2M	'Moravica'	50:50
3M	'Moravica'	30:70
1R	'Rosina'	100:0
2R	'Rosina'	50:50
3R	'Rosina'	30:70

To compare the nutritional value of these products and the influence of preparation technology to stability of bioactive compounds, the mass of purees with sugar before boiling and the marmalades of sweet rowanberries and apples were used for analysis. The moisture content of purees with sugar and the experimental marmalades of sweet rowanberries were determined with a gravimetric method using analytical scales BP-210s (Sartorius). An aliquot of samples (~10 g) was dried in oven TR60 (Naberthern) at 97 °C overnight (Mattila et al., 2006). Measurements were carried out in three replications.

The determination of the organic acids and vitamin C content was made by high performance liquid chromatography (HPLC) method reported by Romero-Rodriguez et al. (1992) and Vanques-Oderiz et al. (1994) by adding some modifications. The part of samples (100 g) was homogenised by a manual blender (Braun). The portion of samples (10 g) was weighted into a volumetric flask (50 ml) and 0.001 M sulphuric acid (~ 30 ml) was added for determination of organic acid content, or 0.1 % oxalic acid (~ 30 ml) – for determination of ascorbic acid content. Mixture was stirred mechanically for 20 minutes and the solution filtered through a paper filter (DP 503 125, Albet) in 50 ml volumetric flask and filled to mark with correspondent acid solution. Acid extract was then filtered through a membrane filter with pore size 0.2 μm (Sartorius) prior to injection into the chromatographic system. Calibration curve was acquired after two repeated HPLC runs of 5 standard solutions of reference materials. Quantifications of the organic acids and ascorbic acid (vitamin C) content of samples were performed in duplicate and were based on peak area measurements. The extract of samples was analysed and content of organic acids and vitamin C determined using HPLC Prominence

(Shimadzu) equipped with Ostion LG-KS H+ column (250 x 8 mm, particle size 10 μm) and an autosampler SIL-20A. Working conditions: the mobile phase – ultra-pure water acidified to pH 2.2 with sulphuric acid. The flow rate was 0.4 ml min^{-1}, column temperature was 30 °C, detection with a UV/VIS

detector SPD-20A (Shimadzu) was at 215 nm for organic acids and at 245 nm for vitamin C, and injection volume of samples – 10 μl. Data were acquired and processed using Shimadzu LabSolutions software (LC-solution Version 1.21 SP1).

The total carotenoids content was analyzed by the spectrophotometric method at 440 nm (Ермаков, 1987) with petroleum ether (boiling temperature range 80-110 ºC) and measured with UV-VIS-NIR spectrophotometer UV-3100PC (Shimadzu) in 10 mm cuvettes. 2-3 grams of homogenized samples were placed in a conic retort (100 ml) and 96 % ethanol (20 ml) was added, and then samples were stirred by a magnetic stirrer for 20 min. Then petroleum ether (25 ml) and water (1 ml) was added and stirring was continued for one more hour. After 3-4 hours the top (yellow) layer was used for the detection of total carotenoids. The carotene equivalent (KE) was found, using graduating curve with $K_2Cr_2O_7$.

The colour (L*a*b*) of samples was measured in CIE L*a*b* colour system by colorimeter ColorTec- PCM. Colour values were recorded as L* (lightness, 0=black, 100=white), a* (-a, greenness, +a, redness) and b* (-b, blueness, +b, yellowness). Values a* and b* are two chromatic components with range from - 120 to +120 (Papadakis et al., 2000). The measurements were repeated on ten randomly selected locations on each sample. Total colour difference (E*) of the marmalades of sweet rowanberries between initial value (the mass of purees with sugar) and after preparation was calculated using the following equation (1):

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

The firmness of the marmalades of sweet rowanberries was measured by texture analyser TA.XT.plus (Stable Microsystems Ltd., Surrey, England) and software Texture Exponent 32. The texture analyser was equipped with a load cell of 50 kg and the probe for the structure analyses SMS P/0.5R. We used the compression test mode with test speed of measurement 1.0 mm s^{-1}. The distance of penetration was 12.0 mm. The results were expressed as maximum force in Newtons (N), and the maximum force required for sample compression was calculated as an average of 10 measurements. Data analysis. All results were given as mean and standard deviation of independent determinations. All statistical analyses were performed with SPSS for Windows (Version 11.0). Differences were considered to be significant at $p<0.05$.

RESULTS AND DISCUSSION

In this study six samples of marmalades of sweet rowanberries and apples in different proportions were prepared (see Table 1) and determined its chemical and physical properties. Both the mass of purees with sugar before boiling and the marmalades of sweet rowanberries and apples were used for analysis. The moisture content is the main parameter of food products, which influences the storage time. The moisture content of the mass of purees with sugar and the experimental marmalades is given in Figure 1. It was determined that the moisture content of mass of purees with sugar is from 57.81±0.08 % to 62.83±0.27 % and the marmalades – from 37.21±0.63 % to 43.88±0.74 %, except the sample 1M, which was prepared from rowanberries „Moravica" (only 26.50±0.77 %).

The organic acids and ascorbic acid (vitamin C) content is very important parameter that determines the quality of berry products. Vitamin C is the least sable of all vitamins and is easily destroyed during processing and storage. The most harmful factors to vitamin C content are the availability of oxygen, prolonged heating in the presence of oxygen and exposure to light. The comparison of the ascorbic acid content in the purees and marmalades of sweet rowanberries with apples is reported in Figure 2.

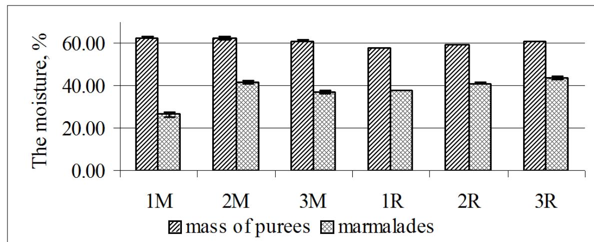

Figure 1. The moisture content of the mass of purees and the marmalades

The ascorbic acid content significantly differed between the puree samples and the marmalades of sweet rowanberries (p=0.000). The highest ascorbic acid contents both before and after heating of purees were detected in sample 1M, which was made only from rowanberry purees without adding apple purees. The ascorbic acid losses during the preparation of the marmalades were from 13.3 % (sample 1R) to 47-49 % (samples 1M and 3R).

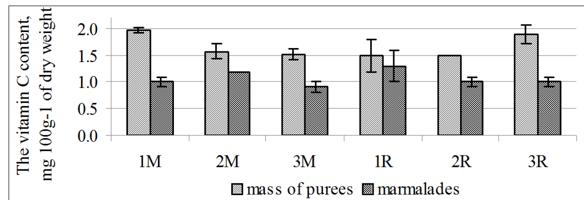

Figure 2. The comparison of ascorbic acid content of the mass of purees and the marmalades

Berries contained many organic acids, which are responsible for total titratable acids content. The amount of organic acids (citric, malic, succinic, and sorbic) of all investigated samples is given in Figures 3.

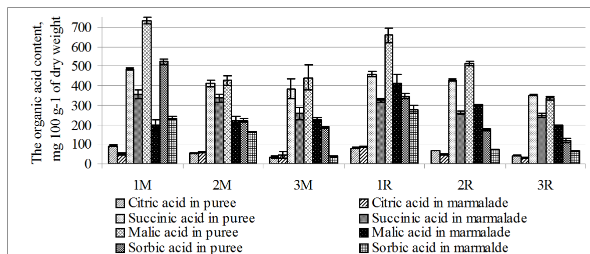

Figure 3. The organic acids content of the purees and marmalades

Malic and succinic acid were the dominant compounds compared with other organic acids in all investigated samples. We observed that during the preparation of the marmalades of sweet rowanberries with apples organic acid content was decreased.

The total carotenoid content of all investigated samples is reported in Figure 4. The differences of the total carotenoid content significantly differed between samples of the mass of purees and the marmalades of sweet rowanberries (p=0.000). The highest total carotenoids content, similar to ascorbic acid content, was in the sample 1M and 1R (made from only rowanberry purees without adding apple purees) and the least in the 3M and 3R (the proportion rowanberries and apples was 30:70). The total carotenoids losses during the preparation of the marmalades were from 72 % to 80 %.

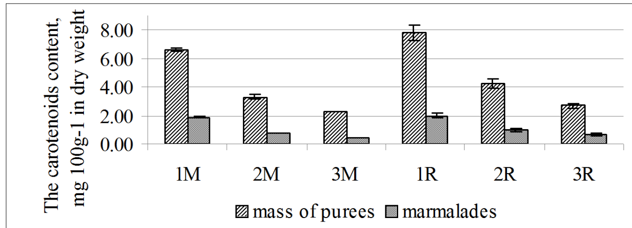

Figure 4. The comparison of the total carotenoids content of the purees and the marmalades

The colour (L*a*b*) measurements was made for the samples of marmalades. The lightness (L*), redness (a*) and yellowness (b*) values of the mass of purees and marmalades are shown in Figure 5. The lightest colour (L* value) was detected at the samples of the mass of purees 3M and 3R (proportion of rowanberries and apples – 30:70), but the darkest (the lowest L* value) and the most red (the highest a* value) sample of the mass of purees was the 1M and 1R (prepared from 100 % rowanberries).

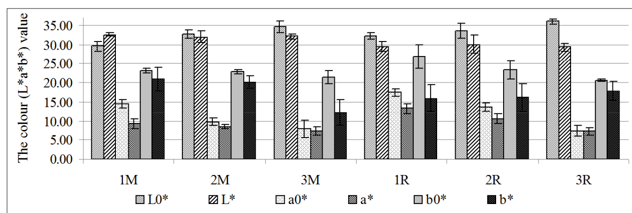

Figure 5. The colour in CIE L*a*b* system of the purees (L*0, a*0, b*0) and the marmalades (L*, a*, b*)

Results showed that all marmalades prepared from rowanberries „Moravica" (samples 1M, 2M and 3M) had higher L* value than marmalades from „Rosina". The darkest (the lowest L* value) and the red (the highest a* value) sample was the marmalade of rowanberries „Rosina" and apples in proportion 30%:70% (sample 3R). The redness and yellowness of all marmalades was slightly decreasing during preparation, mainly during boiling. The total colour difference (E*) between the colour of the purees and the marmalades of sweet rowanberries with apples are given in Figure 6.

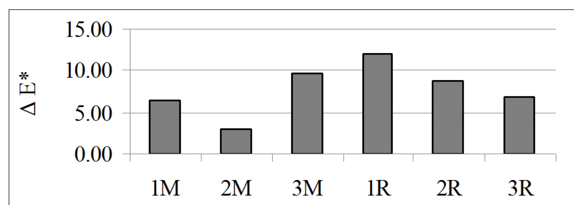

Figure 6. Total colour difference (E*) of experimental samples

The firmness of experimental samples of marmalades is shown in Figure 7. The results of the experiment showed that the marmalade of sweet rowanberries and apples in proportion 30%:70% had a fairly hard texture than others. We observed that the firmness of the marmalades from „Moravica" was increased for 80% replacing 50% rowanberries puree with apples puree and for 125% - replacing 70% rowanberries, but the marmalades from „Rosina" was increased for 10% and 29%, respectively.

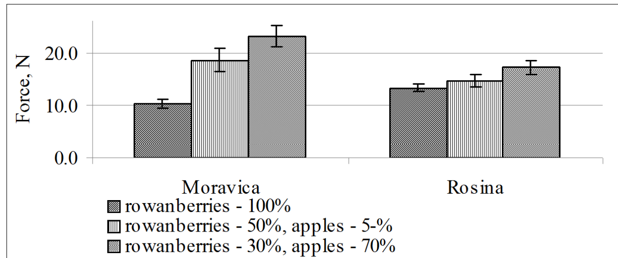

Figure 7. The firmness of the marmalades of rowanberries and apples

CONCLUSION

The present study shows that sweet rowanberries are good raw material for preparation it's in marmalades. The ascorbic acid content losses during preparation of marmalades ranging between 13.3 % and 47-49 % and total carotenoids content losses – between 72 % and 80 %. The firmness of the marmalades of sweet rowanberries was 10.3-13.3 N. The replacing part of rowanberries with apples (50 and 70 %) increased the firmness of marmalade samples to 17.3-21.1 N. The sweet rowanberries purees without adding apple purees could be acceptable for preparation marmalades because these samples showed the highest ascorbic acid and carotenoids content from all investigated samples.

ACKNOWLEDGEMENTS

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EVALUATION AND IDENTIFICATION OF VOLATILE COMPOUNDS OF STRAWBERRIES AND APPLES

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ABSTRACT

Fruit has always been a part of the human diet. The quality of fruit includes many aspects, but flavour is one of the most important quality traits. The flavour of fruit is determined by taste and aroma, it is the result of special assortment and mixture of different metabolites.

The aim of the research is to determine volatile compounds of strawberries and apples.

Fruits and berries harvested from the orchard of Latvia State Fruit Growing institute in 2010 were used for the experiment. Strawberry (*Fragaria x ananassa*) varieties - 'Polka', 'Senga-Sengana', 'Honeoye' and apple varieties – 'Baltais Dzidrais', 'Antonovka', 'Zarja Alatau' were analyzed in the research. The volatiles of strawberries and apple mass were extracted by headspace solid-phase microextraction (HS-SPME) and analyzed using gas chromatograph-mass spectrometer Clarus 500 GC/MS (PerkinElmer). Compounds were identified by comparison of their mass spectra with mass spectral libraries (Nist98), and by calculation of linear retention indexes and comparison with literature data.

The main volatiles in apples were esters and alcohols. The most common combinations of aromatic volatile compounds were acetic acid, butteric acids, hexanoic acids and esters of ethyl-, butyl-, hexyl- alcohols. The flavour of strawberries was determined by a complex mixture of esters, aldehydes, alcohols and sulfur compounds. Primarily methyl and ethyl esters appear to be the most important contributors to strawberry aroma.

Quantitative and qualitative composition of fruit volatile compounds analyzed in the research varies upon the variety. Depending on enzymatic metabolic pathways of a particular fruit, diverse substance mixtures form producing the characteristic aroma of a particular fruit.

KEYWORDS*: strawberry; apple; volatile compound*

INTRODUCTION

The quality of fruit includes many aspects - freshness, colour and absence of decay or physiological disorders and texture (firmness, juiciness and crispness). Although this concept involves aesthetic appeal and mechanical properties associated with quality, it ignores flavour and nutritional quality. Flavour plays an important role in consumer satisfaction and influences further consumption of fruits and foods in general (Pelayo C. et.al. 2003).

The flavour of fruit is determined by aroma and taste, it is the result of special assortment and mixture of different metabolites (Kafkas E., Paydas S., 2007). The aroma profile of a fruit is complex and depends on the combination of all volatile compounds emitted, as well as on the concentration and odour threshold of each individual compound. The contribution of a compound to the aroma depends on its odour threshold (level at which a compound can be detected by smell) and on its concentration in the fruit. (Echeverr G., 2004).

The typical aroma of strawberries comes from not just one or a few impact aroma compounds, but from numerous volatiles present at certain concentrations and in a particular balance among them (Pelayo C., 2003). The volatile compounds produced by strawberry (Fragaria × ananassa, Duch) fruit create aroma and contribute to flavour, thus strongly affecting quality and influencing consumer acceptability (Wang SY., Bunce J.A., 2004). Approximately 360 compounds have been identified in the aroma of strawberries, only

methyl and ethyl esters, furanones, C6 aldehydes and other C6 derivative compounds, diacetyl, acetic acid and other aliphatic acids, linalool, g-dodecalactone, benzaldehyde and some sulfur compounds appear to be the most important contributors to strawberry aroma (Pelayo C., 2003). The biggest part of aromatic volatile compounds in apples is made by esters and alcohols. The most common combinations of aromatic volatile compounds are acetic acids, butteric acids, hexanoic acids and esters of ethyl-, butyl-, hexyl- spirits (Fan X., Mattheis J. P., 1999).

The sensory characteristics of apples can be loosely grouped into three categories: flavour, texture and appearance.

Aroma substances are always more or less volatile compounds, which appear already at extremely tiny concentrations (Baltess V., 1998). About 10 thousand compounds taking part in the taste and smell formation of almost half a thousand products are identified (Morozovs A., 2008). Fruit aroma is usually formed by 200-400 different compounds. They include unbranched acids, alcohols, esters, ketones, aldehydes. In addition, aroma caused by certain components depends on concentration (Baltess V., 1998).

Apparently, it is the concentration and not the class of aroma compounds that is responsible for the large variation in aroma quality among cultivars (Hancock, 1999).

As aroma substances represented in fruits may be decomposed, the optimum aroma of fruits is guaranteed only for a relatively short time. Many fruits are frozen to extend their availability throughout the year, and to further shelf life through lengthy distribution (Skrupskis et al., 2008).

The aim of the research is to determine volatile compounds of frozen strawberries and apples, which further could be used in production of different products for the needs of public catering, e.g. in production of desserts.

MATERIALS AND METHODS

Raw materials

Apples and strawberries harvested from the orchard of Latvia State Institute of Fruit Growing in 2010 were used for the experiment. For analysis three strawberry varieties - 'Polka', 'Senga-Sengana', 'Honeoye' and three apple varieties – 'Baltais Dzidrais', 'Antonovka', 'Zarja Alatau' were used. After harvesting, strawberries were frozen (as whole berries), stored for nine months (-18˚C), after thawing homogenized and analyzed. For apples, before freezing, heat treatment and homogenization were applied to obtain puree. Obtained samples were frozen and stored six months at -18˚C temperature.

Determination of volatile aroma compounds

Volatiles from apple puree and strawberry mass were extracted using solid phase microextraction (SPME). 5 g of puree were weighed in a 20 ml headspace vial and capped with a septum. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) fiber (Supelco Inc., Bellefonte, PA, USA) was used for headspace SPME sampling. SPME parameters were: incubation time 10 min, extraction temperature 40±2 °C, extraction duration 30 min, desorption 15 min, 250 °C. For the analysis of the SPME extracts, a Perkin Elmer Clarus 500 GC/MS and an Elite-Wax ETR (60 m x 0.25 mm i.d.; DF 0.25 μm) was used. Working conditions were: injector 250 °C; transfer line to MSD 260 °C; oven temperature start 40 °C, hold 7 min, programmed from 40 to 160 °C at 6 °C min^{-1} hold 10 min, and from 160 to 210 °C at 15 °C min^{-1}, hold 15 min; carrier gas (He) 1 ml min^{-1}; split ratio 2:1; ionization EI+; acquisition parameters in full scan mode: scanned m/z 50-300. Compounds were identified by comparison of their mass spectra with mass spectral libraries (Nist98), and by calculation of linear retention indexes and comparison with literature data. All analyses were performed in triplicate. As a quantitative measure, the share in the total GC peak area for each compound is given.

Statistical analysis

The differences in the volatile compounds were analyzed and processed by Microsoft Office Excel 2007 programmer.

RESULTS AND DISCUSSION

In current research volatile compounds representing several chemical classes, such as acids, esters, alcohols, terpenes, ketones, aldehydes and others were detected. The volatile aroma compounds in three varieties of strawberries are shown in Table 1. Esters (most characterized by fruity aromatic notes) were the dominant aroma compounds in the all analysed strawberry cultivars. In the variety 'Senga-sengana' esters represented 83.49%, in the variety 'Polka'- 51.46% and in the variety 'Honeoye' -13.53% from total volatile compounds (Table 1.).

Scientific literature confirms that ethyl hexanoate, ethyl butanoate, methyl hexanoate, methyl butanonate, hexyl acetate and hexyl hexanoate are the most important volatiles in 'Honeoye' strawberries and may be the major contributors to its aroma, although the possibility of an underlying minor component strongly influencing the sensory results can never be ruled out (Wang SY., Bunce JA., 2004). When comparing data available in scientific literature with the data of the current research, it can be concluded that the most important volatiles in 'Honeoye' strawberries are hexanoic acid, hexanoic acid ethyl ester, Linalool and nerolidol (Table 1).

Among minor aroma compounds, acetic acid (sour aroma) was the only volatile not detected in 'Senga-sengana' strawberries. The branched esters ethyl-2-methyl propanoate and methyl/ethyl-2-methyl butanoate, the aliphatic acid butanoic acid (sweety aroma) and the ketone 2.3 butanedione or diacetyl (buttery aroma) were considered important contributors to the strawberry flavor by Pelayo C., 2003. In analyzed strawberries of the cultivars 'Polka' and 'Hanoeye' the branched esters butanoic acid 2-methyl ethyl ester, hexanoic acid hexyl ester, decanoic acid ethyl ester, benzoic acid ethyl ester, acetic acid phenyl methyl ester and benzanoic acid 2-hydroxy methyl ester were not detected. The branched esters ethyl acetate, butanoic acid ethyl ester, 1-butanol 3-methyl acetate, hexanoic acid ethyl ester, acetic acid hexyl ester and 2-hexen-1-ol acetate were detected in all three varieties of the researched strawberries. The volatile compounds of alcohol group were not detected of in strawberry variety 'Polka' and terpenes in variety 'Senga-sengana'. The ketone2-butanone 3-methyl represented 1.34% from total volatile compounds and aldehydes (hexanal) 1.57% from total volatile compounds were detected only in the variety 'Hanoeye', in other varieties they were not detected at all. The volatile aroma compounds represented in the researched strawberries are indicated in Table 1. The data ≥ 1% are included in the table.

Table 1Volatile aroma compounds (AU ×105) as measured by SPME-GC-MS in strawberries

Compound	Senga-sengana	Polka	Hanoeye
Acids			
butanoic acid, 2 methyl	35.00567	20.909625	n.d.
hexanoic acid	54.10293	95.64341	131.925725
nonanoic acid	52.06266	41.33044	49.611935
acetic acid	n.d.	26.50764	12.30058
Esters			
ethyl acetate	79.44904	112.99862	18.603375
butanoic acid, ethyl ester	162.42795	111.863145	34.54711
butanoic acid, 2-methyl, ethyl ester	39.66966	n.d.	n.d.
1-butanol, 3-methyl acetate	16.95319	20.161575	10.40533
hexanoic acid, methyl ester	61.765315	60.628155	n.d.

hexanoic acid, ethyl ester	1352.68416	727.74836	165.291545
acetic acid, hexyl ester	110.071385	120.042995	59.56067
2-hexen-1-ol, acetate	40.73437	70.57515	8.649755
octanoic acid, ethyl ester	107.516285	13.664245	n.d.
acetic acid, octyl ester	20.459235	19.086105	n.d.
benzanoic acid, 2-hydroxy, methyl ester	40.90813	n.d.	n.d.
propanoic acid, 2-methyl, methylester	n.d.	n.d.	15.51241
pentafluoropropionic acid, hexyl ester	n.d.	n.d.	22.141285
Methyl Salicylate	n.d.	13.22679	15.07241
Terpenes			
Linalool	234.29034	175.052665	79.67281
nerolidol	n.d.	n.d.	85.31349
Ketones			
3-methyl 2-butanone	n.d.	n.d.	11.206555
Others			
N-Methylcyclohexylamine	55.33722	135.419125	n.d.
benzocyclobutene	n.d.	n.d.	22.96845
2,5-Dimethyl-4-methoxy-3(2H)-furanone	n.d.	n.d.	24.973795
Decanolactone	n.d.	n.d.	39.497775
n.d. – not detected			

Quantity of aroma compounds detected in apple varieties show that the variety 'Antonovka' contains the highest amount of total volatile compounds. Esters are the dominant aroma compounds in the apples of all analyzed varieties**.** The variety 'Antonovka' has the highest quantity of esters (53.42% from total volatile compounds). The most important volatile in the variety 'Antonovka' is acetic acid hexyl ester.

Literature data show that the main groups of compounds are the 2-methylbutanoate esters, particularly ethyl 2-methylbutanoate; they are key contributors to fruit aroma (Rowan D.D., 1999). The biosynthetic origins and interconversions of 2-methylbutyl and 2-methylbutanoate esters in 'Red Delicious' and 'Granny Smith' apples were determined by feeding deuterium-labeled substrates with GC−MS identification of the deuterated aroma volatiles produced. An array of labeled 2-methylbutyl and 2-methylbutanoate esters was produced from each substrate with significant differences in products and product distributions between the two apple cultivars. Novel 2-methyl-(2E)-butenyl esters were identified as biosynthetic products in the aroma of 'Red Delicious' but not 'Granny Smith apples' (Rowan D.D., 1999).

In researched apples of the cultivars 'Baltais Dzidrais', 'Antonovka', 'Zarja Alatau' the acids such as hexanoic acid, acetic acid, octanoic acid were detected. The nonanoic acid was not detected in the variety 'Baltais Dzidrais'. The volatile compounds of alcohol group were not detected in the apple variety 'Zarja Alatau' and ketones in the variety 'Baltais Dzidrais'. The volatile aroma compounds in three varieties of apples are shown

in Table 2. The data ≥ 1% are included in the table.

Table 2 Volatile aroma compounds (AU ×10^5) as measured by SPME-GC-MS in apples

Compound	Antonovka	Zarja Alatau	Baltais Dzidrais
Acids			
hexanoic acid	21.87692	11.67981	8.92
nonanoic acid	304.67811	111.77788	n.d.
acetic acid	55.550025	18.289445	15.42879
octanoic acid	30.837915	10.526915	10.7613
Esters			
ethyl acetate	n.d.	n.d.	7.33479
butanoic acid, ethyl ester	n.d.	330.02694	22.59865
butanoic acid, 2-methyl, ethyl ester	n.d.	95.31399	n.d.
hexanoic acid, ethyl ester	n.d.	125.171705	n.d.
acetic acid, hexyl ester	605.57742		5.58233
hexanoic acid, hexyl ester	115.499205	10.008225	21.90318
acetic acid, butyl ester	284.58984	n.d.	n.d.
pentafluoropropionic acid, hexyl ester	65.91569	62.64378	29.06565
butanoic acid, hexyl ester	108.976195	n.d.	39.40774
butanoic acid, butyl ester	30.73585	n.d.	14.02377
hexanoic acid, butyl ester	36.24729	n.d.	n.d.
valeric acid, tetradecyl ester	114.678275	n.d.	n.d.
butyl caprylate	27.34241	n.d.	n.d.
butanoic acid, 2-methyl, hexyl ester	n.d.	34.54971	n.d.
butanoic acid, 3-hydroxy, ethyl ester	n.d.	19.335555	n.d.
Alcohols			
1-hexanol	227.24372	n.d.	110.73926
1-octanol	29.92012	n.d.	8.09815
8-heptadecanol	n.d.	n.d.	59.00862
Terpenes			
α-farnesene	411.03354	271.49123	5.08334
Ketones			
1-hepten-3-one	27.16893	n.d.	n.d.
Aldehydes			
hexanal		19.75827	6.85326
nonanal		11.65235	11.9581
2-heptenal	40.50862	n.d.	8.99819

2-octenal	28.89863	n.d.	6.67533
Others			
pentane, 3-ethyl-2,2-dimethyl	n.d.	29.660205	n.d.
n.d. – not detected			

In all researched varieties of strawberries and apples esters are the dominant aroma compounds. Scientific literature confirms that under high rates of ethyl ester production, the synthesis of methyl and other esters is limited and deviations from the original aroma profiles are favored. The preferential synthesis of ethyl esters over other esters in the presence of high levels of ethanol is possible because the activity of the enzyme alcohol acyltransferase (AAT) apparently depends more on substrate availability than on substrate specificity (Aharonia et al., 2000). The volatile aroma compounds in apples and strawberries are shown in Fig.1

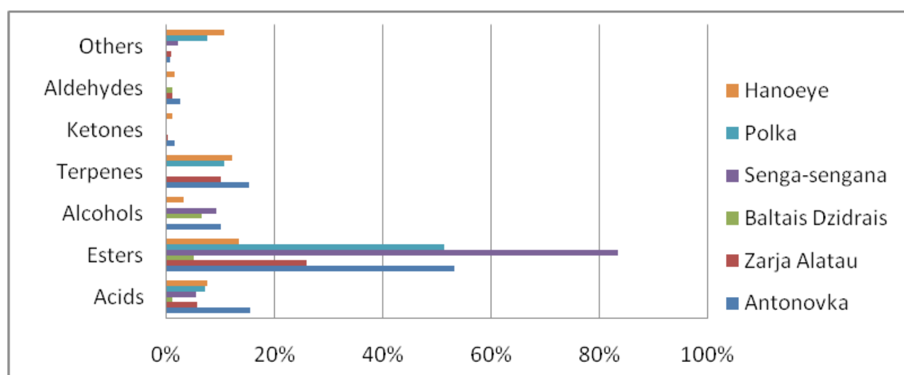

Fig.1. Volatile aroma compounds (*% from total volatile compounds)* in apples and strawberries

Results of the research indicate that aldehydes and ketones are in small amounts of total volatile compounds of strawberry and apple varieties. Esters are the major volatile compound in the researched fruits, particularly in strawberries.

CONCLUSION

It can be concluded that in all researched varieties of strawberries and apples esters were the dominant aroma compounds but the amount of aldehydes and ketones was insignificant. Total volatile compound in the strawberry variety 'Senga-sengana' was the highest, where hexanoic acid ethyl ester was dominant (53.38% *from total volatile compounds).* Total volatile compound in apple variety 'Antonovka' was the highest. The highest volatile compound was in acetic acid and hexyl ester of ester group - 22.83% from total volatile compounds.

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LIGNANS, TOTAL PHENOLS AND ANTIOXIDANT ACTIVITY OF SELECTED CEREAL GRAINS COMPARED TO FLAXSEED

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ABSTRACT

Among broad spectrum of known phenolics, there is a class of non-steroidal estrogenic compounds called phytoestrogens, including lignans as one of the subgroup compounds. Besides the (anti)estrogen effect, lignans are thought to possess antioxidant activity. The richest known source of food lignans is flaxseed. Thus, in this work we determined the lignan content, total phenols and antioxidant activity of selected cereal grains (wheat, barley, rye, and oat) and compared them with flaxseed as the potential enriching ingredient in bakery products. Lignans were quantified by gas chromatography-electron capture detection (GC-ECD), total phenols determined by Folin-Ciocalteu method and antioxidant capacity evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The results show that cereal grains contain more than two hundred times lower amount of lignans than flaxseed, but contain lignan syringaresinol that was not detected in flaxseed. Total phenolic content in cereal grain was found to be in range 256 ± 2.4 to 408 ± 3.6 mg ferulic acid equivalent/100 g, while in flaxseed was 1286 ± 146.8 mg ferulic acid equivalent/100 g. Additionally, flaxseed showed stronger DPPH radical scavenging capacity compared to cereals. The addition of flaxseed could contribute to the overall functional aspect of the bakery products by increasing the level of health promoting components. Future work will show the limits of use, considering the flaxseed laxative properties, content of cyanogenic glucosides and influence on product textural and sensory properties.

KEYWORDS: *cereals; flaxseed; lignans; total phenolic content; antioxidant capacity*

INTRODUCTION

Plant food is known to contain different kind of phenolics, which include simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, tannins, lignans, and lignins (Naczk, Shahidi, 2004). These compounds possess antioxidant properties and can protect against degenerative diseases in which reactive oxygen species are involved (Dykes, Rooney, 2007). Beside antioxidant activity phenolics own other action mechanism with health-promoting and disease-preventing effect, e.g. (anti)estrogen effect. In the Western diet one of the main sources of phytoestrogens are in the form of lignans. Those include food high in fiber such as flaxseed, whole grain products, seeds, nuts, legumes and some fruits and berries (Milder et al., 2005, Blitz et al., 2007). Lignans have been investigated for their health effect in several *in vivo* and *in vitro* studies (Webb, McCullough, 2005; Adlercreutz, 2007), but to our knowledge only few studies examined lignan antioxidant activity (Kitts et al., 1999; Prasad, 2000; Hu et al., 2007). Those studies showed positive correlation of antioxidant activity and pure standards of secoisolaiciresinol and its diglucoside, a type of lignan most abundant in flaxseed, using a variety of *in vitro* methodologies (e.g. chemiluminescence of zymosan-activated polymorphonuclear leukocytes; DPPH stable free radical scavenging; AAPH peroxyl radical-induced DNA nicking; peroxyl radical induced liposome oxidation). The objective of this study was to extract lignans from whole grain cereals (three different varieties of wheat, barley, rye, and oat) and flaxseed and examine its lignan content, total phenolics and DPPH radical scavenging capacity. Since bakery products enriched with flaxseed are on the spot as a functional food, we made a comparison of cereal and flaxseed for overall phenolics and lignan content and their antioxidative activity.

MATERIALS AND METHODS

Materials.

Standards of secoisolariciresinol (>95% purity), pinoresinol (>95%), and lariciresinol (~92%) were a kind gift from Oy Separation Research Ab (Turku, Finland). Syringaresinol (>95%) was purchased from Plantech UK (Berkshire, England). Matairesinol (≥85%), pentafluoropropionic anhydride (PFPA) (≥96%), styrene glycol (≥98.0%), ferulic acid (≥99.0%), enzyme *H. pomatia* β- glucuronidase/sulfatase type H-1, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Folin-Ciocalteu's reagent were purchased from Sigma Aldrich (Taufkirchen, Germany). Methanol, sodium hydroxide, glacial acetic acid, ethyl-acetate, dichloromethane, pyridine, sodium carbonate and *n*-hexane were purchased from J.T.Baker (Griesheim, Germany). The Institute for Seeds and Seedlings (Osijek, Croatia) provided wheat grains: Žitarka, Sana and Divana type. Rye, oats, barley and flaxseed were bought in a local store, and were of unknown variety. Grains and flaxseed were ground in a coffee grinder before analyses.

Sample Preparation and Extraction.

Prior to extraction flaxseed was defatted by Soxhlet extraction in *n*-hexane for 8h (ISO 659:1998). Extraction was done as described by Čukelj et al. (2011). Two hundred mg of cereals and flaxseed was extracted with 5 mL of 70% methanol containing 0.3 M sodium hydroxide at 60 °C, with applying periodical shaking. After 60 minutes the samples were centrifuged at 750 *g*/15 min, and the supernatant was transferred to a fresh tube. The extraction was repeated once more for cereals, and three times more for flaxseed. The supernatants were pooled and pH was adjusted to ~5 using glacial acetic acid and evaporated to dryness under an N_2 stream. Enzymatic hydrolysis was performed with 2300 U of *H. pomatia* β-glucuronidase/sulfatase dissolved in 3 mL of 0.05 M Na-acetate buffer (pH 5). The mixture of freshly prepared enzyme solution and dried supernatant was incubated for 18 h at 37 °C with slow magnetic stirring. After enzymatic hydrolysis the extract was used for determination of lignan and total phenolic content, and DPPH radical scavenging assay.

Determination of Lignan Content.

The enzymatic hydrolysate was applied to a preconditioned SPE cartridge (Varian, Bond Elut – Certify II, 50 mg, 3 mL). After washing the cartridge with Na-acetate buffer, the lignans were eluted with 3 mL of methanol. Methanol was evaporated to dryness. Lignans were quantified by GC-EC detection as described previously (Čukelj et al.). Prior to injection, the sample extracts were derivatized with dichloromethane, pyridine and pentafluoropropionic anhydride (PFPA) at 70 °C for 60 min. The dried samples were reconstituted in *n*-hexane and injected into the Agilent 6890 GC equipped with a ^{63}Ni Micro–ECD. The column used was Rtx-5ms (Restek, USA). The column temperature was raised from 120 °C to 290°C in total 29.5 min run. The injector temperature was 275 °C, detector temperature was 300 °C and the column head pressure was 88.0 kPa. Derivate (1 μL) was injected by the split injection technique (split ratio, 20:1).

Determination of Total Phenolic Content.

Total phenolic content (TPC) was determined spectrophotometrically (UNICAM Heλios β, England) according to Yu et al. (2002)[a], with some modifications. To a 150 μ L aliquot of the enzymatic hydrolysate, 500 μ L Folin–Ciocalteu's reagent, and 1.5 mL 20% Na_2CO_3 was added followed by adjusting the volume to 10 mL with distilled water. The reaction mixture was allowed to stand in dark at ambient temperature for 2 h. The absorbance was measured at 765 nm against a blank sample. Ferulic acid was used to construct standard curve and the results were expressed as mg of ferulic acid equivalents (FAE) per 100 g of sample. All measurements were performed in triplicate.

DPPH Radical Scavenging Assay.

The method described by Zhou ,Yu (2004) was used with modifications in order to assess the DPPH radical-scavenging capacity of cereal and flaxseed extracts. To 3 ml of 0.187 mM 2,2-diphenyl-1- picrylhydrazyl (DPPH) in methanol, 150 uL of enzymatic hydrolysate was added. The free radical scavenging capacity was evaluated by measuring the decrease of absorbance at 520 nm using a UV- Vis spectrophotometer (UNICAM Heλios β, England). The scavenging of DPPH was calculated according to the following equation:

$$\% \text{ scavenging} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

where $Abs_{control}$ and Abs_{sample} are the absorbance values recorded for the control and sample, respectively, after 30 min. In the control, the extract was replaced with an equal volume of Na-acetate buffer. Triplicate tests were conducted for each sample. The scavenging capacity was expressed as μ mole DPPH radical scavenged per 100 g of sample.

RESULTS AND DISCUSSION

GC-ECD method was used for the simultaneous determination of five different lignans in whole grain cereals and defatted flaxseed: secoisolariciresinol (SECO), lariciresinol (LARI), matairesiol (MATA), pinoresinol (PINO), and syringaresinol (SYR) (Table 1.). SECO was not detected in oat, LARI was not detected in rye and SYR was found not to exist in flaxseed. MATA was not detected in any of analyzed samples. The order of total lignan content was as follows: flaxseed (118266 ± 483 μ g/100 g), rye (568 ± 98 μ g/100 g), oat (473 ± 14 g/100 g), Žitarka wheat (444 ± 89 μ g/100 g), Divana wheat (293 ± 56 μ g/100 g), barley (239 ± 36 μ g/100 g), Sana wheat (225 ± 43 μ g/100 g). Previous study by Smeds et al. (2009) showed the total lignan content to be in range: 820 – 2550, 340 - 2270, 2500 – 6700 μ g/100 g in oat, wheat and rye samples, respectively. The difference between two studies can be explained by the other types of lignans analyzed and natural variations among species. Different varieties of wheat showed to contain different amount of lignans confirming the results of previous study on the importance on genotype for differences in lignan content (Smeds et al., 2009). As expected, flaxseed showed significantly higher content of total lignans compared to the cereals, but syringaresinol, the dominant lignan in rye, lacked in flaxseed.

Table 1. Concentrations of lignans (μ g/100 g) in cereal and defatted flaxseed extracts, expressed as mean values of two determinations ± standard deviation

	secoisolariciresinol	lariciresinol	pinoresinol	syringaresinol
Wheat Divana	35.24±3.01	80.88±21.92	53.82±4.86	122.73±29.13
Wheat Sana	21.68±2.53	60.26±10.35	45.17±3.09	97.92±17.76
Wheat Žitarka	78.90±20.53	145.70±37.00	100.49±7.46	119.03±23.90
Rye	21.90±2.38	nd	161.94±3.75	384.05±91.92
Barley	26.06±0.75	124.06±20.93	56.12±5.77	32.60±8.73
Oat	nd	155.14±4.77	290.00±11.46	27.64±1.79
Flaxseed	98677.23±6.06	698.93±39.43	18890.27±449.94	nd

* nd – not detected

The total phenolic content was analysed in extracts obtained by applying methanol extraction with parallel alkaline followed by enzymatic hydrolysis. When analysing TPC in cereals, it is necessary to free bound phenolics, since it has been proven they make more than 60% of all the phenolics (Adom, Liu, 2002). To extract bound phenolic compounds, which are associated with grain cell wall material, alkaline hydrolysis is usual method applied (Adom, Liu, 2002; Siebenhdal et al., 2007). It is considered that the phenolic compounds contribute to overall antioxidant activity. In this study antioxidant activity of whole grain and cereals was evaluated with the widely used DPPH radical. DPPH is used to evaluate the free radical scavenging activity of hydrogen donating antioxidants in many plant extracts (Choi et al., 2007). TPC and DPPH radical scavenging capacity are presented in Figure 1. TPC was similar in all analysed cereals, ranging from 256 ± 2.4 to 408 ± 3.6 mg /100 g of grain, while for flaxseed it was 1286 ±146.8 mg/100 g. In accordance to TPC, DPPH scavenging capacity of flaxseed was more than 2.5 times higher than oat, which had the highest radical scavenging capacity among cereals. As same as for the lignan content, rye showed to have highest TPC content within cereal group, but antioxidant activity was the lowest. On the other hand, oat and barley had the lowest TPC content, but highest DPPH scavenging capacity. This indicates that the presence of other compounds in oat and barley could contribute to its antioxidant activity. No correlation between lignan content and free

radical scavenging activity was observed in this study, although flaxseed possesses highest values of the all three measured parameters.

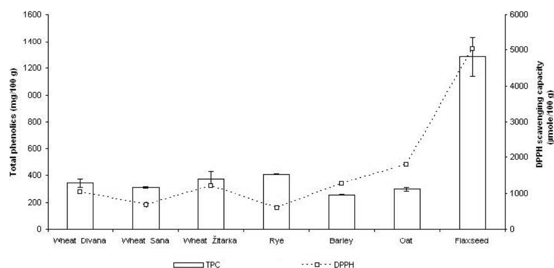

Figure 1. Total phenolic content (TPC; mg FAE/100 g) and DPPH radical scavenging capacity (µ mole/100 g) of whole grain cereal and flaxseed extracts. Values are means of three determinations ± standard deviation.

TPC detected in this study is in accordance with the study of Siebenhdal et al. (2007), where the TPC obtained by alkaline hydrolysis in wholemeal purple wheat was found to be 197 mg/100 g. The results are not comparable with other studies where the TPC values are expressed as gallic acid equivalents (Adom, Liu, 2002; Yu et al., 2002[a]; Zhou, Yu, 2004; Kim et al., 2006) or did not take bound phenolics into account (Liyana-Pathirana, Shahidi, 2007). To evaluate cereal antioxidant activity different assays or testing conditions were previously used, e.g. TOSC assay (Adom, Liu, 2007), ABTS scavenging activity (Zhou, Yu, 2004, Ragaee et al., 2006), ORAC assay (Zhou, Yu, 2004), Fe^{2+} chelating activity (Yu et al., 2002[b]). DPPH was used to evaluate cereal radical scavenging capacity in only few works. Liyana-Pathirana, Shahidi (2007) showed whole wheat extract to scavenge 21000 –21800 µ mole of DPPH/100 g of wheat whole grain. Those results are about 20 times higher than our values for wheat, but are measured after 60 min of assay. Ragaee et al. (2006) showed DPPH scavenging capacity (after 10 min reaction) for hard and soft wheat, barley and rye to be 433, 417, 2100 and 1217 µ mole/100 g, respectively. The discrepancy between results could be result of different testing conditions as well as already mentioned differences between the varieties.

CONCLUSIONS

The findings of this study confirmed flaxseed superior position regarding lignan, total phenolic content and antioxidant activity compared to some mostly used cereals (wheat, oat, rye, barley). Although our results are not fully comparable to previous studies due to the fact that we examined TPC and DPPH assay on extracts obtained by both alkaline and enzymatic hydrolysis, by method primarily used for extraction of lignans from cereals and flaxseed, it can be clearly seen that flaxseed exhibits around 200 times higher total lignan content, 3 times higher total phenol content and more than 2.5 times stronger DPPH scavenging activity compared to whole grain wheat, rye, barley and oat.

Keeping in mind the necessity for increasing food sources rich in bioactive components in everyday diet, cereal and bakery products are being enriched in order to provide health benefits beyond their natural provision. Flaxseed is emerging as functional ingredient being one of the most important sources of phytochemicals among plant foods. Whole grain products already have the potential to enhance quality nutritional intake, but enrichment with flaxseed could lift those product on even higher level making them functional food. Further studies should examine the limits of flaxseed use, taking into account the flaxseed laxative properties, content of cyanogenic glucosides and influence on product textural and sensory properties.

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SAMPLE DISCRIMINATION OF MANDARIN CULTIVARS AND PRODUCTS USING NIR DATA CHEMOMETRIC ANALYSIS

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ABSTRACT

The potential of near-infrared (NIR) spectroscopy for their ability to differentiate between mandarins' cultivar and different products was evaluated.

The non-destructive analysis was conducted using a NIR spectrometer in which the radiation reflected by the fruit products was performed in the near-infrared range from 904 - 1699 nm. Fruit products as juice, marmalade and candied peel from four cultivars, two of mandarins (Citrus unshiu Marcovitch Saigon, and Owari), and two of clementine (Citrus clementine Hernandina and Corsica SRA 63) were analysed. The study was focused on investigation of the possibility of potential distinction of mandarin products produced from different cultivars based on NIR spectrums.

The spectra of fruit products were measured by diffuse trans-reflectance and raw spectra and their second derivative were used. The chemometric procedures applied to the NIR data were partial least squares regression (for discrimination on the basis of different products, and cultivar differentiation) and linear discriminant analysis (LDA) applied to principal component (PC) scores. First two components, PC1 and PC2 gave the highest level of products classification (98%) and fruit cultivars (94.7%), respectively.

The results clearly show the potential for the application of near-infrared spectroscopy for a non- destructive detection of different mandarin cultivars and their final products.

Analyses of the spectrums reveals changes in specific wavelengths of 1387 and 1616 nm which correspond to C–H combinations and the 1-st overtone of the bonds that indicates the advisability of use of NIR for on-line monitoring of product quality and selection. This study showed that near infrared spectroscopy has potential to distinguish mandarin cultivars and their products as well as from which mandarin cultivars it was produced. The finding based on the chemometric methods used in this research, could potentially be utilized to develop a protocol to detect different mandarin cultivars and origin of products in view of cultivar.

KEYWORDS: *NIR; mandarins; chemometrics*

INTRODUCTION

Consumers growing interest in the safety and traceability of food products is a challenge for the development of methods for proving and detection. Methods that require pre-treatments of the sample also must include highly-skilled personnel what is the main reason why fast and non- destructive methods are more and more in use (Oliveri et al., 2011). Among them, near infrared spectroscopy has proven to be a successful analytical method for analysis of a variety of food products (Nicolaï et al., 2007; Reid et al., 2005; Sirisomboon et al., 2007). NIR region (750 – 2500 nm) is very useful spectral fingerprint of food samples in which vibration and overtone combinations of basic bonds (O–H, C–H and N–H) are the main recordable phenomena (Williams et al., 2001; Oliveri et al., 2011). In the food industry, NIR measurements are used in measurement (Hernández Gómez et al., 2006; Liu et al., 2010) evaluation of quality (Sirisomboon et al, 2007), quality prediction (Cayuela et al., 2010), and differentiation of samples (Reid et al, 2005).

In Croatia mandarin is a very popular fruit. Mandarin as other Citrus fruit has nutritional importance due to their particular composition (Mouly et al., 1998; Wang et al., 2008). The Clementine mandarin is another group of high importance and recently is becoming one of the most important mandarins in the world

(Rodrigo and Zacarías, 2006). Mandarines and clementines mandarins are mostly consumed as fresh, but they can be also processed, mostly in the juices and jam and less in the candied peel. In order to protect the consumer, qualitative analysis of the products must be used.

In this paper, the potential of near-infrared (NIR) spectroscopy for their ability to differentiate between mandarins' cultivar and different products (juice, marmalade and candied peel) was evaluated.

MATERIALS AND METHODS

The non-destructive analysis was conducted using a NIR spectrometer in which the radiation reflected by the fruit products was performed in the near-infrared range from 904 - 1699 nm. Fruit products as juice, marmalade and candied peel were analysed from 2 cultivars of mandarins (Citrus Unshiu Marcovitch cv. Saigon and Ovari) and 2 of clementine (Citrus clementina cv. Corsica SRA 63 and Hernandina), purchased at local market in Zagreb (Croatia).

NIR spectra of fruit products were collected in the range of 904 - 1699 nm using a NIR-128-1.7-USB/6.25/50µ m (Control Development, Inc., USA), with installed software Spec32. Each product was recorded in triplicate and their mean value was calculated. The spectra of fruit products were measured by diffuse trans-reflectance and raw spectra and their second derivative were used. NIR spectroscopy is based on the electromagnetic absorption at the near-infrared region but the spectral analysis has to be assisted with various chemometric techniques (Ding & Xu, 1999; Alishahi et al., 2010). Data were analysed using program Statistica v. 8., using principle component analysis (PCA) in order to identify patterns in experimental data and to express the data based on their similarities and differences.

Table 1. Abbreviations used for different products from 4 cultivars of mandarins

cultivar	abbreviation
Citrus Unshiu Marcovitch cv.Saigon	
juice	s_juice
marmalade	s_marm
candied peal	s_cand
Citrus Unshiu Marcovitch cv. Ovari	
juice	o_juice
marmalade	o_marm
candied peal	o_cand
Citrus Clementine Hernandina	
juice	h_juice
marmalade	h_marm
candied peal	h_cand
Citrus Clementine Corsica SRA 63	
juice	c_juice
marmalade	c_marm
candied peal	c_cand

RESULTS AND DISCUSSION

Using NIR process analyzer, each spectrum of different products (juice, marmalade and candied peal)

for 4 cultivars of mandarins was collected. In Fig. 1 are presented mean samples of NIR spectrums (absorbency) for one product (juice) for all 4 cultivars as well as three different products of one cultivar, Citrus Clementine cv. Hernandina.

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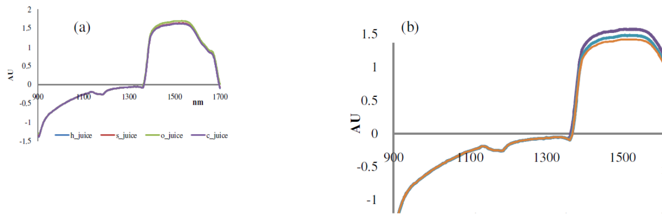

Figure 1. NIR spectra presented with absorbance units, of (a) one product (juice) made from different cultivars of mandarins and (b) different products (juice, marmalade and candid peel) for one cultivar (Citrus Clementine Hernandina)

Analyzing presented spectres in Fig. 1., it seems that all records are very similar with an exception in the range 1400 - 1700 nm, where the height of the curves show slight difference. This observation justifies use of chemometrics in order to detect differences between observed samples.

The chemometric procedures applied to the NIR data were partial least squares regression (for discrimination on the basis of different products, and cultivar differentiation) and linear discriminant analysis (LDA) applied to principal component (PC) scores. The differentiation based on chemometry is presented with Figures 2. and 3.

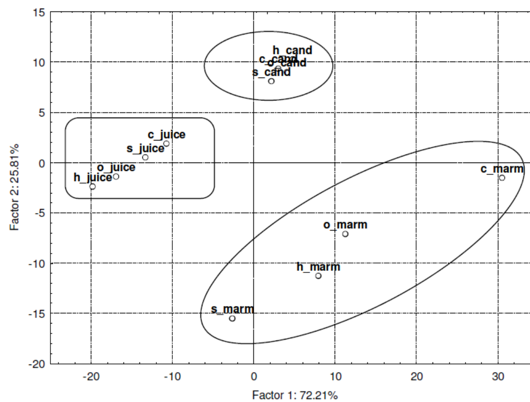

Figure 2. Plot for the first two PC factors, based on spectral data used in differentiation of products made from different cultivars.

From results presented with Fig. 2., can be concluded that the chemometric approach using principal component analysis (PCA) was effective because the data have split into 3 different sets and the sets correspond to observed products (juice; marmalade; candied peel). The PCA scores results indicated discernible differences on the basis of products using the first two PCA scores, where the first two components (PC1 and PC2) gave a high level of classification of mandarins product (98%) made from different cultivars.

In order to test the hypothesis that the samples can also be separated due to the NIR spectra of the cultivar, the PCA scores plot was obtained from the NIR analysis of one product (juice) made from different cultivars (Fig. 3.). In the analysis, two spectrums for each cultivar were analysed. As shown, the differentiation was possible and also effective as the differentiation of the products shown on previous figure. In Fig 3., the first two components (PC1 and PC2) gave the highest level of products classification (99.21%) based on different fruit cultivars, what implies that using the NIR spectra and multivariate exploratory techniques as PCA, it can be detected from which cultivar was produced the observed product, in this case, the juice.

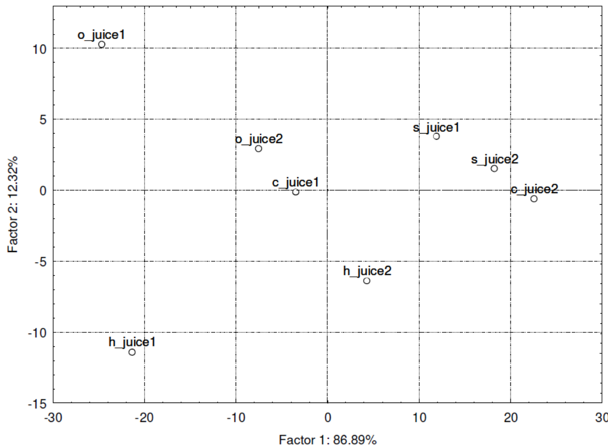

Figure 3. Plot for the first two PC factors, based on spectral data used in differentiation of one product (juice)made from different cultivars.

The results clearly show the potential for the application of near-infrared spectroscopy for a non- destructive detection of different mandarin cultivars and especially in detection of the cultivars in the final products.

Presented results and analyses of the spectrums indicated changes in specific wavelengths of 1387 and 1616 nm (6188 and 7210 cm^{-1}) which corresponds to the C–H combinations and the 1-st overtone of the bonds that indicates the advisability of use of NIR for on-line monitoring of product quality and selection (Alishahi et al., 2010; Liu et al., 2010; Nicolaï et al., 2007). Other authors like Hernández Gómez and co-workers (2006) have investigated mandarins and use of NIR analysis in order to select harvested fruit or to differentiate juice samples as Reid and his co-workers (2005) have done. This paper shows that also other approaches using NIR spectra in product and fruit cultivars can be made.

CONCLUSION

Analyses of the spectrums reveals changes in specific wavelengths of 1387 and 1616 nm which correspond to C–H combinations and the 1-st overtone of the bonds that indicates the advisability of use of NIR for on-line monitoring of product quality and selection. This study showed that near infrared spectroscopy has potential to distinguish mandarin cultivars and their products as well as from which mandarin cultivars it was produced. The finding based on the chemometric methods used in this research, could potentially be utilized to develop a protocol to detect different mandarin cultivars and origin of products in view of cultivar.

The results clearly show the potential for the application of near-infrared spectroscopy for a non- destructive detection of different mandarin cultivars and their final products.

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ESTABLISHMENT OF A PROCESS FOR THE RECOVERY OF A NATURAL YELLOW COLORANT FROM OPUNTIA FRUITS

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ABSTRACT

Cactaceae is considered the most promising family among betalain-bearing plants to be used as source of betaxanthins, water-soluble betalain pigments responsible of the yellow-orange colour in cactus pear fruits. Traditionally, betaxanthins have been defined as condensation products of betalamic acid with different aminoacids. This paper focuses on to develop a processing scheme, paying special attention to the extraction procedure, to obtain a water-soluble natural yellow colorant from *Opuntia* fruits for application in food. Edible yellow *Opuntia* fruits grown in Murcia (Spain) are homogenized and extracted with ethanol, water, and ethanol:water (60:40) solvents. Pigment extract is chemically characterized and individual pigments are identified by chromatography, UV-Vis spectrophotometry, and electrospray ionization mass spectroscopy. A sequential extraction procedure was established being homogenization, stirring, centrifugation, filtration and concentration the main stages. The highest betaxanthin content (210 mg / kg fresh weight) is obtained for the hydroalcoholic extract, and moreover this solvent minimized the presence of mucilage and pectins, which are substances not desired in colorant extracts. A concentrated extract is obtained under rotary evaporation at 35ºC and reduced pressure (6 kPa), with a betaxanthin concentration of 4.40 g / kg fresh weight. The individual pigment analysis by RP-HPLC with photodiode array and mass-spectral detection revealed that proline-betaxanthin (indicaxanthin) is clearly dominant, while all other betaxanthins were present in comparatively low quantities. The pigment stability is checked at 4 and 25ºC, and in the degradation pattern first-order kinetics are observed.

KEYWORDS: *Opuntia; betalains; betaxanthins; natural food colorant*

INTRODUCTION

Every food designer knows that consumers judge a product not only on its flavour, but on its appearance as well. Colour mainly defines the aesthetic value of food, predetermines consumer´s expectation and modulates appetite (Bayarri et al., 2001). One important class of ingredients exists solely to enhance the appearance of what we eat: food colours. Colour is probably the most important factor in the acceptance of food products because it is used as an indicator of quality. In the last years synthetic colorants are increasingly being perceived as undesirable or harmful by consumers (Downham and Collins, 2000) and there is growing interest in the development of natural colorants for use in the food industry, which has been encouraged by a strong consumer demand for natural products. Natural colours are commonly used in the food industry and this natural trend has become of increasing importance among consumers. Food and drink manufacturers are needed of a full range of natural colours to suit all applications, and this demand provides the opportunity for innovation and research in new sources of natural colorants (Adam-Burrows, 2009).

Opuntia fruits are widely consumed in Central and Southern America, Italy, Spain, Israel, Egypt and South Africa. These plants may be found as spontaneous vegetation, although cultivated cactus pears also reach the market. The main interest in *Opuntia* fruits is attributed to the betalains they content due to the strong demand of the food industry for colorants obtained from natural sources (Stintzing et al., 2002; Castellar et al., 2003). Some authors have also recently reported that these pigments, which are of a polyphenolic nature, possess antioxidant activity (Butera et al., 2002), and taking into account the strong demand of consumers for naturally derived products, associated with an image of health and quality, these fruits should be taken also in consideration.

Betalains are characteristic plant pigments of the order Caryophyllales. They can be divided into two structural groups, the yellow betaxanthins and the red-purple betacyanins, both of which have as their basic structure betalamic acid, whose chromophore is a 1,7–diazaheptamethinium system (Piatelli, 1976), while they differ mainly in the radicals bonded to the main structure. Their colour is due to the conjugation of a substituted aromatic nucleus to the diaza system which shifts the absorption maximum from around 535 nm in betacyanins to near 480 nm in betaxanthins. In cactus pear (*Opuntia* sp.) these pigments are responsible of the purple, red, orange and yellow colours. Betalains are recognized as natural food colorants (Wissgott and Bortlik, 1996) and in contrast to other natural pigments, their appearance is maintained over a wide pH range from 4 to 7. This property makes them ideal pigments for colouring low acid foodstuff. Untill now, only red beet (*Beta vulgaris*) has been used as a source of betalains and other sources have not been considered for industrial use, despite the fact that several studies have been made concerning their presence and their stability in *Opuntia* fruits (Castellar et al., 2006). Recently there has been increasing interest in betalains since some studies have pointed to their possible antioxidant effects (Fernández-López et al., 2010), and taking into account that antioxidants are an increasingly important ingredient in food processing, this would enhance the added commercial value of *Opuntia* fruits. The aim of this work was to obtain a concentrated extract of betaxanthins from *Opuntia* fruits to be used as natural food colorant. A processing scheme was proposed paying special attention to the extraction procedure and to the chemical characterization of the concentrated extract.

MATERIALS & METHODS

Plant Material.

Cactus pear fruits (*Opuntia ficus-indica* L.) yellow-skinned, were harvested in Murcia (Spain) when characteristic mature skin colour became manifest. The fruits were hand-picked, separated into peel and pulp tissues, weighed, homogenized in a T25 basic ultra-turrax (Ika®-Werke, Staufen, Germany), frozen and stored at -25ºC until use.

Chemicals.

Acetonitrile and ethanol were of HPLC grade and were purchased from Lab Scan (Dublin, Ireland). Water was purified in a Milli-Q water purification system from Millipore (Bedford, MA, USA). All other chemicals employed were of analytical grade.

Pigment extraction.

The homogenized pulp was magnetically stirred for 20 min in darkness using a 1/5 (w/v) ratio of fruit/solvent. Water, ethanol and ethanol:water (60:40 v/v) were used as solvents. After stirring, the samples were centrifuged at 15000g at 10ºC for 10 min in a Z383K Hermle centrifuge (Wehingen, Germany) to remove the vegetal tissue residue. Supernatants were concentrated using a vacuum Büchi Rotavapor R-200 (Flawil, Switzerland). Temperature was controlled at 30ºC and vacuum at 6 kPa. The ethanol was completely removed after the concentration process.

Spectrophotometric analysis.

The visible spectra (380-780 nm) of the extracts were recorded using an Agilent 8453 UV-visible spectrophotometer (Waldbronn, Germany). The total betaxanthin content, was referred to indicaxanthin (molecular mass = 308 g mol^{-1}) applying a mean molecular extinction coefficient (ε = 48,000 L mol^{-1} cm^{-1}) (Kugler et al., 2007). Concentration of individuals betaxanthins were calculated by multiplying the spectrophotometrically assessed value with the relative chromatogram area of the particular betaxanthin at 470 nm.

Chemical analysis.

The soluble solid content (ºBrix) was measured using a Zeiss Opton (Japan) refractometer at 20ºC. Acidity was determined by titration with 0.1N NaOH and expressed as meq kg^{-1}. The pH was measured with a Crison micropH200 pH meter (Madrid, Spain). Viscosity (cP) was determined in a Canon-Fenske viscosimeter. The water content was determined using a Karl Fischer titrator, Mettler, DL38 (Toledo, Spain). All measurements were performed in triplicate.

HPLC analysis.

The pigment analyses were performed in a Waters modular liquid chromatographic system (Waters, Milford, MA, USA) equipped with two M510 pumps, a 717 plus autosampler and a M996 photodiode array detector (PDA). HPLC was run by Millenium 2010 Chromatography Manager data system. An Atlantis dC_{18} 5 µm, 25 cm x 4.6 mm i.d. column (Waters, Milford, MA, USA) was used. Elution was carried out following the method previously proposed (Fernández-López and Almela, 2001), using a gradient between 175 mM acetic acid in water and 175 mM acetic acid in acetonitrile as mobile phase. The flow rate was 1 ml min^{-1}. Betaxanthins were monitored at 470 nm.

Betaxanthin stability.

Storage stability studies of the concentrated extract were performed at 25 and 4ºC. Samples were withdrawn at different time intervals and spectrophotometrically analyzed. Betaxanthin contents were determined in triplicate for each sample. The storage stability was expressed in terms of half-life time ($t_{1/2}$). Half-life time values were determined by calculating the ratio of the betaxanthin content in the initial sample (B_0) and in the samples maintained at 4 or 25ºC during time (B_t). The natural logarithm of ratio B_0/B_t was plotted against the time (days) of storage. The slope of the graph through the origin obtained by connecting the data points was equated with k, from which the half-life values ($t_{1/2} = \ln 2/k$) were deduced.

Colour measurements.

Based on absorption measurements covering the range from 380 to 780 nm, objective colour (CIELab) was calculated. Chromaticity (C^*) [$C^* = (a^{*2} + b^{*2})^{1/2}$] and hue angle ($h^o$) [$h^o = \arctan(b^*/a^*)$] were calculated from a^*- and b^*-values at D_{65} and an observer angle of 10º. The hue angles were expressed on a 360º-colour wheel where 0º/360º presents purplish-red, 90º yellow, 180º bluish-green, and 270º blue.

RESULTS & DISCUSSION

The design of a new industrial process requires an appropriate approach to consider the different aspects from which to assess the situation, this is a key factor in achieving success. We are working with fruits, so the first steps of the processing scheme are going to imply the selection, classification, washing and conditioning (glochid elimination and pulp separation) of the *Opuntia* fruits. Now, the plant material is ready to undertake the pigment extraction. Initially, the pulp is homogenized at high speed in a blender and before the addition of the solvent, the mixture is magnetically stirred to facilitate the pigment extraction to the liquid phase. Three different solvents were used: water, ethanol and ethanol-water 60:40 (V/V). The selection of the more appropriate solvent is very important in the process in order to obtain a highly-pigmented extract minimizing the presence of pectins and mucilage, which are in a high concentration in cactus pear fruits. By means of centrifugation and subsequent filtration the plant residue, mainly composed of pectins, cellulose and mucilage is discarded, and a clarified pigment extract is obtained. Table 1 shows the main chemical characteristics of the extracts depending on the solvent used. The pH value was around 7.0 in the three extracts, similar to other pH values (5.3-7.1) reported for *Opuntia ficus-indica* fruits (Saénz, 2000), confirming *Opuntia* fruits as low-acid food. The refractive index, which depends on the presence of sugars, showed low variability between extracts, and density ranged from 0.83 to 1.00 showing the lowest value for the ethanolic extract. As regards the pigment content, the hydroalcoholic solvent extracted the highest level of betaxanthins (210.12 mg/kg fresh fruit) and so, this solvent was chosen as the most efficient because it can recover the pigments completely from the pulp without co-extracting polysaccharides and other alcohol-insoluble solids. This simplifies the further purification of the pigment extract. This level of betaxanthins is higher than those reported for Italian cactus pears (Butera et al., 2002) and it is similar than content found in some Mexican cultivars (Castellanos-Santiago and Yahia, 2008).

Table 1. Chemical characteristics of the pigment extractsa

Extraction solvent	pH	Refractive index	Density (g/mL)	Betaxanthins (mg indicaxanthin / kg fresh fruit)
Water	6.89 ± 0.03	1.371 ± 0.002	1.00 ± 0.01	181.93 ± 4.24
Ethanol	7.48 ± 0.04	1.371 ± 0.001	0.85 ± 0.02	172.50 ± 7.38
Ethanol:Water (60:40 V/V)	7.29 ± 0.04	1.363 ± 0.001	0.93 ± 0.02	210.12 ± 6.32

[a]Each value is the mean ± SD of three determinations

In order to obtain a concentrated betaxanthin extract to be used as commercial food colorant, a 21-fold concentrated extract was obtained under rotary evaporation at 35°C and reduced pressure (6 kPa). It is important to offer a concentrated product to reduce water activity, thus increasing the stability of the pigments, minimizing the risk of microbial contamination and extending shelf life of the colorant. Also, transport and storage are made easier. Table 2 summarizes the results of analyses performed on the concentrated betaxanthin extract. It is remarkable the high colour strength, reported as the absorbance at 480 nm of a 100-fold diluted extract. The titratable acidity (0.22 g $\times L^{-1}$) is lower than that reported for *Opuntia ficus-indica* concentrated juice, which ranged between 7.4 and 8.1 g $\times L^{-1}$ (Saénz et al., 1998; Sáenz, 2000). The density (1.04 g $\times mL^{-1}$) was lower than previously reported for concentrated juice (1.29 g $\times mL^{-1}$) (Sáenz et al., 1998). These differences could be attributed to the use of ethanol in the extraction solvent, which leads to cutting down sugars in the extract.

Table 2. Chemical characterization of the concentrated betaxanthin extract from Opuntia fruitsa

Parameter	Value
pH	5.83 ± 0.03
Titratable acidity (*g citric acid* $\cdot L^{-1}$)	0.22 ± 0.05
Density ($g \cdot mL^{-1}$)	1.04 ± 0.04
Colour strength (*OD 480 nm, 1% V/V sol.*)	0.18 ± 0.02
Betaxanthin concentration (*g indicaxanthin* $\cdot L^{-1}$)	0.27 ± 0.01

[a]Each value is the mean ± SD of three determinations

At this point, a natural colorant extract has been obtained. The flow diagram describing the overall extraction process, from the raw material till obtaining the colorant extract, is depicted in Figure 1. The extraction process comprises the following stages: fruit conditioning, broken down into selection, classification, washing, glochid elimination and pulp separation; after that, pigment extraction is performed.

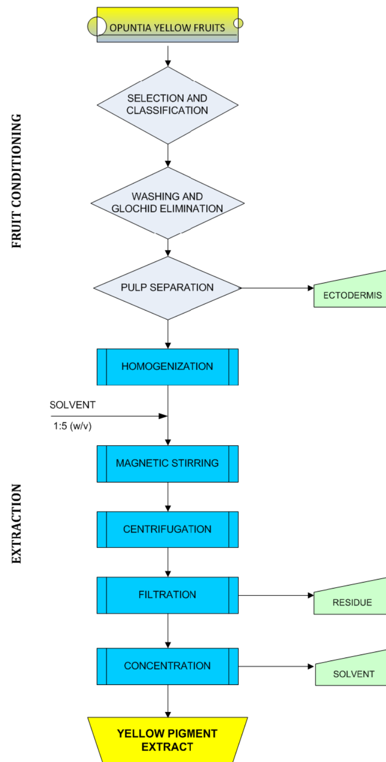

Figure 1. Flowchart representing the extraction process

The by-products from betaxanthin extraction include the ectodermis of *Opuntia* fruits, the pellet resulting after centrifugation of the pigment extract, and the ethanol-water mixture obtained after the rotary evaporation of the initial pigment extract. The ectodermis constitutes the 42-48 % of the whole fruit, and the main compounds in the ectodermis include cellulose, glucose and proteins. The ectodermis presents a potential use as biosorbent, which could serve as a cost effective means of treating effluents charged with toxic heavy metals (Barrera et al., 2006). The pellet resulting of centrifugation represents a 10-12 % of the pulp extracted and it is mainly constituted by pectins, mucilage and the seeds typical of these fruits. The solvent obtained in the concentration process can be recycled in order to reduce ethanol purchases, to shrink the volume of disposable waste and to decrease waste treatment costs.

The HPLC analysis of the betaxanthin extract (Fig. 2) revealed the presence of a mean peak, with a maximum absorbance at 479 nm and a *m/z* ratio $[M+H^+]=309$, which was identified as indicaxanthin 41(proline-betaxanthin). All other betaxanthins were present in comparatively low quantities.

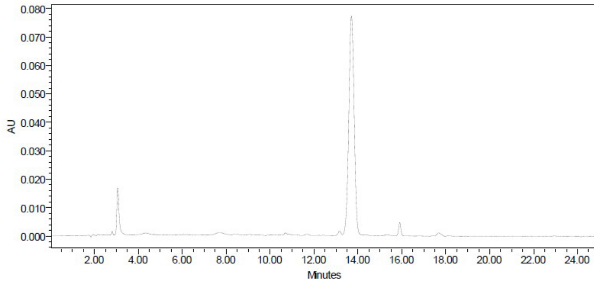

Figure 2. Chromatogram (479 nm) obtained for the HPLC analysis of the pigment extract from *Opuntia* fruits and chemical structure for indicaxanthin (t_R=13.8 min)

To check the storage stability, two assays were performed at 4 and 25ºC, respectively. Fig. 3 shows the behaviour of betaxanthin content. First order kinetics were observed, with half-life time values (t1/2) of 80 and 15 days at 4 and 25ºC, respectively. These data are lower than those of betacyanin extracts in the same storage conditions (Castellar et al., 2006).

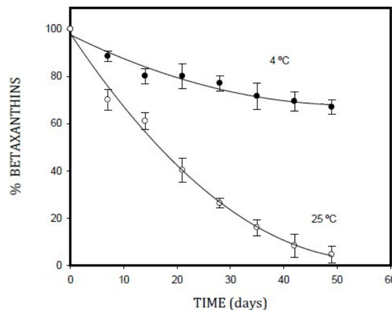

Figure 3. Storage stability of the yellow pigment extract from *Opuntia* fruits

With the CIELab parameters, the colour of plant extracts can be measured precisely, determined unambiguously, and differences to a colour standard can be quantified. Thus, CIELab colour measurements are used extensively in the food industry and are gaining greater use as descriptors for colour specification in food manufacturers. The results of the colour measurements (CIELab parameters) for the pigment extracts are shown in Table 3. It is remarkable the high lightness (L*) in both of them, the similar value of the hue angle (hº), while the chromaticity (C*) was lower in the concentrated extract.

Table 3. Yellow-pigment extracts colour analysis

Colour parameter	Initial pigment extract	Concentrated pigment extract
a*	5.13	-0.78
b*	96.54	68.49
L*	88.24	92.72
C*	96.67	68.49
h°	3.04	-0.66

CONCLUSION

Opuntia fruits can be used as source of betaxanthins, natural water-soluble yellow pigments. In this paper, a simple and reliable betaxanthin extraction process is established, which provides a concentrated natural yellow extract of high colour strength. HPLC analysis of the pigment extract revealed only one major betaxanthin identified as indicaxanthin. It offers new opportunities for the use of betaxanthins as a natural food colorant.

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USE OF CHEMOMETRIC ANALYSIS OF NIRS DATA AND ANTIOXIDANT CAPACITY DURING THE PRODUCTION OF WINE PROŠEK FROM CV. POŠIP

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ABSTRACT

Near infrared spectroscopy (NIRS) is a fast and non-destructive analytical method. In association with chemometric modelling, it becomes a powerful tool for application in food industry. In this work was applied for analysis of dessert wine Prošek produced by semi scaled fermentation from dried grapes of cv. Pošip. Samples of raw material and dessert wine Prošek during different stages of production were analysed.

NIR spectrums and the antioxidant capacity of the samples beginning from the grape must till the Prošek aged for 2 years were observed. In this paper was used the range of NIR (899–1699 nm, respectively 8495–4495 cm^{-1}) mainly observing vibrations of C–H, O–H, S–H and N–H bonds. In the evaluation of antioxidative capacity of the samples, the Briggs–Rauscher reaction was used as a method based on the inhibitory effects of antioxidants, which is applied near the pH of the fluids in the stomach (pH ≈2).

The model allows further discrimination between samples according to the fermentation stages and maturity of the wine. The main features of the spectra are absorption bands at 1610 and 1670 nm which are related to the first overtone of the C–H stretch and 917 nm which are related to the 3 C–H overtone and 2 overtone of the O–H stretch of H_2O and a combination of stretch and determination of the O–H group in H_2O and ethanol, respectively. PC1 and PC2, the first two components, gave the highest level of classification (96%) based on the wine ageing stage and the content of antioxidant components.

NIR, as a non-destructive method, has shown the potential for determining antioxidative capacity in must and dessert wine. This paper has presented that NIRS with chemometric analysis is a useful tool for quality control and on-line applications. NIRS can be used qualitatively to detect, to identify, and to qualify raw materials-grape and to control final products, Prošek.

KEYWORDS: *dessert wine; antioxidant activity, BR reaction; NIRs; chemometrics*

INTRODUCTION

Wine is an important component in Mediterranean dietary tradition and is considered as an important health benefactor since it is rich with antioxidant compounds. The antioxidant capacity of wine (related with phenolic compounds) depends on the grape variety, vineyard location, cultivation system, climate, soil type, vine cultivation practices, harvesting time, production process and ageing (Shahidi and Naczk, 1995) and grape maturity (Le Moigne et al., 2008). Epidemiology studies have observed that a diet rich in polyphenolic compounds may provide a positive effect due to their antioxidant properties (Frankel, Waterhouse, and Teissedre, 1995; Hertog, et al., 1993) resulting with bether wellbeing (Fernàndez-Pachòs et al., 2004; Landrault et al., 2001). Höner and Cervellati (2002) and Höner and co-workers (2002a) reported a new method for measuring the antioxidant activit y based on the inhibitory effect of antioxidants on the Briggs-Rauscher reaction (BR). This oscillation reaction is interesting because of the pH of the mixture (pH ≈ 2) which is similar to the pH of human digestion in the stomach. An interesting source of antioxidants is wine. The most famous dessert wine in Croatia, traditionall y produced in Dalmatia, is Prošek. Prošek is mostly consumed as appetitive or with a dessert what is also a positive health issue. The high sugar content of Prošek is achieved naturally from dried grape pomace. Wines with high sugar content are particularl y appreciated by the consumers for their sweetness, smooth mouthfeel and characteristic flavour.

Over recent years, consumers have become increasingly interested of the consumed uptake on human and environmental health (Forbes et al., 2009). Next consumer interest is the authenticity of foods (Lesschaeve and Noble, 2005) and, in particular, of wine what has been extensively investigated because wine is an easily adulterated product due to its strong chemical basis (high alcohol content, low pH) and its availability throughout the world (Moret et al., 1994). To insure quality and safe product for the costumer, new methods are proposed, among them is also use of near infrared analysis (Müller and Steinhart, 2007; Parpinello et al., 2009; Williams et al., 1988).

Near infrared region covers the range of electromagnetic spectrum between 780 and 2500 nm. The basic principle in near-infrared spectroscopy (NIRS) is that the examined sample is irradiated with NIR radiation and the reflected or transmitted radiation is recorded in the form of a spectrum (Nicolaï et al., 2007).

NIR-spectroscopy is suitable for simple matrices such as yoghurt or beverages to determine the content of macronutrient (protein, fat, carbohydrates). At least it is possible to control for specific production standards, so tedious reference methods need only be used if deviations from these quality standards occur during production (Azizian et al., 2004; Prevolnik et al., 2004). NIRS is a fast and non-destructive analytical method.

This paper has discusses the ability of NIRS in monitoring must, in production and in ageing of dessert Prošek wine. Samples of raw material and dessert wine Prošek during different stages of production were analysed. The samples beginning from the grape must till the Prošek aged for 2 years were observed analysing the antioxidant capacity using BR reaction and recording the NIR spectra of each grape, must or wine stage. In order to obtain the results that are based on multivariate analysis, this information needs to be as clear as possible. The inter-relationships between chemical properties and sensorial evaluation can provide reliable results (Williams et al., 1988; Bueno et al., 2010) that could be used in the production and market positioning of the wine (Parpinello et al., 2009; Charters & Pettigrew, 2007).

MATERIALS AND METHODS

Dessert wine Prošek, was produced by semi scaled fermentation from dried grapes of cv. Pošip in the harvest of 2008. 490 kg of grapes (27°Brix) of cv. Pošip were picked from the vineyards of Korčula and put to dry in glasshouse. Grapes were dried at a temperature from 25 to 45 °C in the glasshouse for 5 days. Grape mass after drying was 323 kg (31°Brix). Dried grapes were crushed, destemmed and sulphited. After 4 hours of skin contact at 15 °C, the must was racked into 25-litre glass container. Must was inoculated with selected yeast (Lalvin EC1118, Saccharomyces cerevisiae var. bayanus, 40 g/hL, Danstar Ferment AG, Zng Switzerland and VIN 13, Anchor yeast) and two different alcoholic fermentation was done, each in triplicate. After one month Prošek was racked, sulphited and left to mature.

NIR analysis

Applied was the range of NIR (904–1699 nm, respectively 11062–5886 cm) what gives the possibility to observe vibrations of C–H, O–H, S–H and N–H bonds. The spectra of samples were measured by diffuse trans-reflectance and raw spectra and their second derivative were used. NIR spectroscopy is based on the electromagnetic absorption at the near-infrared region but the spectral analysis has to be assisted with various chemometric techniques (Ding & Xu, 1999; Alishahi et al., 2010). NIR spectra of grape, must and wine samples were collected in the range of 904–1699 nm using a Control Development, Inc., NIR-128-1.7-USB/6.25/50 μ m, with installed Control Development software Spec32.

Briggs-Rauscher reaction

In the evaluation of antioxidative capacity of the samples, the Briggs–Rauscher reaction was used as a method based on the inhibitory effects of antioxidants, which is applied near the pH of the fluids in the stomach (pH ≈ 2). BR mixture is prepared by mixing three colourless solutions: solution A was 8.6 % hydrogen peroxide; second solution (B) was prepared by dissolving 4.3 g potassium iodate in 100 ml distilled water with addition of 0.5 ml sulphuric acid; and the third solution (C) was a mixture of 1.5 g malonic acid, 0.4 g manganese (II) sulphate monohydrate and 0.1 g starch dissolved in 100 ml of distilled water (Shakhashiri,

1985; Gajdoš Kljusurić et al., 2005). All three solutions (A, B and C) in equal volume ratios are mixed at temperature of 25 ± 0.1 °C (Höner et al. 2002; Gajdoš Kljusurić et al., 2005). In 15ml BR mixture is added 1 ml sample containing antioxidants. The reaction is followed potentiometrically and the inhibition time (IT) is recorded. IT, or time of no oscillations, is proportional to concentration of antioxidant.

Data were analysed using program Statistica v. 8., using principle component analysis (PCA) in order to identify patterns in experimental data and to express the data based on their similarities and differences.

RESULTS AND DISCUSSION

Table 1. Average values for the inhibition time with corresponding standard deviations, for grape, must and dessert wine Prošek, from cv. Pošip in vintage 2008

No	Sample	Stage of production	Yeast inoculation	Inhibition time (s)
1	Grape	in the harvest	-	359.7 ± 13.4
2	Must	off-vine drying	-	651.3 ± 19.9
3	Wine Prošek	first racking	VIN13	657.1 ± 23.1
4	Wine Prošek	first racking	EC1118	660.4 ± 22.2
5	Wine Prošek	6 months ageing	VIN13	761.6 ± 25.4
6	Wine Prošek	6 months ageing	EC1118	778.2 ± 18.9
7	Wine Prošek	2 years of ageing	VIN13	905.2 ± 20.1
8	Wine Prošek	2 years of ageing	EC1118	931.9 ± 28.7

Table 1 presents results of evaluation of antioxidative capacity of the samples based on Briggs–Rauscher reaction in different stage of production and type of yeast inoculation to induce alcoholic fermentation. Höner et al. (2002; 2002a) have proven that the oscillating Briggs-Rauscher reaction is suitable as an analytical method to measure relative in vitro activities of white wines (2002a) as well as fruits and vegetables (2002). Numerous studies have shown a linear correlation between antioxidant activity and phenolic content (Di Majo et al., 2008; Frankel et al., 1995; Gajdoš Kljusurić et al., 2005; Höner et al., 2002 and 2002a; Landrault et al., 2001). According the results, the ageing of wine increases the inhibition time, what is an indication for incensement of the phenolic content and is also in accordance with previous studies conducted by Fernàndez-Pachò et al. (2004) and Forbes et al. (2009).

The content of alcohol and reducing sugar was also determined (data not presented). For wines Prošek, the content of alcohol ranged from 15.3-16.4 vol % and of reducing sugar ranged from 29.9 to 66.8 g/L.

Using NIR process analyzer, each spectrum of different grape, must and stage for Prošek wine from cv. Pošip was recorded. Each sample was recorded in triplicate and the mean value was calculated. In Fig. 1 are presented mean samples of NIR spectrums (1 derivative).

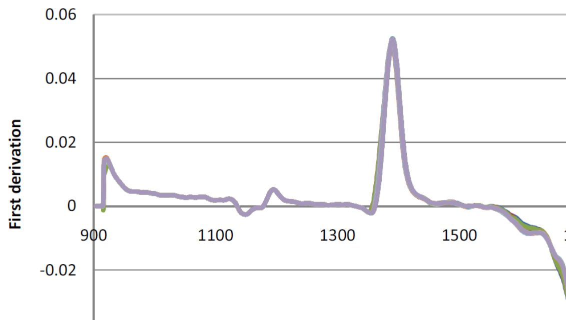

Figure 1. NIR absorbance spectra (1st derivative). of samples analysed during the production of dessert wine Prošek (cv. Pošip)

The model allows further discrimination between samples according to the production stages and maturity of wine. The main features of the spectra are absorption bands at 1610 and 1670 nm which are related to the first overtone of the CH stretch (Moret et al., 1994; Müller and Steinhart, 2007; Nicolaï et al., 2007) and 917 nm which are related to the 3rd CH overtone and 2nd overtone of the OH stretch of H_2O and a combination of stretch and determination of the OH group in H_2O and ethanol (Alishahi et al., 2010; Charters and Pettigrew, 2007), respectivel y. PC1 and PC2, the first two components, gave the highest level of classification (96%) based on the stage of production, yeast inoculation, and the content of antioxidant components.

Analyzing presented spectres in Fig. 1., it seems that all records are very similar with an exception in the range 1400-1700 nm, where the curves show slight differences. Presented results are in accordance with the study conducted by Cozzolino et al. (2004). The range of the used NIR instrument is significantly narrower then the instruments usually used (with a range from 400 nm to 2500 nm), but to ensure the data differentiation between the samples that represent all stage in the wine production, for the further analysis, to the set of spectrum data were assigned the values of the antioxidant capacit y of each sample (table 1). This observation justifies use of chemometrics in order to detect differences between observed samples.

The chemometric procedure was applied on the NIR and antioxidant data to accomplish the factor analysis (FA) and principal component (PC) scores. Factor analysis as a multivariate exploratory technique is used to describe variability among observed variables in terms of a potentially lower the number of observed variables (factors). Input data were NIR spectrums, content of alcohol, reducing sugar and antioxidant activity. The factor analysis has indicated that antioxidant activity, content of alcohol and reducing sugars positive correlate with NIR spectrum based on the loading factor (alcohol = 0.77; reducing sugar = 0.93 and antiox. activity = 0.82). But only the antioxidant activity has been placed in the factor (Factor 1) with the largest range of the spectrum (range for alcohol and reducing sugar from 1650-1687 nm and antiox. activity = 1637-1687 nm) what is an indication which data should be observed as input data for the next multivariate tool – principle component analysis.

The differentiation based on chemometry of the observed samples, including as input data filtered data set based on NIR spectrum and antioxidant activity of Prošek wines is presented with Figure 2.

Similar projection of the first two factors is gained when instead of antioxidant activity, the content of alcohol or reducing sugar was utilised as input variable including NIRs (not presented in the paper).

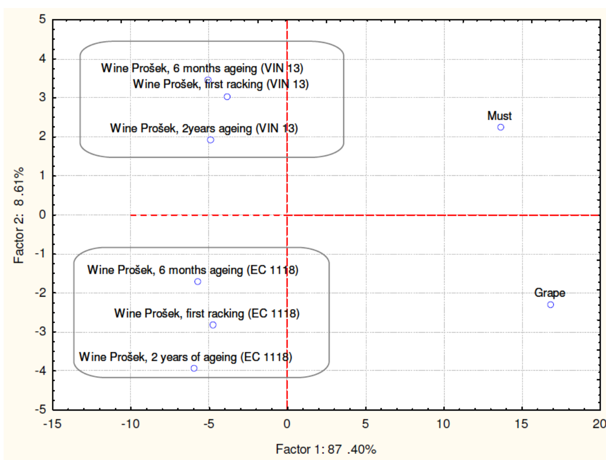

Figure 2. PCA used in differentiation of the product stage and type of yeast inoculation.

From results presented in Fig. 2., can be concluded that the chemometric approach using principal component anal ysis (PCA) was effective because the data have split toward the stage of the product, grape, must and wine (after the decantation and after 2 years) as well as according the yeasts added (VIN 13 and EC 1118). First two components (factors), gave the highest level of products classification (>96%) with the emphasis on the added yeasts.

The PCA score results indicated the highest loading around 1650 nm what has also shown the best

correlation with the antioxidant capacity what implies the possibility to predict the antioxidant activity what other authors (Cozzolino et al., 2010) have proven using NIR spectra in prediction phenolic compound in red wines. In summary, this work annotated the advisability of use of NIR for on-line monitoring of product quality and selection (Alishahi et al., 2010; Nicolaï et al., 2007). It has also demonstrated the potential of NIR spectroscopy for detection of the product stage as well as the possibility to use it in forecasting of antioxidant capacity based on the inhibition time (according the BR reaction).

CONCLUSION

The antioxidant activity measured using the oscillating Briggs-Rauscher reaction linearl y increases over time (wine ageing).

Near infrared spectroscopy showed promise as a rapid, non-destructive method in detection of the product stage, distinguishing grape from fermenting must and dessert Prošek wine as well as to detect the basic yeasts added.

Adding the data of the antioxidant capacit y to the NIR data, the chemometric approach has shown the potential for determining antioxidative capacity in fermenting must and dessert wine Prošek based on their NIR spectra with a special indication of the wavelength around 1655 nm. This paper has presented that NIRS with chemometric analysis is a useful tool for quality control and on-line applications. NIRS can be used qualitatively to detect, to identify, and to qualify raw materials-grape and to control final products, dessert wine Prošek.

ACKNOWLEDGEMENTS

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ANTIOXIDANT AND ANTI-MICROBIAL PROPERTIES OF CAMELLIA SINENSIS PRODUCTS

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ABSTRACT

Products based on *Camellia sinensis* plant include white, green, yellow, oolong and black tea. White and green tea present completely unfermented products, due to enzyme inactivation during production. Oolong and black tea are products in which deliberate oxidation of polyphenols is induced, while yellow tea presents the rarest and least investigated type of teas in which a slight oxidation might occur due to the lack of enzyme inactivation phase during production. All of these products present a very rich source of bioactive compounds, especially polyphenols, which impart positive health effects but there is a lack of data on their anti-microbial properties.

Bioactive composition (total phenols and flavonoids) of tested teas was determined spectrophotometrically. Additionally, antioxidant capacity was determined using three different assays (DPPH, ABTS, FRAP). Anti-microbial activity was tested on following test microorganisms: *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Candida albicans* 11 and *Salmonella* sp. 3064, by disc-diffusion and turbidimetric method on microtiter plates.

In total, nine different teas were tested in this study (white tea – Pai Mu Tan superior; yellow tea – Yin zhen; green tea – Matcha, Twinings, Gyokuro, Long Jing; oolong – Formosa Fine oolong; black tea – Twinings English breakfast, Pu erh). Among tested teas, Twinings green tea possessed the highest content of total phenols (2560,23 mg/L GAE) and flavonoids (1920,20 mg/L GAE), while Pu-erh showed the lowest contents (675,36 and 185,76 mg/L GAE, respectively). Antioxidant capacity was in compliance with total phenol and flavonoid content. Bacteriostatic activity of Twinings English breakfast black tea against *Salmonella* sp. 3064 and Gyokuro green tea against *S. aureus* 3048 was detected.

The obtained results confirmed high polyphenolic content and antioxidant capacity of tested teas. Despite the high content of bioactive compounds in all samples, only the Twinings English breakfast (black tea) and Gyokuro (green tea) showed bacteriostatic properties.

KEYWORDS: *Camellia sinensis; polyphenols; antioxidant capacity; anti-microbial properties*

INTRODUCTION

Recently, researchers have paid particular attention to the biologically active ingredients, especially alkaloids and polyphenols in food and beverages due to their positive effects on human health. Tea is one of the most commonly consumed beverages throughout the world. Beside the attractive aroma and specific taste, its popularity is also a result of its potential health benefits. Numerous epidemiological studies link tea consumption to a reduction of the risk of cardiovascular diseases (Hertog et al., 1993; Young et al., 1967), high cholesterol levels (Maron et al., 2003; Vinson et al., 2004), diabetes (Vinson, Zhang, 2005), arthritis (Haggi et al., 1999), ostheoporosis (Hegarty et al. 2000) and dental caries (Otake et al. 1991). These beneficial effects of tea have been attributed to the antioxidant properties of polyphenolic compounds, particularly of the catechin derivatives: (-)-epicatechin, (-)-epigallocatechin gallate,(-)-epigallocatechin, (-)-epicatechin gallate, (+)-gallocatechin, and (+)-catechin (Roberts, Wood, 1953). In addition to the polyphenols, tea leaves are an important source of methylxanthines (caffeine, theobromine and theophylline) as well as amino acids (theanine), minerals and trace elements such as potassium,magnesium, calcium, nickel and zinc, which are essential to human health (Fernandez et al., 2002). Methylxanthines have physiological and pharmacological effects on some body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory and

renal systems (Nehlig et al, 1992; Spiller, 1998). Several types of tea are produced from the leaves of Camellia sinensis: white tea, yellow tea, green tea (both unoxidized), Oolong tea (semi-oxidized), black tea (oxidized) and Pu erh tea (oxidized and microbiologically fermented). Oxidation of tea leaves induces enzymatic oxidation of catechins and leads to formation of two major pigments in black tea, theaflavins and thearubigins, which contribute to characteristic bright orange–red colour of black tea (Coggon et al., 1973).

Although the chemical composition and various beneficial health effects of different types of tea has been extensively studied, the number of the studies on the antimicrobial properties of tea on human pathogens, especially yeasts, is insufficient. Most of the studies are focused on application on antimicrobial properties of green tea extracts in order to prolong shelf life of food products (Chiu, Lai, 2010; Perumalla, Hettiarachchy, 2011; Martín-Diana et al., 2008; Kumudavally et al., 2008) or inhibition of *Streptococcus mutans* (Tsai et al, 2008), microorganism present in human oral cavity, causing a tooth decay. Therefore, the aim of this study was to determine total phenols and flavonoids in nine teas belonging to different categories, depending on the oxidation level and to compare it to the antimictobial activity tesed on four different microorganisms (*Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Candida albicans* 11 and *Salmonella* sp. 3064). Antioxidant capacity was also determined, applying three different assays (DPPH, ABTS, FRAP).

MATERIALS AND METHODS

Sample preparation

Nine different teas (white tea – Pai Mu Tan superior; yellow tea – Yin zhen; green tea – Matcha, Twinings, Gyokuro, Long Jing; oolong – Formosa Fine oolong; black tea – Twinings English breakfast, Pu erh) were tested in this study. In order to simulate household brewing conditions, teas were prepared using an aqueous extraction procedure. Tea samples (2.0 g) were poured with 200 mL of water heated to 80°C and stirred with a glass rod for 3 min. Tea extracts were filtered through a tea strainer.

Determination of total phenol and total flavonoid content

Total phenol content (TPC) in teas was determined spectrophotometrically according to the modified Folin–Ciocalteu method described by Lachman et al. (1998). Briefly, to a volume of 0.5 mL of a sample, 2.5 mL Folin–Ciocalteu's reagent, 30 mL distilled water and 7.5 mL of 20% Na_2CO_3 was added and diluted to 50 mL with distilled water. After 2 h, the absorbance was measured at 765 nm against blank. To determine total flavonoid content (TFC), formaldehyde was added to tea extracts causing precipitation of flavonoids from the solution. Precipitated flavonoids were separated from the solution by filtration and non-flavonoid phenols remaining in the filtrate were determined by Folin– Ciocalteu method. TFC was calculated as the difference between TPC and non-flavonoids. The results were expressed as mg/L of gallic acid equivalents (GAEs) (Kramling, Singleton, 1969).

Determination of antioxidant capacity

DPPH (2,2-diphenyl- 1-picrylhydrazyl) assay

This method is based on the reduction of stable DPPH radical by antioxidants in the solution. In the presence of antioxidants the purple colour of the DPPH radical solution changes to a bright yellow and the intensity of this change can be monitored spectrophotometrically (Blois, 1958). The samples were analysed according to the method reported by Brand-Williams et al. (1995). In brief, a volume of 3.8 mL of DPPH solution was added to 200 μ L of sample and the absorbance was measured at 517 nm after 30 minutes. All measurements were performed in triplicate and expressed as Trolox equivalents.

FRAP (Ferric reducing/Antioxidant power) assay.

The ferric reducing/antioxidant power (FRAP) assay was performed according to a standard procedure by Benzie and Strain (1996). This method is based on the reduction of ferric-2,4,6-tripyridyl-S-triazine (Fe^3TPTZ) complex into ferrous (Fe^{2+}) form, in the presence of an antioxidant, which results in the development of an intense blue colour with absorption maximum at 593 nm. Measurements were performed as follows: a volume of 1.9 mL of freshly prepared FRAP reagent was added to 100 mL of extract. Absorbance

readings were taken after 4 min, at 593 nm, against the blank. All measurements were performed in triplicate and the results are expressed as mM Fe(II).

ABTS [2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid)] radical cation decolorization assay

ABTS radical cation decolourization assay was performed according to the method described by Re *et al.*, 1999. This method measures the relative ability of various antioxidants to scavenge the free [2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid)] radical cation ($ABTS^{\bullet+}$). A 20 μ L aliquot of extract was added to 2.0 mL of the diluted $ABTS^{\bullet+}$ solution, and the absorbance readings were taken after exactly 6min against the blank containing ethanol instead of the sample. The results, obtained from triplicate analyses, were expressed as Trolox equivalents.

Antimicrobial activity

Antimicrobial activity of the teas was tested on test microorganisms: *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Candida albicans* 11 and *Salmonella* sp. 3064 (Collection of microorganisms of Laboratory for General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia), by disc-diffusion and turbidimetric method on microtiter plates. For disc-diffusion method, zone of inhibition was determined using 100 μL of prepared solution of nine different teas. Inhibition zones around the filter disks were examined during 48 h and the values were expressed as an average of three measures (Markov et al., 2011).

Turbidimetric method was performed according to Leboš et al. (2008) and Babić et al. (2011). The results were expressed as inhibition of the growth of test microorganisms during 48 hours at 37°C/28°C for bacteria/yeast.

RESULTS AND DISCUSSION

Products based on *C. sinensis* are known for its bioactive content as confirmed in our previous studies (Komes et al., 2010; Horžić et al., 2009). In this study, total phenol content (TPC) and total flavonoids content (TFC) of nine teas of five different types were determined spectrophotometrically by Folin- Ciocalteu method and presented on Fig. 1. As can be seen the highest TPC was determined in Matcha (2230.16 ± 99.87 mg/L), Twinings (2560.23 ± 119.19 mg/L) and Gyokuro (1720.46 ± 75.23 mg/L) teas, all belonging to green teas. They are followed by Twinings English breakfast (1705.05 ± 59,98), a type of black bagged tea. The lowest TPC was recorded in following order: Yin Zhen yellow tea (1096.97 ± 40.11 mg/L)>Pai Mu Tan superior white tea (795.36 ± 25.68 mg/L) > Pu erh black tea (675.36 ± 13.45 mg/L). Very similar trend was observed in the TPC of tested teas, which correlated very well with obtained results for TPC (r=0,957). The presented results show that TPC and TFC are influenced by the type of the tea i.e. a degree of oxidation.

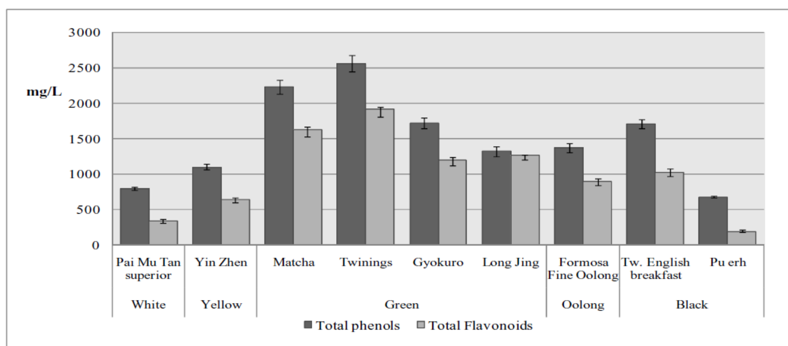

Figure 1. Total phenols and flavonoids in tested teas

Antioxidant capacity of tested teas, determined by three different assays (ABTS, DPPH and FRAP) is presented in Figure 2. ABTS method revealed Twinings green tea as the most potent antioxidant among tested teas (16,99 ± 1,54 mmol/L Trolox) and Pu erh black tea as the weakest (1,53 ± 0,13 mmol/L Trolox). However DPPH and FRAP methods gave different rankings of the tested samples – DPPH revealed Yin Zhen yellow tea as the sample with the highest antioxidant capacity , while FRAP showed the highest antioxidant capacity for Matcha green tea (18.55 ± 2.01 mmol/L Fe(II)). According to DPPH and FRAP, lowest ranked sample was again Pu erh black tea (2,84 ± 0.44 mmol/L Trolox and 1,80 ± 0.02 mmol/L Fe(II), respectively). Correlation between TPC, TFC and results of antioxidant capacity assays was calculated. Both TPC and TFC showed a very good correlation with results of ABTS assay (r=0,915 and 0,869 respectively). FRAP showed moderate correlation with TPC (r= 0,637) and TFC (0,612853), while DPPH showed low correlation (r<0.5)

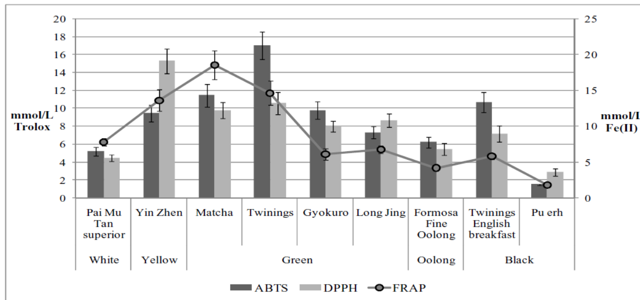

Figure 2. Antioxidant capacity of tested teas, determined by three different assays (ABTS, DPPH and FRAP)

In preliminary studies results obtained by disc-diffusion method showed that all investegated tea have slight inhibitory activities on the growth of tested microorganisms (Table 1), while results of the turbidimetric method proved inhibitory effect only of the Gyokuro and Twinings English breakfast tea on the growth of bacteria (Fig. 3).

Table 1. Size of the Zone of growth inhibition (mm)

	Zone of growth inhibition (mm)			
	E. coli 3014	*S. aureus* 3048	*Salmonella* sp. 3064	*C. albicans* 11
Pai Mu Tan superior	8	8	9	8
Yin Zhen	9	8	8	9
Matcha	8	7	9	9
Twinings	9	8	9	8
Gyokuro	8	18	8	9
Long Jing	9	8	9	8
Formosa Fine Oolong	7	9	8	8
Twinings English breakfast	9	9	16	9
Pu erh	8	8	9	8

Size of the zones of growth inhibition for each test microorganism was correlated with TPC, TFC, ABTS, DPPH, and FRAP. All of the obtained correlation factors were lower then 0.5, except for the correlation between DPPH results and size of growth inhibition for *E. coli* (r=0.581) and *C. albicans* (r=0.519). Percentage of growth inhibition of *S. aureus* 3048, and *Salmonella* spp. 3064 in the presence of Gyokuro and Twinings English breakfast tea during 48 hours was 22,1-4,6 %, and 26,0 – 7,2 %, respectively, and the results are presented in Table 2 (other data not shown). The obtained results suggest that only black tea – Twinings English breakfast and Gyokuro tea showed bacteriostatic properties.

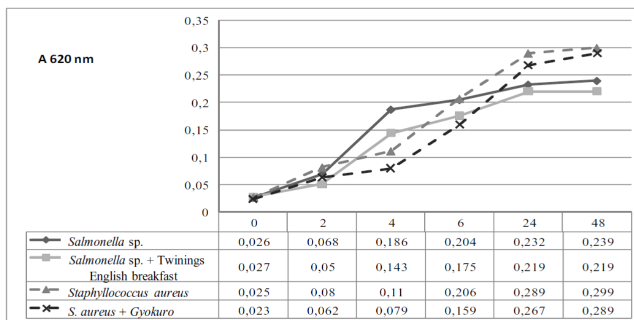

Figure 3. Results of the antimicrobial activity determined by turbidimetric method

Table 2. Percentage of growth inhibition of *S. aureus* 3048, and *Salmonella* spp. 3064 in the presence tea extracts

Time (h)	**% inhibition** (mean±SD)	
	S. aureus 3048 + Gyokuro	*Salmonella sp.* 3064 + Twinings English breakfast
2	22,1 ± 0,5	26,0 ± 0,3
4	28,3 ± 0,9	23,1 ± 0,8
6	23,0 ± 1,1	15,3 ± 0,6
24	8,2 ± 0,7	6,1 ± 0,4
48	4,6 ± 0,6	7,2 ± 0,3

CONCLUSION

All tested teas showed a high total phenolic and flavonoid content, as well as high antioxidant capacity determined by three different methods (ABTS, DPPH, FRAP). However, in spite of their high bioactive potential, tested teas did not show a good antimicrobial activity, which was proven with very low correlation factors between size of growth inhibition for all four test microorganisms and above mentioned parameters of studied teas.

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CONCENTRATIONS OF L-TRYPTOPHAN, INDOLE-3-ACETIC ACID AND 2-AMINOACETOPHENONE IN MALVASIA FROM ISTRIA WINES

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ABSTRACT

The phenomenon of untypical aging off-flavor, associated with higher concentrations of 2- aminoacetophenone (2-AAP) in white wines, can stem from some technological treatments applied in grape and wine production. In order to find out if a wine is liable to untypical aging off-flavor, it is necessary to determine the concentrations of indole-3-acetic acid and L-tryptophan, which are the precursors of 2-aminoacetophenone. The purpose of this investigation was to determine the influence of soil type and different yeast strains on the levels of L-tryptophan, indole-3-acetic acid and 2- aminoacetophenone in the Malvasia from Istria must and wine. In the 2007 and 2009. grapes were harvested from two different soil type locations (terra Rosa, terra Bianca), in Istria wine growing region, Croatia, and separately vinified. Vinification was carried up with 4 different wine strains ((Fermol Cryoarome, Fermol Assosiee, Uvaferm CS2, Anchor VIN 13). Young wine samples were taken for analysis after completed alcoholic fermentation. Analyses of L-tryptophan and indole-3-acetic acid were performed using Agilent 1100 equipped with autosampler and fluorescence detector. Separation was achieved on Luna C_{18} (endcapped) column (5 μm packing, 250 x 4.6 mm i.d.), (Phenomenex, USA), protected with guard column from same material (Phenomenex, USA). 2-aminoacetophenone (2-AAP) analyses were performed using gas chromatography-mass spectrometry (GC-MS). 2-AAP was extracted from wine by direct immersion solid phase microextraction (DISPME). Divinylbenzene / carboxen / polydimethylsiloxane SPME fibres were used. Chemical analyses of compared musts and wines in both investigated years showed differences in L-tryptophan, ranging from 260,2 up to 1065,6 μg/L, and indole-3-acetic acid concentrations, ranging from 26,1 up to 71,3 μg/L, that depend more upon the soil type than yeast strain used. Different soil type and yeast strains also influenced 2-aminoacetophenone concentrations in Malvasia wines, ranging between 0,3-1,2 μg/L.

KEYWORDS: *indole-3-acetic acid; L- tryptophan; 2-aminoacetopheno; wine; soil type, yeast*

INTRODUCTION

The phenomenon of untypical aging off-flavor, associated with higher concentrations of 2- aminoacetophenone (2-AAP) in white wines, can stem from some technological treatments applied in grape and wine production. In order to find out if a wine is liable to untypical aging off-flavor, it is necessary to determine the concentrations of indole-3-acetic acid and L-tryptophan, which are the precursors of 2-aminoacetophenone (Christoph et al.,1998). It is assumed that UTA is the result of a stress reaction in grape. As a consequence, the IAA concentrations in must have been considered as stress indicator with increasing stress leading to higher IAA concentrations (Müller, 2000). Studies by Christoph et al. (1998) have shown that 2-APP can be formed by an oxidative degradation of IAA, which is triggered by a sulfuration, a measure indispensable for white wine making. It could be demonstrated that pyrole ring cleavage of IAA by superoxide radicals, which can be generated by aerobic oxidation of sulfite during storage of sulfurized wines, leads in several steps to N-formyl-2-aminoacetophenone (FAP) which is further decomposed to 2-AAP (Hoenicke, 2002). The response of Trp in grapes to stress conditions is yet unknown. Grossweiner (1984) found out that Trp levels may be reduced by enhanced UV-B radiation. Investigations by Linsenmeier et al. (2004) pointed out that the Trp concentrations of must essentially depended on weather; hot years resulted in low values, while

in cool years values were very high. During alcoholic fermentation the level of free Trp decreased, the consumption by the yeast was between 60 % and 100 % (Linsenmeier et al., 2004). Utilization of Trp by the yeast was also reported by Berger and Haller (1969), and Sponholz (1991). Results by Ough et al. (1991) pointed out that certain amino acids such as leucine, isoleucine, phenylalanine and tryptophan are preferentially incorporated by yeast. According to Arias-Gil et al. (2007) tryptophan was totally consumed in all fermentations, independently of the concentration of amino acids added to the must. The purpose of this investigation was to determine the influence of soil type and different yeast strains on the levels of L-tryptophan, indole-3-acetic acid and 2-aminoacetophenone in the Malvasia from Istria must and wine.

MATERIALS AND METHODS

Malvasia from Istria grapes from two vine growing producers (Pilato, Rossi) having vines (planted in 1994, Guyot trained, grafted on 420A rootstock), on different soil type locations in Istria wine growing region (terra Rosa, terra Bianca), harvested in 2007 and 2009 year, was used to produce the wines by traditional vinification for white wine. For the winemaking a lot of 300 kg of grapes was de-stemmed, crushed and separated in three 100 L stainless-steel tanks for fermentation. Alcoholic fermentation was carried without yeast inoculation (spontaneous fermentation) and by use of 4 commercial yeast strains (Fermol Cryoarome, Fermol Assosiee, Uvaferm CS2, Anchor VIN 13). All treatments were carried out in triplicate. After completion of alcoholic fermentation, the wines were racked, sulfited with 100 mg/L of SO_2 and stored under cellar condition. Basic chemical analyses of must and wine were done using methods proposed by O.I.V. (2007). Extraction of free IAA and tryptophan were done by solid phase extraction (SPE) using AccuBond II Env (Agilent) cartridges. Analyses of free IAA and tryptophan were performed using Agilent 1100 equipped with autosampler and fluorescence detector. Separation was achieved on Luna C_{18} (endcapped) column (5 μm packing, 250 x 4.6 mm i.d.), (Phenomenex, USA), protected with guard column from same material (Phenomenex, USA). 2-aminoacetophenone (2-AAP) analyses were performed using gas chromatography-mass spectrometry (GC-MS). 2-AAP was extracted from wine by direct immersion solid phase microextraction (DI-SPME) method by Fan et al. (2007). Divinylbenzene / carboxen / polydimethylsiloxane SPME fibres were used. One-way analysis of variance (ANOVA) and Least Significant Difference (LSD) comparison test of SAS (SAS Institute, Cary, NC, USA) were used to interpret differences in means, if any, at the 95% confidence level.

RESULTS AND DISCUSSION

Table 1 shows basic chemical composition of musts, from two production years, used in this investigation. As it can be seen there were no marked differences between samples from two types of soil. Must sugar concentration and total acidity pointed out that at the time of vintage grape was ripe, while the NTU values and FAN concentrations were relatively low. The free IAA concentration in both Malvasia musts and examined years was relatively similar ranging from 3.2 μg/L to 4.3 μg/L, what is in accordance whit data published by Hoenicke et al. (2001). During the vintage period from 1996-1999 they found out only traces of free IAA (<3 μg/L) in the examined grape musts. In the wines the free IAA concentrations were as expected much higher indicating either a neosynthesis by the yeast or hydrolysis of conjugated IAA during fermentation. In both investigated years indigenous yeasts favored the neosynthesis of IAA and lead to higher IAA concentrations in the fermentation medium. Hoenicke et al. (2002) found a significant correlation between the quantity of Trp in must and IAA in wine. As shown in table 2. during alcoholic fermentation the level of Trp decreased, the utilization by yeast ranged from 65-75% in wines produced from grapes grown in Terra Rosa and 69%-89% in wines produced from grapes grown in Terra Bianca. 2- AAP concentrations were detected in all wine samples ranging from 0,3 up to 1,2 μg/L. In both investigated years wines from Terra Bianca had higher amounts of 2-AAP compared to wines from Terra Rossa. Type of yeast had no marked influence in 2-AAP production and no correlation was found between IAA concentrations and 2-AAP amounts in the wine. Bely et al. (1990) found that minimum FAN concentration of 140 mg/L was needed for satisfactory fermentation. Our results showed that even with relatively low FAN concentrations (70-98 mg/L) all yeast strains managed to complete fermentation of Malvasia must. According to Gessner et al. (1998) nitrogen

deficiency has been considered as a factor causing UTA (Gessner et al., 1998). Probably insufficient nitrogen supply is one of the reasons for 2-AAP appearance in analyzed Malvasia wines. Even though concentrations in some samples were higher than 2- AAP odour threshold of about 1 µg/L no negative aromas like painty, mothball and medicinal were detected by a panel of 8 professional wine judges. The main reason for this is probably characteristic cultivar typical fruity aroma of Malvasia wines.

Table 1. Chemical composition of Malvasia must from two different soil type

Compounds	Terra Rossa		Terra Bianca	
	2007	2009	2007	2009
Sugar °Oe	89	83	92	82
pH	3.25	3,50	3.30	3,34
Total acidity (g/L)*	6.9	4,6	6.4	5,6
FAN (mg/L)	85	98	70	87
NTU	89	55	76	57
Indole -3-acetic acid (µg/L)	4.3	4,1	3.2	3,4
Tryptophan (µg/L)	3305	3489	2611	2817

Table 2. IAA, tryptophan and 2-aminoacetophenon concentrations in Malvasia wines from two soil type (terra rossa, terra bianca)

Compounds		Terra Rossa						Terra Bianca					
		Indigenous yeasts	Fermol assosiee	Fermol cryoarome	Anchor VIN13	Uvaferm CS2	LSD	Indigenous yeasts	Fermol assosiee	Fermol cryoarome	Anchor VIN13	Uvaferm CS2	LSD
Indole -3-acetic acid (µg/L)	2007	40.2	32.9	31.8	30.2	30.5	5%=4,6	67.1	28.9	30.2	26.1	25.4	5%=5.8
	2009	48,1	42,5	33,4	40,8	29,6	5%= 3,2	57,2	33,8	39,8	21,3	27,6	5%= 2,1
Tryptophan (µg/L)	2007	1044.6	1007.8	828.2	1131.5	880.5	5%=97.56	621.6	693.6	665.1	729.3	726.2	5%=35.83
	2009	985,6	1003,4	954,7	1021,5	893,1	5%= 62,12	782,9	769,2	638,1	855,6	695,8	5%= 33,73
2.AAP (µg/L)	2007	0,3	0,4	0,4	0,3	0,5	n.s.	1,0	1,2	0,9	0,9	0,7	5%= 0,21
	2009	0,6	0,6	0,5	0,5	0,6	n.s.	0,8	1,1	0,9	1,0	0,8	n.s.

CONCLUSIONS

Results of this study indicate the possible connection between tryptophan and its metabolite indole-3- acetic acid as the results pointed out significant increase in indole-3-acetic acid concentrations and decrease of Trp concentrations during alcoholic fermentation. Commercial yeasts used in this investigation varied in tryptophan consumption during alcoholic fermentation but more pronounced differences in Trp utilization was between soil types examined. More intense use of tryptophan was noticed during fermentation of must from Terra Bianca. Significantly higher concentrations of IAA were noticed in wines produced by indigenous yeasts. 2-AAP concentrations varied between wines produced from grapes grown on different type of soil, while yeast had no influence on 2-AAP synthesize.These preliminary results show the complexity of the interactions involved and the need for further research, including more detailed chemical analysis.

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SCREENING OF FRUITS AND VEGETABLES FOR OLIGOSACCHARIDES INTENDED FOR PREBIOTIC EXPLOITATION

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ABSTRACT

Interest in functional non-digestible oligosaccharides (NDOs) as prebiotic compounds is motivated by their diverse industrial applications. The health benefits of functional oligosaccharides are well known and far reaching. Before the health promoting properties of prebiotics can be claimed, it is important to analyze their content and composition in foodstuffs. The objective of the present study was to provide precise information of NDOs content in selected fruits and vegetables grown in the Republic of Macedonia, which are usually part of everyday diet. In addition different types of FOS (1-kestose (GF_2), nystose (GF_3) and 1^F-β fructofuranosylnystose (GF_4)) were quantified. The FOS were extracted with 85% ethanol in water bath with constant shaking at 50 °C for 1 h. The extracts were evaporated in a vacuum rotary evaporator and analyzed by high performance liquid chromatography (HPLC). Most of the studied fruits contained low amount of total FOS. The highest content of FOS was found in nectarine and watermelon (0.89 and 0.81 g/100 g fm). Vegetables with the highest quantity of FOS included scallion, white onion, garlic, leek and spring garlic (3.32, 2.01, 1.20, 1.07 and 0.98 g/100 g fm). Out of the 73 samples of fruits and vegetables evaluated for the composition of NDOs, white onion (2.24 g/100 g fm) and scallion (4.10 g/100 g fm) were found to have reasonably high proportion of total FOS.

KEYWORDS: *functional food; prebiotics; non-digestible oligosaccharides; fruits; vegetables*

INTRODUCTION

Over the last decade, drastic changes have taken place in the image and assessment of the importance of the daily diet. Foods are no longer judged only in terms of taste and immediate nutritional needs but also in terms of their ability to improve the health and well-being of consumers. Since the late 1990s, the scientists showed great interest in the health properties of non- digestible oligosaccharides (NDOs) as prebiotic compounds. Many scientific papers have been published describing the effect of prebiotics on human health. Prebiotics have been claimed to improve some physiological functions in humans such as relieving constipation (Robertfroid, 1993), decreasing the risk of osteoporosis by increasing mineral absorption, especially of calcium (Van den Heuvel et al., 1999), and atherosclerosis by lowering the synthesis of triglycerides and reducing plasma cholesterol concentrations (Davidson, Maki, 1999).

Because of several scientific claims of benefits of these carbohydrates with a "bioactive" or "functional" action in intestinal health, the food industry has increased the inclusion of NDOs in their products (Bengmark, 2010; Singh, Singh, 2010). However, the consumption of natural sources of NDOs, such as fruits and vegetables, has advantages for its lower price in comparison to industrialized products. Moreover, natural sources offer combined intake with other nutrients such as vitamins, and minerals.

Nutrient composition of fruits and vegetables is very complex and difficult to evaluate. Levels of plant metabolites are strongly affected by genetic and environmental factors as well as transportation and storage conditions. Growth factors such as light, temperature, humidity, type of soil, application of fertilizers, damage caused by microorganisms and insects, stress induced by UV radiation, heavy metals, and pesticides all alter metabolite composition of plants (Orcutt, Nilsen, 2000). Before vegetables appear on a supermarket shelf they have been handled by plant growers, transporters, packagers, storehouse operators, distributors, and/or processors. The chemical and physical changes that occur in vegetables during these stages can lead to loss of potentially beneficial components (MacEvily, Peltoa, 2003).

There are not much published data on oligosaccharide contents in foods and even less on oligosaccharide consumption of any population in the world (Trumbo, Shimakawa, 2009). In the Republic of Macedonia, there is not much information on the different carbohydrate fractions in foods or on the biological availability of carbohydrates when consumed. A need for such information is particularly important in case of sugars, due to the increasing interest of nutritionists in the role of these components in the diet. Quantifying each individual carbohydrate and evaluating foods on case-by-case basis is the current tendency of worldwide databases (Greenfield, Southgate, 2003).

Before the health promoting properties of prebiotics can be claimed, it is important to analyze their content and composition in food staffs. The objective of the present study was to provide precise information of FOSs content, including 1-kestose (GF_2), nystose (GF_3) and 1^F-β fructofuranosylnystose (GF_4) in selected fruits and vegetables grown in the Republic of Macedonia, which are usually part of everyday diet.

MATERIALS AND METHODS

Samples and sample preparation

The selected foods for analysis were 32 fruits and 41 vegetables grown and commonly consumed in the Republic of Macedonia. Fresh food samples (fruits and vegetables) were collected from local green markets and grocery stores or directly from producers. Approximately 500 g of each sample was chosen at random. The edible part was cut into small pieces and dried at 60 °C in a vacuum oven (Heraeus Instruments vacutherm VT 6025, Germany) until constant dry mass was reached. Dried samples were ground with a laboratory grinder to a particle size less than 1 mm before extraction.

Extraction of fructooligosaccharides (FOS)

Fructooligosaccharides were extracted according to Espinosa-Martos and co-workers (2006). The samples (400 mg) were extracted with 40 mL ethanol (85 %, v/v). Extractions were performed in screw-capped tubes, at 50 °C in a water bath with constant shaking for 1 hour. After cooling at room temperature, the samples were centrifuged at 3000x*g* for 15 min. 10 mL of supernatants were evaporated in a vacuum rotary evaporator at 50 °C until the samples were completely dried. The extracts were redissolved in deionised water (1.5 mL) and passed through 0.45 μm filters (Econofilter, Agilent Technologies, USA) just before high-performance liquid chromatography (HPLC) analyses.

Dry matter determination

Approximately 3-5 g of each fresh tissue were weighted in triplicate from each sample and dried at 60 °C in a vacuum oven to constant mass. Moisture content was calculated as difference between fresh and dried mass of the samples.

Determination of fructooligosaccharides by HPLC

Twenty microliters of FOS extracts were filtered throgh 0.45 μm filters and injected into Agilent 1200 HPLC (USA) fitted with a Zorbax carbohydrate analysis column (4.6 x 150 mm, 5 μm particle size) and a Zorbax NH_2 guard column (4.6 x 12.5 mm) (Agilent, USA). The mobile phase was 75:25 (v/v) acetonitrile:water and the flow rate was kept constant at 1.5 mL/min. The column temperature was kept at 30 °C. FOS were identified by their retention time and quantified by comparison with known oligosaccharide standars. Peaks were eluted by the increasing order of molecular weight. Thus, 1-kestose was first (Rt=6.4 min), then the nystose (Rt=8.5 min) and 1^F-β fructofuranosylnystose (Rt=9.5 min). The concentration of sugars was calculated from the peak height of detector response.

RESULTS AND DISCUSSION

Quantifying oligosaccharides levels in fruits and vegetables is a challenging area as foods contain a complex mixture of these compounds of varying degree of polymerization (DP) from two to 60 units. This study provides comprehensive information on the content of FOS in 32 fruits and 41 vegetables produced and

commonly consumed in the Republic of Macedonia. The content of individual fractions of fructooligosaccharides (FOS), such as GF_2, 1-kestose; GF_3, nystose; and GF_4, 1F-β-fructofuranosylnystose are presented in Table 1.

Table 1. Oligosaccharides content in common Macedonian fruits

sample	moisture	GF_2	GF_3	GF_4	Total FOS
		(g/100 g fresh mass of edible sample)			
apple, Golden Delicious	87	0.07	nd	nd	0.07
apple, Idared	85	0.09	nd	nd	0.09
apple, Petrovka	87	0.11	0.11	0.07	0.29
apricot	87	0.08	nd	nd	0.08
blackberry	71	nd	nd	nd	0
blueberry	85	0.18	0.32	tr	0.50
cherry	86	0.22	0.10	nd	0.32
currant, black	78	0.08	nd	nd	0.08
currant, red	83	0.15	0.20	tr	0.35
fig, common	73	tr	0.09	nd	0.09
fig, wild green	88	0.19	0.11	tr	0.20
grape, red Vranec	74	0.08	tr	nd	0.08
grape, white	74	nd	tr	nd	0
medlar	67	nd	nd	nd	0
melon, honeydew	87	nd	0.09	nd	0.09
melon, Polidor	88	0.19	0.11	tr	0.30
mulberry, black	82	0.13	0.17	tr	0.30
mulberry, white	79	0.16	0.19	tr	0.35
nectarine	88	0.18	0.65	0.06	0.89
pear	83	0.15	0.32	0.09	0.56
peach, yellow-green	86	nd	tr	nd	0
plum, cherry	83	nd	0.09	nd	0.09
plum, Ciruela	88	nd	0.08	nd	0.08
plum, red	86	0.12	nd	nd	0.12
pomegranate	76	nd	0.11	nd	0.11
pumpkin	87	tr	nd	nd	0
quince	75	tr	nd	nd	0
raspberry	84	0.32	0.12	0.07	0.51
sour cherry	77	0.11	0.22	tr	0.33
strawberry, common	91	nd	nd	nd	0
strawberry, woodland	82	0.09	nd	nd	0.09
watermelon	90	0.29	0.44	0.08	0.81

GF_2 - 1-kestose; GF_3 - nystose; GF_4 - 1^F-β fructofuranosylnystose; nd-not detected;
tr-trace amount <0.05 g/100 g fm

Most fresh fruits contained low amount of FOS. The content of total FOS in a descending order was nectarine > watermelon > pear > raspberry > blueberry (0.89, 0.81, 0.52, 0.51 and 0.50 g/100 g fm). The fruits with the highest amount of GF_2 included raspberry 0.32, watermelon 0.29 and cherry 0.22 g/100 g fm. GF_3 was present in 18 fruit samples ranging from 0.08 g/100 g fm in Ciruela plum to 0.65 g/100 g fm in nectarine. GF_4 was present only in five fruits with the highest amount of 0.08 g/100 g fm in watermelon.

Only limited data for oligosaccharides content in fruits and vegetables are available in the literature for comparison. The level of FOS in the nectarine was found higher than that reported by Muir et al., 2009 (0.59 g/100 g fm). On the other hand, in the peach we have not found any FOS, while Muir et al., 2009 reported their presence in traces, and Campbell et al., 1997 found 0.4 g/100 g. No published data on oligosaccharides content in Petrovka apple, black and red currant, common and wild fig, red grape Vranec, medlar, black and white mulberry, cherry plum, pomegranate, quince and strawberry woodland are available for comparison. These findings on the FOS amounts and constituents can be used for development of a national fruit database.

The contents of individual fractions of FOS in vegetables are presented in Table 2. The content of total FOS in a descending order was scallion > onion > garlic > leek > spring garlic (3.32, 2.01, 1.20, 1.07 and 0.98 g/100 g fm).

Table 2. Oligosaccharides content in common Macedonian vegetables

sample	moisture	GF_2	GF_3	GF_4	Total FOS
		(g/100 g fresh mass of edible sample)			
artichoke, Jerusalem	63	0.11	0.73	tr	0.84
beans, yellow	92	nd	nd	nd	0
beetroot	90	0.15	0.21	nd	0.36
Brussels sprouts	81	0.35	0.43	0.04	0.82
broccoli	86	0.06	0.72	nd	0.78
cabbage, common	88	nd	0.82	nd	0.82
cabbage, red	91	nd	0.61	nd	0.61
carrot	90	nd	nd	nd	0
cauliflower	90	nd	nd	nd	0
celery, bulb	89	nd	nd	nd	0
celery, leaves	84	nd	nd	nd	0
chard	91	nd	nd	nd	0
chicory	59	0.09	0.21	0.09	0.39
daikon	95	tr	nd	nd	0
dandelion, bulb	62	0.54	0.11	0.08	0.73
eggplant	93	nd	nd	nd	0
fennel, bulb	92	0.19	0.36	0.06	0.61
fennel, leaves	88	0.08	0.13	nd	0.21
garlic	63	0.69	0.52	0.09	1.20
garlic, spring	70	0.50	0.41	0.07	0.98
kale	88	0.07	0.44	nd	0.51
kohlrabi	90	nd	0.18	nd	0.18
leek	90	0.95	0.12	tr	1.07
lettuce, green	91	nd	tr	nd	0
lettuce, red	91	0.23	0.14	nd	0.37
mushroom, button	92	0.06	0.22	tr	0.28
mushroom, oyster	92	tr	0.15	tr	0.15
mushroom, Lisicarka	88	0.09	0.26	tr	0.35
okra	87	tr	0.23	tr	0.23
onion, white	88	1.15	0.77	0.09	2.01
parsnip	83	0.27	0.12	tr	0.39
peas	67	nd	nd	nd	0
pepper, red	88	nd	nd	nd	0
radish	96	nd	tr	nd	0
rocket	88	nd	nd	nd	0
scallion	84	1.85	1.36	0.11	3.32
tomato, common	92	nd	nd	nd	0
tomato, cherry	92	nd	nd	nd	0
tomato, strawberry	81	nd	nd	nd	0
zucchini, common	96	nd	nd	nd	0
zucchini, green	93	tr	nd	nd	0

GF_2 - 1-kestose; GF_3 - nystose; GF_4-1^F-β fructofuranosylnystose; nd-not detected; tr-trace amount <0.05 g/100 g fm

A medium level of FOS was found in Jerusalem artichoke, broccoli, Brussels sprouts, common and red cabbage, dandelion and fennel bulb and kale in range from 0.50 to 0.84 g/100 g fm, mainly GF_3. Undetectable to low amounts of FOS were found in other vegetables.

No published data on FOS content in yellow beans, red cabbage, celery leaves, chard, kohlrabi, mushroom oyster and Lisicarka, parsnip, rucola and tomato strawberry were available. From these vegetables parsnip, red cabbage, kohlrabi, mushrooms oyster and Lisicarka showed presence of FOS (0.39; 0.61; 0.18; 0.15 and 0.35 g/100g fm of total FOS, respectively).

A typical chromatographic profile of the scallion extract is shown in Figure 1.

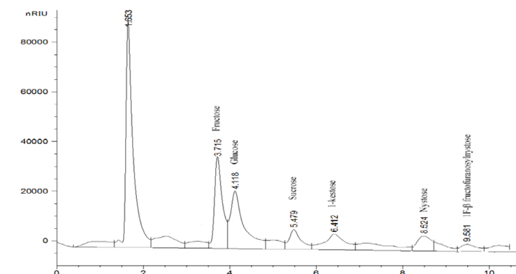

Figure 1. HPLC chromatogram profile illustrating the scallion extract

Figure 2 illustrates the distribution of total FOS in selected fruits (nectarine and watermelon) and vegetables (garlic, leek, white onion and scallion) as a potential source for prebiotic extraction. As can be seen, scallion and white onion were found to have reasonably high proportion of FOS.

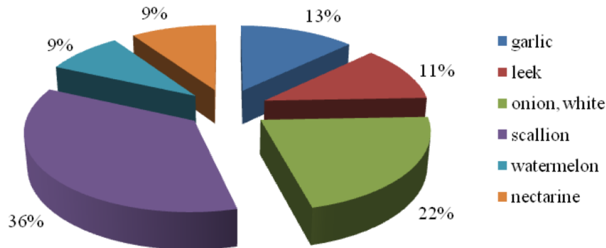

Figure 2. Distribution of total FOS in selected fruits and vegetables

CONCLUSION

The present study provides more detailed information about FOS level in a wide range of fruits and vegetables available in the Republic of Macedonia and considerably expanded our understanding of food composition. Furthermore, this database on FOS content may better facilitate nutritional formulation and diet selection for higher FOS consumption. The results of this study support evaluating foods on a case-by-case basis rather than developing generalizations on the relative proportions of FOS fractions according to food groups. Out of the 73 samples of fruits and vegetables evaluated for the composition of FOS, white onion and scallion were found to have relatively high proportion of FOS, which may be exploited for commercial extraction of FOS, and can be used as ingredients for the development of functional foods.

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ANTIOXIDANT ACTIVITY AND PHYSICOCHEMICAL CHARACTERISTICS OF MULTIFLORAL HONEY PRODUCED IN CROATIA

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ABSTRACT

Many studies indicated that the antioxidant activity of honey varies widely, depending on the floral source. Antioxidant activities of some unifloral honey produced in Croatia were reported in a few studies, while antioxidant capacity of multifloral honey seems unknown. The aim of this study was to determine the antioxidant activity and physicochemical characteristics of multifloral honey produced in Croatia during three different seasons. In 55 samples of multifloral honey, the physicochemical parameters were determined according to International regulatory standards and Croatian legislation. Physicochemical parameters (water content, electrical conductivity, total reducing sugars content, sucrose content, acidity, prolin content, diastase and invertase activity) were determined by standard analytical methods. Phenolics were determined by modified Folin-Ciocalteu method, antiradical activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and potential antioxidant activity using the ferric reducing antioxidant power (FRAP) method. Principal component analysis (PCA) was used for comparison of the obtained results between three seasons. The results of physicochemical analyses showed that values of investigated parameters lie within the limits set by national and international honey-profiling criteria. Phenolic compounds appear to be responsible for antioxidant capacity of multifloral honey. Multifloral honey samples from season 2007 differ from the honey samples from seasons 2008 and 2009 mainly on the basis of total reducing sugars content and prolin content.

KEYWORDS: *antioxidant activity; honey; physicochemical parameters*

INTRODUCTION

Honey has quite complex chemical composition, so there are basically no two samples alike. Its highly variable sensorial and physicochemical characteristics are due to diverse climatic and environmental conditions and various origins of plants from which it is harvested. It is essentially a concentrated aqueous solution of inverted sugar, but it also contains around 200 substances which form a mixture of other sacharides, enzymes, amino and organic acids, polyphenols, carotenoid-like substances, Maillard-reaction products, vitamins and minerals (Gheldof et al., 2002). Some of these compounds (phenolics, vitamin C, Maillard-reaction products, some amino acids etc.) give the bioactive properties to honey (Beretta et al., 2005). This makes honey more than just a nourishment of high value, but a valuable dietary source of antioxidants. Besides that, honey contains phenolic acids and their derivatives - flavonoid aglycones (pinobanksin, chrizin, galangin, luteolin, kaempferol, myricetin, quercetin etc.), an antioxidant pool that by acting synergistically can explain many of the biological or therapeutic properties of honey (Gil et al., 1995; Hermosin et al., 2003).

Antioxidant activity has recently been determined in various foodstuffs by many scientists and research groups around the world. Numerous chemical compounds show different antioxidant activity which depends on their origin, chemical structure, bioavailability etc. It is proven that they have synergistic effects and protective properties against various degenerative disorders including cancer, stroke, cardiovascular, Alzheimer's and Parkinson's disease (Giasson et al., 2002; Ndhlala et al., 2006; Kawasaki et al., 2008; Abdel-Hameed, 2009).

Because of its diverse and complex composition, even honey samples from the same botanical origin can show different antioxidant activity. Results from several studies show that honey types that are light in color (acacia, lime) show lower values for some parameters of antioxidant activity than honey

types that are darker (forest, chestnut, spruce, fir) (Blasa et al., 2006; Bertoncelj et al., 2007). Increased browning of honey during heating correlates very well with its antioxidant activity (Turkmen et al. 2006).

Melisopalynological analysis is at the moment the only recognized method for determination of botanical origin of honey and is therefore an important part of honey analysis. It is easy to comprehend that the majority of analytical efforts until now have focused on characterization of some potential markers of botanical, geographical and seasonal origin, for example: amino acids, proteins, minerals, volatiles, sugar composition and recently phenolics. Because of that, physicochemical parameters are important in determination of botanical and/or geographical origin, and also in providing the information if honey sample complies the conditions given by certain regulations.

In this study, the antioxidant activity and eight different physicochemical parameters of multifloral honey samples, produced in Croatia during three different seasons, were determined. The objective was to identify possible differences between seasons, to characterise honey samples by their physicochemical characteristics and to determine their antioxidant capacity.

MATERIALS AND METHODS

Multifloral honey samples were obtained directly from beekeepers from different locations across Croatia. Samples were stored, before and during the analyses, in glass containers at room temperature. All of the chemicals and reagents used were of analytical grade and purchased from Sigma (St.Louis, MO, USA) and Merck (Darmstadt, Germany).

In all samples, the physicochemical parameters were determined according to the national regulations and methods proposed by the International Honey Commission (IHC) (IHC, 2002; Croatian Regulations, 2009). The Folin–Ciocalteu method as modified by Beretta et al. (2005) and Bertoncelj at al. (2007) was used to determine total phenolic content. Antioxidant activity of multifloral honey samples was determined by FRAP (the ferric reducing/antioxidant power) and DPPH (1,1-diphenyl-2- picrylhydrazyl) assays, also according to Beretta et al. (2005) and Bertoncelj at al. (2007). Obtained results were evaluated using the principal components analysis (PCA) (StatSoft, 2010).

RESULTS AND DISCUSSION

Multifloral honey samples from season 2007 (n = 22), 2008 (n = 13) and 2009 (n = 20) were investigated for physicochemical characteristics, phenolic content and antioxidant activity. Results of physicochemical parameters are shown in Table 1 and Table 2. The average values of water content, electrical conductivity, total reducing sugars content, sucrose content and acidity, for samples from each of the investigated season, lie within the limits set by Croatian and international honey-profiling criteria (IHC, 2002; Croatian Regulations, 2009) (Table 1).

Table 1. Physicochemical parameters of the honey samples (n = 55) from three different seasons

season	statistics	*w* (water content) (%)	electrical conductivity (mS/cm)	*w* (total reducing sugars) (%)	*w* (sucrose) (%)	acidity (meq/kg)
	mean ± SD	17.76 ± 1.43	0.66 ± 0.21	62.03 ± 5.24	2.95 ± 1.07	27.36 ± 8.05
2007	range	15.60 - 21.24	0.27 - 1.24	53.14 - 78.52	1.87 - 6.10	12.01 - 42.71
(*n* =22)	CV (%)	8.05	31.82	8.45	36.27	29.42
	mean ± SD	16.97 ± 0.98	0.46 ± 0.17	70.35 ± 1.88	1.41 ± 1.51	24.06 ± 7.12
2008	range	14.92 - 18.20	0.25 - 0.84	66.76 - 73.48	0.00 - 3.62	12.95 - 37.77
(*n* =13)	CV (%)	5.77	36.96	2.67	107.09	29.59
	mean ± SD	17.32 ± 1.69	0.53 ± 0.17	69.38 ± 2.16	0.95 ± 1.33	23.91 ± 5.51
2009	range	14.92 - 20.52	0.23 - 0.76	62.86 - 71.68	0.00 - 4.67	14.71 - 37.38
(*n* =20)	CV (%)	9.76	32.08	3.11	144.00	23.04

Exception are the values of diastase activity of honey samples from season 2008, which are mostly below 8 diastase units, indicating that those honey samples were heated during processing or storage (Table 2). As additional criterions of honey ripeness and freshness indicators, prolin content and invertase activity were determined. Average values of invertase number (IN) of investigated multifloral honey samples, obtained during this study, were between values for acacia honey (4.04 IN) and chestnut honey (25.61) obtained by Bonvehi et al. (2000). Prolin content is in some cases used as a criterion of sugar adulteration if this value is below 180 mg/kg. Prolin content in all of the investigated multifloral honey samples is above this value (Table 2).

Table 2. Prolin content, diastase and invertase activity of the honey samples (n = 55) from three different seasons

season	statistics	*w* (proline) (mg/kg)	diastase activity (DN)	invertase activity (IN)
2007 (*n* =22)	mean ± SD	503.88 ± 304.06	27.18 ± 13.04	25.75 ± 6.61
	range	74.03 - 1189.04	5.12 - 41.91	12.27 - 37.29
	CV (%)	60.34	47.98	25.67
2008 (*n* =13)	mean ± SD	731.96 ± 304.78	6.47 ± 3.29	16.73 ± 11.29
	range	297.78 - 1340.37	3.54 - 13.55	1.13 - 34.00
	CV (%)	41.64	50.85	6.69
2009 (*n* =20)	mean ± SD	730.10 ± 49.66	19.26 ± 8.76	15.37 ± 8.14
	range	648.14 - 814.75	4.10 - 39.00	2.70 - 28.40
	CV (%)	6.80	45.48	52.96

In order to evaluate antioxidant capacity of multifloral honey samples from three different seasons, total phenolics, antioxidant and antiradical activities were determined (Table 3). The highest average value of phenolic content, expressed as mg of gallic acid equivalents (GAE) per kg of honey, was determined in multifloral samples from season 2007 (225.61 mg GAE/kg of honey) and the lowest in samples from season 2008 (193.76 mg GAE/kg of honey). Bertoncelj et al. (2007) investigated Slovenian multifloral honey samples and determined the average total phenolics value of 157.3 $mg_{gallic\ acid}$/kg, while Beretta et al. (2005) determined in Italian multifloral honey samples the total phenolics value of 170.4 $mg_{gallic\ acid}$/kg. The highest average FRAP value obtained in this study, expressed as μM Fe(II) of the 10 % (w/v) honey solution, was 405.58 (season 2007) and the lowest 295.40 (season 2009). Bertoncelj et al. (2007) and Beretta et al. (2005) reported the average FRAP value in multifloral honey samples of 224.8 and 361.9 μM Fe(II), respectively. For determination of free radical scavenging activity, the DPPH method with the stable organic radical 1,1-diphenyl-2-picrylhydrazyl is used and results were expressed as IC_{50}, the amount of antioxidant or concentration of honey solution necessary to decrease the initial concentration of DPPH by 50 %. This means that the lower the IC_{50} value is, the higher is the antioxidant activity of honey sample (Molyneux, 2004). The lowest average DPPH-IC_{50} value in this study was obtained in season 2009 (12.20 mg/mL), and the highest in season 2008 (17.12 mg/mL). Bertoncelj et al. (2007) found that DPPH-IC_{50} average value for Slovenian multifloral honey samples was 10.7 mg/mL, while Beretta et al. (2005) determined in Italian multifloral honey samples the average DPPH-IC_{50} value of 5.32 mg/mL. The significant correlation was found between phenolic content and antioxidant activity determined by DPPH and FRAP assay in multifloral honey samples from season 2007, 2008, and 2009 (r = 0.9592 and r = 0.9382, r = 0.8892 and r = 0.9265, r = 0.8271 and r = 0.9503, respectively).

Table 3. Total phenol content, FRAP values and antiradical power (DPPH) of the honey samples (n = 55) from three different seasons

season	statistics	phenol content (mg GAE/kg honey)	FRAP value (μM Fe(II))	DPPH-IC_{50} (mg/mL)
	mean ± SD	225.61 ± 79.35	405.58 ± 155.85	14.52 ± 4.22
2007	range	109.14 - 440.33	156.72 - 673.14	5.50 - 23.60
(*n* =22)	CV (%)	35.17	38.43	29.06
	mean ± SD	193.76 ± 55.85	364.28 ± 181.97	17.12 ± 8.93
2008	range	116.67 - 293.01	112.81 - 682.81	5.28 - 33.87
(*n* =13)	CV (%)	28.82	49.95	52.16
	mean ± SD	201.14 ± 28.83	295.40 ± 77.21	12.20 ± 3.50
2009	range	141.14 - 247.81	106.32 - 453.90	7.39 - 22.85
(*n* =20)	CV (%)	14.33	26.14	28.69

In order to present all of the obtained data with highlights to their differences and similarities between three investigated seasons, principal components analysis (PCA) was used (StatSoft, 2010). As shown in Figure 1, the multifloral honey samples from season 2007 are separated from samples from seasons 2009 and 2008 on the first principal component (PC-1; 80.72 % of the variance). On the second principal component (PC-2; 19.28 % of the variance) samples from each investigated season are divided in separate groups (Figure 1).

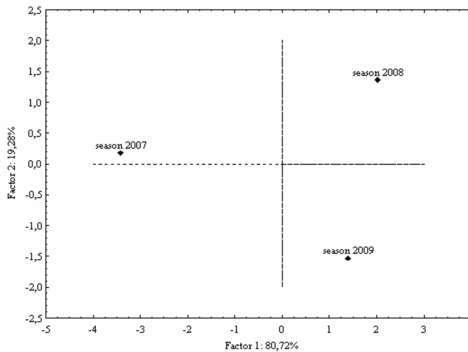

Figure 1. Plot of PC-1 vs. PC-2 for honey samples from three different seasons

Projection of the investigated variables on first two components is shown in Figure 2. The highest loading on first principal component (Table 4) had reducing sugars content (0.9999) and prolin content (0.9952) as confirmed by the PCA plot in Figure 2. This indicates that samples from season 2007 differ from other samples (seasons 2008 and 2009), primarily because of the total reducing sugars content and prolin content. The highest loading on second principal component (Table 4) had DPPH (0.9904) and FRAP values (0.6980), and also diastase activity (-0.5253) indicating that antioxidant activity and diastase activity are parameters responsible for distinction between samples from three investigated seasons. PCA analysis upholds indications based on low diastase units achieved by physicochemical analyses, that samples from 2008 are heated during processing or storage.

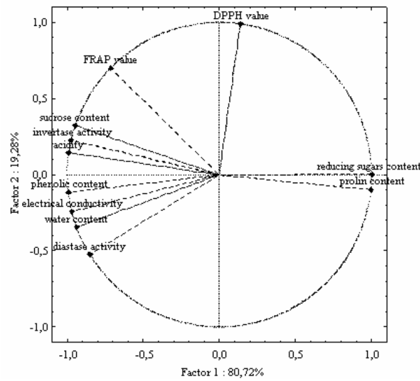

Figure 2. Plot of PC-1 vs. PC-2 for physicochemical parameters, phenolic content and antioxidant activity of honey samples from three different seasons

Table 4. Factor variable correlations (factor loadings)

Variable	Factor 1	Factor 2
electrical conductivity	-0,969741	-0,244136
water content	-0,938529	-0,345201
reducing sugars content	0,999999	0,001342
sucrose content	-0,947060	0,321058
acidity	-0,989659	0,143441
prolin content	0,995165	-0,098217
diastase activity	-0,850915	-0,525304
invertase activity	-0,974504	0,224371
phenolic content	-0,993080	-0,117444
DPPH value	0,137871	0,990450
FRAP value	-0,716104	0,697994

CONCLUSION

Physicochemical parameters of multifloral honey samples produced in Croatia in season 2007, 2008 and 2009 were in accordance to national and international honey-profiling criteria. Multifloral honey is reach source of phenolic compounds and high correlations between phenolic content and antioxidant activities indicate that phenolics are responsible for its antioxidant capacity. Multifloral honey samples from season 2007 differ from honey samples from seasons 2008 and 2009 mainly on the basis of total reducing sugars and prolin content.

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ANTIMICROBIAL EFFECT OF LACTIC ACID BACTERIA FROM MACEDONIAN WINES

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ABSTRACT

The antimicrobial effect of lactic acid bacteria has been documented. Lactic acid bacteria have potential for use in biopreservation. This group of bacteria have several genera and are associated with many different foods and drinks. They have a long history of application in many fermented foods because of their beneficial influence on nutritional, organoleptic and shelf-life characteristic. In fermented foods lactic acid bacteria display numerous antimicrobial activities (De Vuyst and Leroy, 2007).

The antimicrobial activity of 111 isolates from Macedonian red and white wines was tested in this study. The isolates were inoculated in MRS broth. After 48h at 300C MRS plates were inoculated with each broth and each of the isolate was tested for his inhibition activity against the rest by using the toothpicks method. Subsequent to 48h of incubation at 300C the active isolates were noted and percentage of efficiency was calculated. The percent was in the range of 0-7.2. The highest percent 7.2, 5.4, 4.5 and 4.5 showed isolates 181, 152', 81 and 154', respectively. It was low compared to other studies (Yurdugül, 2002; Yurdugül and Bozoglu, 2008). That may be a result of the characteristics of the wine or the grape which has specific landmark of the geographic area. The activity of the isolates had bacteriostatic character and established atypical zones.

KEYWORDS: *Lactic acid bacteria; antimicrobial activity, Macedonian wines*

INTRODUCTION

The antimicrobial effect of lactic acid bacteria (LAB) has been appreciated by man for more than 10 000 years and has enabled him to extend the shelf life of many foods through fermentation processes (Nes et al., 1996). The antimicrobial effect of LAB is mainly due to their lactic acid and organic production, causing the pH of the growth environment to decrease (Nes et al.,1996; Caplice and Fitzgerald, 1999; Kuipers et al., 2000). They also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins (Nes et al., 1996; Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999; Rodríguez et al., 2003). They are further known to produce bioactive molecules such as ethanol, formic acid, fatty acids, reuterin and reutericyclin (De Vuyst and Leroy, 2007) which also have antimicrobial effect, but the antimicrobial ribosomaly synthesized peptides, generally termed bacteriocins, have received special attention (Nes et al., 1996; Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999; Rodríguez et al., 2003). Bacteriocins produced by LAB are small, ribosomally synthesized, antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their bacteriocin(s) (Klaenhammer, 1988; De Vuyst and Vandamme, 1994; Cotter et al., 2005). The antibacterial action towards undesirable bacteria, bacteriocins are believed to contibute to the competitiveness of the producer cells (Vogel et al., 1993). Activity against Gram-negative bacteria such as E. coli and Salmonella has been shown, but usually only when the integrity of the outer membrane has been compromised (Stevens et al., 1991).

Bacteriocins are classified into three main groups: lantibiotics (Class I), non-lantibiotics, small heat-stable peptides (Class II) and large heat-labile protein (Class III) (Nes et al., 1996; O'Sullivan et al., 2002).

Bacteriocin activity is frequently found associated with large aggregates in cell free extracts. These aggregates include not only proteinaceous material but, most probably, also lipids and other macromolecules which could affect the bacteriocin activity (Nes et al., 1996).

The aim of this project was to test the antimicrobial activity of isolates from Macedonian wines. Each of them was tested with the rest. The one that would show the highest inhibition percent may be used in further researches.

MATERIALS AND METHODS

isolates: 111 isolates from Macedonian red and white wines were used. They were isolated during the malolactic fermentation. Red wine samples were: Vranec, Caberne Sauvignon, Syrah, Merlot and white wine: Rizling, Muskat Timjanika, Smederevka, Sauvignon Blank.

medium: MRS (Fluka) broth and agar enriched with Tween 80 (Merck) were used for cultivation. inhibition test: Each isolate was inoculated in MRS broth for 48 h at 300C. Then 0.1 ml of the broth was inoculated on MRS agar plate with L-stick method and incubated at 300C for 60 min. Afterwards the plate was inoculated with the rest of the isolates with the toothpick method and incubated at 300C for 48h. The anaerobic condition was obtained by putting an upper layer of agar.

RESULTS AND DISCUSSION

The results from the inhibition test are established in the following table (Tab.1). The isolates that have shown inhibition on the lawn isolate, after 48h incubation, have been noted.

Table 1. Results from the inhibition test.

Lawn isolate	Isolates that have shown inhibition	Lawn isolate	Isolates that have shown inhibition
2	0	**146**	202
3	0	**148**	241
4	0	**149**	202
5	0	**150**	11, 181
5’	0	**151**	3, 64
6	257, 261	**152**	176, 152’
7	0	**154**	73, 81, 181
8	0	**155**	107
9	119к	**157**	252б
10	81, 187, 159’	**175**	68
10’	149’	**176**	0
11	0	**177**	159’
12	0	**178**	205
13	27, 67, 68, 81, 151, 178, 183, 158’	**179**	0
15	119б	**180**	151’
18	150’	**181**	26
19	0	**182**	146
20	66, 137	**183**	178, 202
21	148, 151, 181	**184**	187
23	5’, 9, 25, 28, 45, 120, 178, 179, 181, 184, 186, 150’, 152’, 155’, 156’, 205, 240, 252б, 262	**185**	259, 156’
24	7, 28, 61, 62, 63, 67, 69, 120, 177, 187, 149’, 151’, 154’, 208, 240, 253, 259	**186**	149
25	262, 150	**187**	138, 145
26	71, 144, 261	**149’**	241, 181
27	158’, 159’, 240	**150’**	25
28	252к	**151’**	61
37	120	**152’**	182
38	37, 183	**153’**	155’
40	0	**154’**	155, 176
45	0	**155’**	150

Lawn isolate	Isolates that have shown inhibition	Lawn isolate	Isolates that have shown inhibition
2	0	**146**	202
3	0	**148**	241
4	0	**149**	202
5	0	**150**	11, 181
5'	0	**151**	3, 64
6	257, 261	**152**	176, 152'
7	0	**154**	73, 81, 181
8	0	**155**	107
9	119к	**157**	252б
10	81, 187, 159'	**175**	68
10'	149'	**176**	0
11	0	**177**	159'
12	0	**178**	205
13	27, 67, 68, 81, 151, 178, 183, 158'	**179**	0
15	119б	**180**	151'
18	150'	**181**	26
19	0	**182**	146
20	66, 137	**183**	178, 202
21	148, 151, 181	**184**	187
23	5', 9, 25, 28, 45, 120, 178, 179, 181, 184, 186, 150', 152', 155', 156', 205, 240, 252б, 262	**185**	259, 156'
24	7, 28, 61, 62, 63, 67, 69, 120, 177, 187, 149', 151', 154', 208, 240, 253, 259	**186**	149
25	262, 150	**187**	138, 145
26	71, 144, 261	**149'**	241, 181
27	158', 159', 240	**150'**	25
28	252к	**151'**	61
37	120	**152'**	182
38	37, 183	**153'**	155'
40	0	**154'**	155, 176
45	0	**155'**	150

According to the number of zones, established by each isolate, percentage of efficiency has been calculated (Fig. 1). The percent was in the range of 0-7.2. The highest percent 7.2, 5.4, 4.5 and 4.5 showed isolates 181, 152', 81 and 154', respectively. Some of the zones of inhibition are shown on Figures 2 and 3.

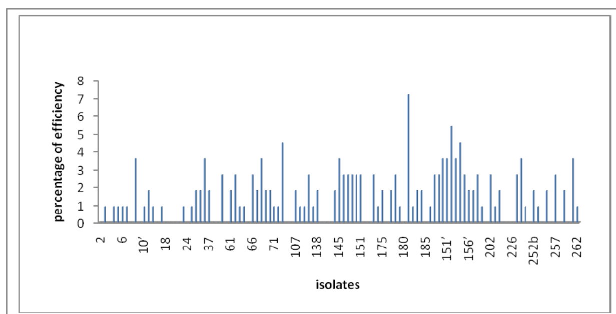

Figure 1. Percentage of efficiency for each isolate.

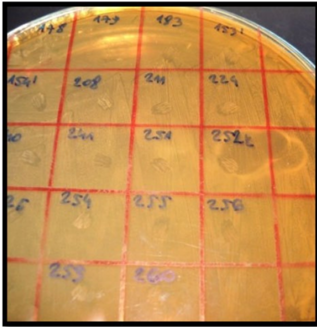

Figure 2. Inhibition zone of isolate 252k

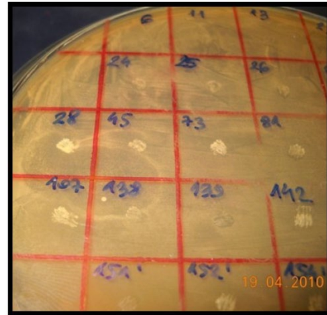

Figure 3. Inhibition zone of isolates 28 and 45 against isolate 63. against isolate 28.

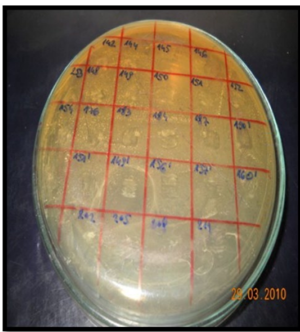

Figure 4. Inhibition zone of isolate 149'

Figure 5. Inhibition zone of isolates 138 and 145 against isolate 10'. against isolate 187.

Yurdugül (2002) and Yurdugül and Bozoglu (2008) performed inhibition test with 120 isolates from wines from the Cappadocia region of Turkey. 88 isolates were effective in the range of 20-41%, but only 16 of them were LAB. Among these isolates, one showing a significant inhibitory potential on other isolates was selected and monitored for inhibitory activities. This isolate was capable of stopping the growth and activity of malolactic isolates and his cell free extract was as well effective on malolactic bacteria.

In our project the percentage of efficiency is significantly lower when compared to that in the studies of Yurdugül (2002) and Yurdugül and Bozoglu (2008). The reason for that could be the characterictics of the used wine samples and of course the characteristics of the primary feedstock, the grape, which has specific landmark of the geographic area. There was also synergism between the isolates that had inhibition capability and established atypical zones. The activity of the isolates had bacteriostatic character.

According to Tadesse et al. (2005), Lactobacillus isolates resulted in the highest diameter of inhibition zone than the other LAB isolates on the test strains. Test microorganisms were Salmonela spp., Shigella flexneri,

Staphylococcus aureus and Esherichia coli O157:H7. Pediococcus isolates also showed relatively large zone of inhibition, while Leuconostoc isolates showed some inhibition activity. The authors claim that the antimicrobial activity depends on the culture growth medium. In addition, the diameter of the zone of inhibition from the culture filtrate of Lactobacillus isolates, grown in LAPTg broth, on some test strains was significantly higher than that from culture filtrate of Lactobacillus isolates grown in MRS broth. LAB might produce more metabolites with antimicrobial property when grown in a less nutitious medium and the zones might not be as a result of the produced acid.

Among the tested isolates for their inhibition activity, Lactobacillus plantarum OL2, OL9 and OL15 and Enterococcus faecaecalis OL20 showed highest inhibition activity. The Lactobacillus isolates were identified according to the growth temperature, disability of growing in 10% NaCl MRS medium, production of L-lactic acid with no gas production from glucose and according to API 50 CHL kits. The Enterococcus isolates were aslo identified according to the growth temperature, pH and API 20 STREP kits. The rest of the Lactobacillus and Enterococcus sp. isolates showed inhibition toward 1-3 isolates. None of the Lactococcus isolates showed inhibition against the used inhibitor strains. The cell free supernatants of Lactobacillus plantarum OL9 was capable to inhibit the growth of Erwinia chrysanthemi ATCC 1254 (Gram-negative bacteria associated with vegetable spoilage), (Kacem и сор., 2005).

CONCLUSION

The microorganisms found in wine depend on the geographic area of origin and of the characteristics of the grape, must and wine. The percentage of efficiency of inhibition of the isolates was in the range of 0-7.2. The activity of the cell free supernatant of the most active isolates could be investigated in the further work.

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IDENTIFICATION OF POLYPHENOLS AND METHYLXANTHINES IN TEAS, HERBAL INFUSIONS, COFFEE AND COCOA PRODUCTS BY NEAR INFRARED SPECTROSCOPY (NIRS) COMPARED TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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ABSTRACT

Bioactive compounds of plant origin are widely used in food industry as functional ingredients. Tea, medicinal plants and coffee are among the richest sources of bioactive compounds (polyphenols and methylxanthines), proven to exhibit positive health effects. Application of bioactive compounds imply development of advanced analytical techniques which enable the identification of new phytochemicals, characterization of their chemical structure, quantification, quality control, etc.

High performance liquid chromatography (HPLC) is the most extensively used but quite time consuming analytical technique for quantification of bioactive compounds. Therefore, use of near infrared spectroscopy (NIRS) was evaluated as the alternative. Six types of „true" teas (black, white, oolong), six herbal infusions and four different coffee brews were analyzed by HPLC technique in order to evaluate the contents of most representative polyphenolic compounds and methylxanthines. The results of HPLC analysis were compared to the ones obtained by NIRS and in order to determine whether the NIRS can be used as an identification technique for the analysis of bioactive compounds. The obtained results revealed a variability in epigallocatechin gallate (EGCG) and caffeine content of true teas. Green tea (*Sencha*) contained the highest content of EGCG (226.88 mg/L), while black tea (*Bherjan*) contained the highest content of caffeine (358.23 mg/L). The content of caffeine and chlorogenic acid in coffee was affected by brewing technique. Instant coffee contained the highest content of caffeine (8.09 g/L). Rosmarinic acid was identified only in herbal infusions. The principal component analysis of NIRS spectra confirmed the possibility to identify the chosen polyphenols and methylxanthines using this analytical technique, and revealed a high correlation of the NIRS spectra with the results of HPLC analysis.

The obtained results imply on the potential of using NIRS for the rapid identification of various phytochemicals in food products.

KEYWORDS: *caffeine; high performance liquid chromatography; NIRS; polyphenols; teas*

INTRODUCTION

Teas, medicinal plants and coffee commonly consumed world wide contain different chemical substances that display a broad spectrum of biological activities, which enables to induce positive effects by treatment of many human diseases. The use of plant extracts as functional ingredients in various food, beverage and cosmetic applications is gaining growing interest for scientists, as well as for consumers and food manufacturers. In the last few decades, numerous screening studies of various plant materials have been performed, in order to find naturally occurring antioxidants for use in food or medicinal preparations, as replacement for potentially harmful synthetic additives (Reische et al., 1998). The dominant majority of biologically active plant compounds with antioxidative properties are flavonoids or other phenolics and methylxanthines, especially caffeine who does not display antioxidant properties but exibit strong stimulative effects on central nervous and other body sytems. Quality control is becoming more and more important, since every single component in a mixture of different plant materials has to be quantified and its characteristics, representa-

tive and specific ingredients identified. The exact knowledge of the bioactive composition of plant derived materials enables to develop a new generation of standardized, effect-optimized mono- and multi-extract preparations, which fulfil today's standards for quality, safety and efficiency of medicinal drugs (Wagner, Ulrich-Merzenich, 2009). In the past few years numerous different methods of analysis have been employed to determine the contents of bioactive compounds. Teas, medicinal plants and coffee have been extensively studied for the presence of bioacive compounds but emphasis has been given to chemical analytical determinations, mostly using spectrophotometric and HPLC analyses. However, chemical analytical methods usually request an extensive consumption of reagent and the use of sophisticated equipment, they are time-consuming and require qualified human resources. Near Infrared Reflectance Spectroscopy (NIRS) is a non-destructive and a rapid qualitative and quantitative analysis method (Wilson, 1994). This technique has already been used by Hall et al. (1988), for measuring the theaflavin and moisture contents as well as for the prediction of black tea quality as assessed by tea tasters. Attempts have been made also to describe the quality as defined by tasters of Chinese tea or Japanese tea by NIRS prediction of total polyphenols, total nitrogen, tannin, and total free amino acid content (Yan et al., 1990; Ikegaya, 1990; Goto et al., 1991). Schulz et al. (1999) also reported on the successful NIRS analysis of caffeine and theobromine in tea (*Camellia sinensis*) and the application of NIRS for the authentication of the two different coffee bean varieties "Arabica" and "Robusta" (Schulz, 2004).

The aim of this study was to employ a NIRS-method for the rapid identification of several representative bioactive compounds (epigallocatechin-gallate (EGCG), caffeine, chlorogenic acid and rosmarinic acid) of tea, herbal infusions and coffee brews and to determine the contents of these compounds using HPLC analysis. The potential of using NIRS as an analytical technique for the identification of bioactive compounds in various plant derived substartes were compared to HPLC analysis using principal component analysis.

MATERIALS AND METHODS

Preparation of coffee, tea and plant extracts

Three green teas (Sencha, Bancha, Rose of the Orient), two black teas (Bherjan, Pettiagala), white tea (Pai Mu Tan superior) and roasted matè tea were purchased at a special market - House of tea. Dried plant material of lemon balm (*Melissa officinalis L.*), thyme (*Thymus serpyllum* L.), sage (Salvia officinalis L.) and peppermint (*Mentha piperita* L.), were purchased at a local medicinal plant manufacture. In order to simulate beverage brewing, teas, herbal infusions and coffees were prepared using an aqueous extraction procedure. The extraction of teas was carried out by pouring 200 mL of distilled water hated to 80°C over the plant samples (2 g) at room temperature, while for the preparation of herbal infusions 200 mL of boiling distilled water was poured over 2 g of ground plant at room temperature. After extraction (3 min for teas and 10 min for herbal infusions), the extracts were filtered through a tea strainer.

Four different coffee beverages were prepared (espresso, filter, instant, turkish (boiled) coffee), using coffee samples purchased at a local market (7 g/100 mL of water for boiled, filter and instant, and 7g/30 mL of water for espresso) and apparatus specially suited for that particular type of brew.

HPLC analysis of phenolic compounds and methyxanthines

All analyzed samples were filtered through a 0.45 μm filter (Nylon Membranes, Supelco, Bellefonte, USA) before the HPLC analysis. 20 μL of each sample was injected for HPLC analysis using a Varian Pro Star Solvent Delivery System 230 (Varian, Walnut Creek, USA) and a Photodiode Array detector Varian Pro Star 330 (Varian, Walnut Creek, USA) by using a reversed-phase Zorbax C-18 column (Phenomenex, USA) (150 × 4.6 mm, 2.6 μm i.d.). The solvents consisted of 2% formic acid in water (solvent A) and methanol (solvent B) at a flow rate of 1.0 mL/min. The elution was performed with a gradient starting at 2% B to reach 32% B at 20 min, 40% B at 30 min and 95% B at 40 min, and becoming isocratic for 5 min. Chromatograms were recorded at 278 nm. Detection was performed with a Photodiode Array Detector by scanning between 200 and 400 nm, with a resolution of 1.2 nm. Phenolic compounds and caffeine were identified by comparing the retention times and spectral data with those of standards. The data acquisition and treatment were conducted using Star Chromatography Workstation Version 5 software. All analyses were repeated three time. The

results were analyzed statistically using the Statistica 8.0 to determine the average value and standard error.

Data acquisition of NIR spectra

The technique of NIR spectroscopy is based on the electromagnetic absorption at the near-infrared region. NIR spectra were collected continuously over the range of 904-1699 nm using a Control Development, Inc., NIR-128-1.7-USB/6.25/50μm with an optical fiber using installed Control Development software Spec 32. The mean of the five spectra which were collected from the same sample was used in the following analysis step. The temperature was kept around 25 °C and the humidity at a steady level in the laboratory.

NIR spectra analysis and statistical analysis

NIR spectra were recorded in Excel format and imported to STATISTICA v.8 for analysis. The original spectra by Savitsky-Golay "smooth" algorithm for data filtering were imported. The spectrums were analysed using principal component analysis (PCA) based on the correlation matrix between the values of the characteristics.

RESULTS AND DISCUSSION

The contents of several polyphenolic compounds and caffeine as the most representative bioactive compounds of analyzed plant materials, determined using HPLC analysis are displayed in Table 1.

As can be seen EGCG is the second most abundant bioactive compound in the analyzed samples, which can be found only in „real" teas derived from the *Camellia sinensis* plant. The highest content of EGCG was determined in green teas (226.95 mg/L in Sencha and 137.33 mg/L in Bancha) followed by white tea (124.72 mg/L in Pai mu Tan), while the lowest content of EGCG was detected in black tea (70.35 mg/L in Pettiagala). Chlorogenic acid is the only compound found in all analyzed samples, which is in agreement with the fact that this hydroxycinnamic acid with strong antioxidative properties is one of the most widely spread bioactive secondary metabolites in plant materials belonging to the group of polyphenolic compounds. The highest content of chlorogenic acid was determined in coffee brews (88.36 in filter – 105.73 mg/L in turkish brew). Rosmarinic acid is a common constituent of all *Lamiaceae* species, so it is not surprising that this compound has been found only in herbal infusions prepared from the plants derived from this botanical family. Belonging to the class of hydroxycinnamic acids, this compound exhibits significant biological activity owing to its antioxidative properties. Rosmarinic acid is the most abundant compound among the plants analyzed in this study, and its content ranges from (128.33 mg/L) in sage to (362.25 mg/L) in lemon balm.

Caffeine was detected in teas and coffees, which confirm that herbal infusions prepared from various medicinal and aromatic plants do not contain this stimulating substance. This finding supports the frequent cosumption of herbal infusions by some consumers rather than teas or coffee due to their high conent of caffeine and its negative effects upon consumption. Namely, caffeine exerts pharmacological effects on the central nervous system, the heart, the renal system, the peripheral and central vasculature, the gastrointestinal system, and the respiratory system (Lawrence, 1986). The strong pharmacological effects of caffeine have led to consumer demand for caffeine-free coffee beverages. As can be seen the highest content of caffeine was determined in instant coffee (212.88 mg/L), followed by Bherjan black tea (188.26 mg/L) and Pai mu tan white tea (182.35 mg/L), while roasted matè infusion prepared from the leaves of south-american plant *Ilex paraguariensis,* exhibits the lowest content of caffeine (36.00 mg/L). The content of caffeine is varying significantly depending on the brewing technique, so it can be seen that instant and espresso coffees contain the highest, and filter coffee the lowest caffeine content. This is apart from the brewing technique also a results of the processing technique of instant coffee which is produced by concentrating the coffee brew and the spray-drying which leads to higher concentrations of bioactive compound in that coffee type.

Table 1. The content of polyphenols and methylxanthines in the analyzed teas, herbal infusions and coffee brews determined by using HPLC analysis

	Polyphenols			Methylxanthines
	EGCG mg/L	Chlorogenic acid mg/L	Rosmarinic acid mg/L	Caffeine mg/L
Teas				
Sencha	226.95 ± 13.45	32.13 ± 2.11	n.d.	132.04 ± 4.45
Bancha	137.33 ± 8.78	22.64 ± 1.78	n.d.	101.13 ± 9.87
Rose of the Orient	100.50 ± 6.45	44.99 ± 0.48	n.d.	107.15 ± 3.65
Bherjan	86.48 ± 5.13	54.26 ± 2.87	n.d.	188.26 ± 11.25
Pettiagala	70.35 ± 2.15	51.17 ± 4.36	n.d.	131.83 ± 7.36
Pai mu tan	124.72 ± 9.87	34.85 ± 2.65	n.d.	182.35 ± 8.25
Roasted Maté	n.d.	29.51 ± 1.45	n.d.	36.00 ± 0.12
Herbal infusions				
Lemon balm	n.d.	18.76 ± 0.87	362.25 ± 13.54	n.d.
Peppermint	n.d.	19.65 ± 1.01	282.76 ± 22.58	n.d.
Thyme	n.d.	22.09 ± 1.45	200.88 ± 19.65	n.d.
Sage	n.d.	17.04 ± 0.64	128.33 ± 7.89	n.d.
Coffee brews				
Instant	n.d.	100.46 ± 6.54	n.d.	212.88 ± 15.78
Turkish/boiled	n.d.	105.73 ± 1.56	n.d.	120.53 ± 7.89
Espresso	n.d.	96.133 ± 5.15	n.d.	182.92 ± 5.64
Filter	n.d.	88.36 ± 3.47	n.d.	98.66 ± 7.28

n.d. – not detected

Most of the analytical methods used for determination of polyphenols content are time-consuming, due to requiring sample preparation. Opposite to that near infrared reflectance spectroscopy is a fast, accurate and non-destructive technique. Since 1990s, the attempts have been made to simultaneously predict alkaloids and phenolic substances in green tea leaves using near infrared spectroscopy (Chen et al., 2008). In this work teas, herbal infusions and coffee brews listed in Table 1. were analysed using NIR spectroscopy. Since the NIR spectra do not provide specific discriminatory peaks additional analysis is necessary.

Analysis of the spectrums revealed wave lengths specific for each group of analysed samples; range for 1370-1390 for teas corresponding to C-H bonds, range form 920-950 1390 for herbal infusion corresponding to C-H 2. overtone and O-H bonds and 1340-1350 for coffee brews corresponding to C- H bonds. The aim of this study was to test if NIR could be useful for identification of polyphenolic compound is teas and coffees. The principal component analysis (PCA) was applied to perform a preliminary study of the data structure; whose results are presented in Figure 1. Sums of factors percent contribution on each graph show that over 95% of data was taken into analysis.

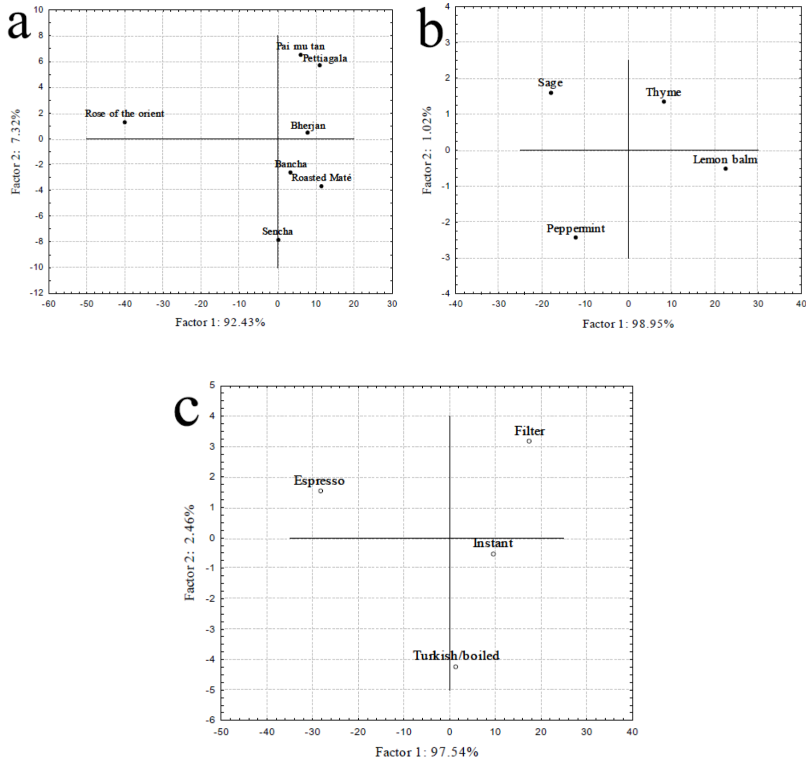

Figure 1. Results of PCA analysis for (a) teas, (b) herbal infusions, (c) coffee brews

Analysis of the NIR spectra of teas (Figure 1a) showed that analyzed samples could be grouped in two classes with the exception of Rose of the Orient tea. When compared with the results of HPLC analysis it becomes obvious that the first group of teas (Pai mu Tan, Pettiagala, Bherjan) located in the positive region of Factor 2, is characterized with the highest content of caffeine and the lowest content of EGCG among the analyzed teas. Similarly, it can be observed that Sencha and Bancha grouped at the lower part of Figure 1a contain the highest EGCG content and moderate caffeine content. Among herbal infusions (Figure 1b) and coffee brews (Figure 1c) significant differences between the sampleswere observed. This is in agreement with the contents of the main representatives of bioactive compounds for these two groups of products, rosmarinic acid in herbal infusions and caffeine in coffee brews determined by the HPLC analysis. Namely it can be seen (Table 1), that the contents of both rosmarinic acid and caffeine vary significantly depending on the plant or coffee brew technique, which explains their scattered appearance on Figures 1 b&c.

CONCLUSIONS

The results obtained by the HPLC analysis revealed that the content of specific bioactive compounds of the evaluated teas, herbal infusions and coffee brews depends on the type of plant material and the brewing technique of coffee. Based on the PCA analysis of NIR spectra it can be concluded that NIR spectroscopy could be used for rapid analysis and distinguishing of tea and coffee samples. For more accurate quantitative

determination of bioactive compounds composition of tested samples additional NIR calibration would be necessary.

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CHARACTERIZATION OF OLIVE VARIETIES FROM ALBANIA: FATTY ACID PROFILE AND TOTAL PHENOLIC CONTENT

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ABSTRACT

The research study presented in this paper, the first of its kind in Albania, characterizes the fatty acid profile and total phenolic content of some olive varieties, namely, *Boçi*, *Ulli i Kuq, Ulli i bardhe Lezha*, *Sterbjak* and *Micka* all harvested during crop year 2010-2011.

Annual production capacity of the country is 56 000 tons of olive fruits and 7000 tons of olive oil. Fatty acid (FA) profiles to the studied olive cultivars exhibit a great variation in oleic acid, from 66.32 ± 0.14 % (Ulli i Kuq) to 75.13 ± 0.31 % (Sterbjak), values which are within the normal range for such FA. The content of linoleic acid varies from 7.23 ± 0.04 % (Sterbjak) to whereas the content of linolenic acid varies from 0.45 ± 0.01 % (Ulli i Kuq) to 0.96 ± 0.01 % (Sterbjak). All studied olive varieties revealed moderate levels of palmitic acid, which varied between 10.76 ± 0.05 % (Sterbjak) and 13.05 ± 0.02 % (Boçi). From a nutritional point of view, it is worth noticing that the Sterbjak variety has an n-6/n-3 ratio of 7.68, while the Ulli i bardhe Lezha goes to 10.20.

The Total Phenolic Content for the studied olive cultivars varied from 63.02 ± 5.63 GA mg/kg olive oil (Ulli i kuq) to 322.05 ± 3.97 GA mg/kg olive oil Ulli i bardhe Lezha such variation reflect differentness among studied olive varieties.

KEYWORDS: *Albanian Native Olive Cultivars; Sterbjak; Ulli i Bardhe Lezha, Ulli i Kuq, Fatty acid Profiles*

INTRODUCTION

The origin of olive trees in Albania is not different from the road of the distribution of this tree in Mediterranean region. Archaeological evidences on the agriculture activities such as wheat cobs, truss grapes, olive lop are stamped in stones and coins of the Illyrian tribes. Olive tree is planted mainly in the Western Lowland, by penetrating the mainland through the river valleys (Kafazi, Muço, 1984; Thomaj, Panajoti, 2003). The analyzed olive cultivars belong to the regions of Tirana and Lezha, in the Central and Northern part of Country. The actual annual producing capacity is 56 000 tons of olive fruits and 7000 tons of Olive oil (FAOSTAT, 2011). Recent genetic studies concluded that Albania owns 22 native olive cultivars (Bacu-Grazhdani et al, 2008). They are grouped according to their region of cultivation and classified as primary and secondary cultivars based on cultivar distribution (Thomaj, Panajoti, 2003).

Olive oil is a vegetable oil, extracted by the olive fruits, that can be consumed without prior refining treatment (Angerosa et al, 2006; Aparicio, Luna, 2002). Triglycerides are its major components and represent more than 98 % of the total weight. The remaining part are non-saponifiable chemical compounds such as sterols, polyphenols, alcohols, waxes, hydrocarbons, etc (Servilli, Montedoro, 2003; Mannina et al, 2003). Its composition depends on numerous factors such as the interaction between the cultivar and the environment, cultivation techniques, fruit ripeness and the oil extraction system (Aparicio, Luna, 2002). The characterisation of fatty acid profiles of olive oils from different olive cultivars is usually proposed as a methodology to differentiate these products according to their cultivar and geographical origin (Aparicio, Luna, 2002; Mannina et al, 2003).

In this study, are analysed the fatty acid composition and total polyphenol content of five olive cultivars: *Boçi, Ulli i bardhe Lezha (UbL)*, *Sterbjak*, *Ulli i Kuq* and *Micka* which belong to the Tirana and Lezha regions. These cultivars are mainly used in the oil production, although *Boçi* is used also in parallel as table olives. The *UbL* is the most planted by 2 % of the total of 6 million olive trees.

The scope of the study presented herein is linked with chemical evaluation of the Monovarietal Virgin Olive Oils. Assessment of quality parameters and nutritional value of olive oils from studied cultivars is presented in this paper. Nowadays, an attempt to modify the national fund of olive tree is an ongoing process. Such pioneering study will allow for the identification of native cultivars that produce good quality olive oil and that are very well adapted to the pedo-climatic conditions in Albania.

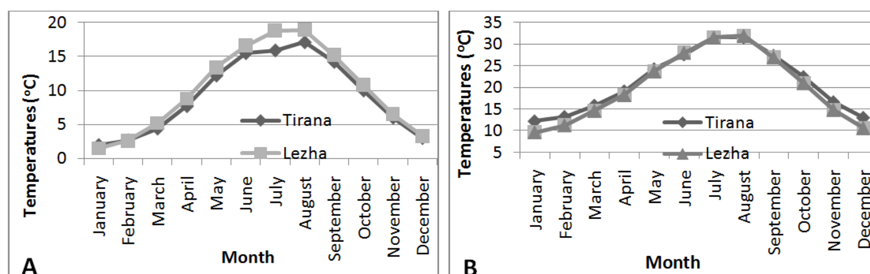

Figure 1: Monthly minimum (A) and maximum (B) average temperatures of Tirana and Lezha regions

MATERIALS AND METHODS

Sample collection and oil extraction

Native olive varieties, namely: *Boçi*, *Ulli i Bardhe Lezha*, *Sterbjak, Ulli i Kuq* and *Micka* were harvested in their main area of Tirana and Lezha (Table 1). The climatic characteristics of the production areas, in terms of temperature, are reported in Figure 1.

Oil extraction was performed with a SPREMOLIVA Press (an Italian TEM technology, Italy). The extraction condition was cold, mechanical pressing. SPREMOLIVA apply discontinuous process with 20-25 kg/cycle. After extraction the oil samples were stored in the dark at 4 oC until analysis.

Table 1: Olive varieties, harvesting and extraction day

Cultivar name	Harvesting day	Extraction day	Region
Boçi	25/10/2010	25/10	Tirana
Ulli kuq	13/11/2010	16/11	Tirana
Micka	11/11/2010	16/11	Tirana
Ulli i Bardhe Lezha	10/11/2010	13/11	Lezha
Sterbjaku	10/11/2010	13/11	Lezha

Chemicals

The chemical reagents were analytical grade, from Sigma-Aldrich Chemie (Steinheim, Germany). Internal standard C 15:0 was purchased from Sigma-Aldrich. Gallic acid and Folin-Ciocalteau reagent were supplied by Fluka Chemie GmbH (Buchs, Switzerland).

Determination of fatty acid profiles

Fatty acid methyl esters (FAME) were prepared through direct acidic transesterification, as originally proposed by Lepage and Roy (1984) and later modified by Carvalho and Malcata (2005), using Pentadecanoic acid (C 15:0) as internal standard. The assay of FAME was carried out with a HP-6890 Gas chromatograph,

equipped with a Flame Ionization Detector (GC-FID). Separation was achieved in a SP-2380 capillary column (60 m × 0.25 mm × 0.20 m) from Supelco. Hydrogen was used as carrier gas at a flow rate of 1.0 mL/min. The temperatures in the injector and detector were 240 °C and 260 °C, respectively. The injection was performed in split mode (1:50). Oven temperature was set to 150 °C increased to 200 °C at a rate of 1 °C/min and held at 200 °C for 20 min. Calculations were performed according to AOCS Official Method Ce 1b-89 (AOCS, 1994).

Determination of Total Polyphenol Content

Fractionation of olive oils

The method used to perform the fractionation of oils was proposed by Kalantzakis *et al* (*2006*). Briefly, samples were dissolved in n-hexane (Sigma, Germany) and extracted with a methanol/water mixture (60:40, vol/vol). After dissolving 2.5 g of oil with 5 mL n-hexane, 5 mL of methanol/water 60:40 were added, and the mixture was shaken vigorously and centrifuged at 3500 rpm for 10 min. The polar fraction was used, as it was, for further analysis.

Colorimetric determination of total polyphenol content

The Colorimetric method was used to determine the total polyphenol content (TPC) of samples, according to method proposed by Kalantzakis *et al.* (2006). An UV-VIS Mini-1240 Spectrophotometer (Shimadzu) was used at 725 nm. Results were expressed as Gallic acid equivalent (mg/kg olive oil), calculated from the following calibration curve, determined by linear regression.

Statistical analysis

The complete data were evaluated by randomized block design, with three replicates from fatty acid analysis and duplicates for TPC values. Results were displayed as mean values and standard error (n=3). Significance of the differences among the values was determined by analysis of variance using One-way ANOVA test. The level of significance was determined at $P < 0.05$. The employed statistical program was SPSS 17.0 Statistics 2008 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Fatty acid profiles

Olive cultivars studied are described in Table 2. One-way ANOVA analysis showed that fatty acid profiles of the seven olive cultivars were statistically significantly different. Results revealed that, in what concerns Palmitic acid (PA), the cultivars can be grouped in two groups: (i) those with lower PA content, such as Sterbjak (10.76 ± 0.05 %) and Micka (10.79 ± 0.01 %) cultivars, and those with higher PA content, such as UbL (12.85 ± 0.01 %), Ulli i kuq (12.95 ± 0.03 %), and Boçi (13.05 ± 0.02 %).

The oleic acid (OA) content was: 67.65 ± 0.01 % (Boçi), 75.13 ± 0.31 % (Sterbjak), 70.84 ± 0.07 % (UbL); 66.26 ± 0.14 % (Ulli i kuq) and Micka 73.471 ± 0.14 %. Cluster analysis of the studied cultivars regarding the FA profiles indicate that olive cultivars are grouped in two groups; those with low content of OA such as: Boçi and Ulli i Kuq; and with high content of OA: UbL, Sterbjak and Micka.

Linoleic acid (LA) content showed high variation among the studied cultivars; olive cultivars such as Sterbjak (7.23 ± 0.04 %), Micka (9.10±0.02 %) and UbL (8.69 ± 0.00 %), presented low content of LA while the others Ulli i kuq (13.29 ± 0.02 %) and Boçi (12.47 ± 0.01 %), presented high contents – almost two-fold the amount in some cases. The -Linolenic acid (ALA) is below 1 %, a condition for the quality of the Extra Virgin Olive Oils (EEC 2568/91). The ALA content varied according to the following ascending order: 0.45 ± 0.01 % (Ulli i kuq), 0.64±0.00 % (Boçi), 0.86 ± 0.00 % (UbL), 0.94 ± 0.01 % (Sterbjak) and 0.54±0.00 % (Micka).

A high content of ALA contributes to the n-6/n-3 ratio, a very important value for the nutritional evaluation of lipids of different origin. Regarding such ratio, the Sterbjak cultivar show a n-6/n-3 ratio of 7.68, followed

by 10.12 for UbL, while the remaining cultivars show higher values, 16.98 (Micka) and 19.5 for Boçi.

Table 2: Fatty acid profile (in % of total FA), Iodine Value, n-6/n-3 and 18:1/18:2 ratios of the olive cultivars

Formula	Boçi	UbL	Sterbjak	Ulli i kuq	Micka
14:0	ND	ND	ND	ND	N.D
16:0	13.05±0.009‡	12.85±0.003‡	10.76±0.028‡	12.95±0.02‡	10.799±0.010
16:1(n-9)	0.15±0.00‡	0.12±0.00‡	0.146±0.003‡	0.096±0.008‡	0.023±0.040
16:1(n-7)	0.63±0.00‡	0.97±0.00‡	0.646±0.003‡	0.826±0.003‡	0.606±0.003
17:0	0.12±0.00‡	0.00±0.00‡	0.00±0.00‡	0.15±0.00‡	0.084±0.001
17:1 (n-7)	0.223±0.003‡	0.00±0.00‡	0.00±0.00‡	0.25±0.00‡	0.175±0.003
18:0	2.113±0.003‡	2.536±0.003‡	2.206±0.006‡	2.643±0.008‡	2.306±0.005
18:1(n-9)cis	67.643±0.003‡	70.843±0.039‡	75.123±0.81‡	66.26±0.081‡	73.471±0.140
18:1(n-7)	2.24±0.00‡	2.49±0.00‡	2.10±0.01‡	2.28±0.025‡	2.271±0.031
18:2(n-6)trans	ND	ND	ND	ND	ND
18:2 (n-6)cis	12.467±0.008‡	8.686±0.003‡	7.233±0.021‡	13.286±0.008‡	9.098±0.022
20:0	0.346±0.006‡	0.423±0.006‡	0.363±0.012‡	0.41±0.005‡	0.347±0.008
18:3 (n-3)	0.64±0.00‡	0.86±0.00‡	0.943±0.006	0.443±0.003	0.538±0.003
20:1 (n-9)	0.24±0.01^{NS}	0.176±0.048^{NS}	0.236±0.008^{NS}	0.246±0.033^{NS}	0.208±0.025
22:0	0.116±0.008‡	0.056±0.003‡	0.063±0.003‡	0.153±0.012‡	0.050±0.087
Σ–SFA	15.75	15.87	13.39	16.27	13.587
Σ–MUFA	70.41	74.60	78.25	69.95	76.752
Σ–PUFA	13.09	9.72	8.17	13.73	9.663
n-6/n-3	19.52	10.12	7.68	29.8	16.976
18:1/18:2	5.38	8.15	10.39	4.99	8.075
MUFAs/SFAs	4.47	4.70	5.83	4.30	5.649
MUFAs/PUFAs	5.38	7.67	9.57	5.09	7.943
Iodine Value	85.807	82.765	83.881	85.71	84.05

End note 1: SFA= Saturated Fatty Acids; PUFA = Polyunsaturated Fatty Acids; MUFA= Monounsaturated Fatty Acids; ‡-Statistically significant among the different olive cultivars (p<0.005); and †-Statistically significant (p<0.05); NS-Statistically Non Significant among olive cultivars.

End note 1: SFA= Saturated Fatty Acids; PUFA = Polyunsaturated Fatty Acids; MUFA= Monounsaturated Fatty Acids; ‡-Statistically significant among the different olive cultivars (p<0.005); and †-Statistically significant (p<0.05); NS-Statistically Non Significant among olive cultivars.

Comparison of the FA profiles of the olive oils from Albanian native cultivars with those in neighbouring countries: Italy, Greece (Aparicio, Luna 2002; Pinelli et al. 2003; Paz Aguilera et al. 2005) and Northern Africa (Haddada et al. 2008) gives indication that they are comparable with Italian and Greek olive cultivars (Paz Aguilera, et al. 2005). For example, the level of palmitic acid in the studied native cultivars is comparable with Italian cultivars Leccino (14.3 %) and Moraiolo (10.5 %); Spanish cultivars Arbequina (14.3 %); Lechin (10.5 %) and Redondilla (12.5 %), and Greek cultivar Koreiniki (13.3 %). The level of oleic acid in Albanian cultivars is comparable with Frantoio (78.2 %), Arbequina (75.3 %) and Koreiniki (71.9 %) (Aparicio, Luna, 2002). Related to the linoleic acid trends is not as even since the Albanian olive cultivars present content differences. While the Boçi cultivar presents high content of linoleic acid comparable to the Spanish cultivars Redondilla and Lechin (Aparicio, Luna, 2002).

Oxidation stability

Analysis of the ratio 18:1/18:2 is an indication that refers to the oil oxidation stability. The lowest value proposed is 18:1/18:2 ≥ 7 (Kiritsakis et al. 1998). The results show that the Monovarietal olive oils from 8.08 (Micka) and 10.39 (Sterbjak) have acceptable oxidation stability, whereas the 4.99 (Ulli i kuq); and 5.38 (Boçi) cultivars have ratios under the minimum threshold meaning a minimal oxidation stability. The ratio between monounsaturated and saturated fatty acids of the studied cultivars had an average value of 4.86, whereas the ratio between monounsaturated and polyunsaturated fatty acids presented an average value of 7.09 (Table 2), which are relatively low; however, the high phenol content could indicate that oil quality was maintained without lipid deterioration.

The estimation of oil stability for the analyzed olive cultivars was analyzed based on the proposed equation for Iodine Value (Kiritsakis et al. 1998). The Iodine Values recorded for the seven cultivars are in the referred range of 80-90 for olive oils. The Iodine Value belongs vary from Ulli bardhe Lezha (82.76), to the max value Boçi (85.81) cultivars.

Phenolic compounds

Quantitative determination of phenolic compounds in olive oil was performed according to the colorimetric method (Kalantzakis et al. 2006). The amount of phenolic compounds in olive oil varies from 50 to 1000 mg/kg and depends on several factors such as: climate, and extraction technology, degree of maturation (Servilli, Montedoro, 2003) and cultivar (Montedoro et al. 1992).

The results for the five olive cultivars (Figure 2) reveal that the highest value belongs to the UbL cultivar 322.05 ± 3.97 mg/kg olive oil, and the lowest value belongs to Ulli i kuq by 63.02 ± 3.98 mg/kg olive oil. The results show that the polyphenol content of the studied olive oils had significant differences ($p<0.05$) among the cultivars. According to the classification for the TPC level of the olive oils proposed by Montedoro et al (1992) the studied cultivars can be classified as: "low" (50-200 mg/kg) Ulli i kuq, Sterbjak and Micka cultivars; "medium" (200-500 mg/kg) UbL and Boçi cultivars. The results obtained for the studied cultivars can be related mainly with the cultivar differences.

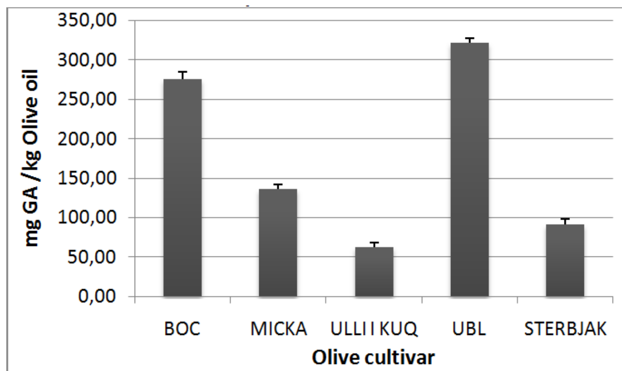

Figure 2: TPC values of the studied olive cultivars (as Gallic acid Equivalent mg/kg olive oil)

The actual stage of agriculture in Albania does not have premise for irrigation of the olive plantations. Furthermore, concerning the maximum and minimum temperature values in the different regions, respectively, give evidence that differences are not significant. Hence, differences reported in TPC content are not due to climatic conditions, but they might come from the cultivar caracteristics. The TPC values of UbL and Boçi are comparable with Koreiniki (Greece), Picual (Spain) and Frantoio (Italy) olive cultivars (Paz Aguilera et al. 2005).

CONCLUSIONS

The results presented show significant differences in the chemical composition of the studied cultivars. Those variations, observed in fatty acid composition and phenolic compounds, are probably due to both genetic factors and environmental conditions. By comparison with results from literature, it can be concluded that the levels of fatty acids in the oils of the studied cultivars are similar to those found in the group of olive cultivars typical from Northern Mediterranean regions. The nutritional profile of Sterbjak cultivar is highly interesting, as well as the UbL olive cultivar, due to its n-6/n-3 ratio and total phenolic content.

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THE EFFECT OF EXTRACTION CONDITIONS ON THE TOTAL PHENOLIC CONTENT, TANNIN CONTENT AND ANTIRADICAL ACTIVITY OF CAROB EXTRACTS

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ABSTRACT

The main goal of conducted study was to enhance extraction rates of phenolics from carob pods. Namely, carob fiber is rich in amount and variety of phenolic antioxidant substances, and therefore, could be used as the source of natural antioxidants in everyday nutrition or in creating novel functional foods with increased content of antioxidative bioactive compounds. Four extraction procedures described in literature (reflux cooking, shaking overnight at room temperature, shaking at 37 °C, ultrasound extraction) and three types of solvents (water, 50% ethanol, 80% ethanol) were evaluated regarding total phenolic content, tannin content and antiradical activity of obtained extracts. The procedures with the highest extraction yields were modified in order to additionally increase extraction efficiency. Reflux cooking and ultrasound extraction were found to be the most efficient procedures for extraction of antioxidants from milled carob pods resulting in the highest total phenolic content and the highest antiradical activity of obtained extracts. In order to additionally increase the yields, duration of mentioned procedures was extended several times until maximum yields were achieved. Finally, 60-minute reflux cooking of carob pods with water was found to be the most efficient way for extraction of phenolic antioxidants from carob, resulting in the following yields: total phenols/tannins: 993.32±49.95/539.30±32.93 g tannic acid equivalents/100g dry matter; Trolox equivalent antioxidant capacity: 15631.62±369.10 mg Trolox equivalents/100 g dry matter. Application of high temperature in combination with polar solvent was found to be the most efficient way for extraction of antioxidants from carob pods, probably due to the fact that the majority of carob phenolics are bound to the dietary fiber fraction and cannot be extracted when mild extraction conditions are applied. The extension of extraction time was stopped at 60 minutes since further prolongation did not significantly influence obtained extraction yields.

KEYWORDS: *carob; antiradical activity; extraction; phenols*

INTRODUCTION

Carob (*Ceratonia siliqua* L.) is a beanlike fruit that grows widely in the Mediterranean region. The carob pod is about 20 cm long, very sweet, intensively flavored and spongy, and therefore has been used in a diet since ancient times. Its nutritive composition is mainly characterized with low fat content and high amounts of soluble carbohydrates (40 - 60 % - -mainly sucrose), dietary fibers, and certain essential minerals, especially Ca (Yousif and Alghzawi, 2000). Carob´s application in food industry is mainly focused on the extraction of carob been gum which is added to variety of products as thickener, stabilizer or flavorant (Bouzouita et al., 2006). Carob seeds are also important in terms of their medicinal value since carob gum has been found to treat infantile diarrhea and to control hyperlipidemia (high cholesterol) while carob germ, , has been found to be suitable both as a major dietary item and for medical use due to its good amino acid profile and high content of Ω-6 PUFA (Dakia, 2011). In spite of its high nutritional value, the use of deseeded carob pod is generally low, resulting in its very low economical market potential. More recent literature data indicate that carob pods contain very high amounts of nonessential polyphenolic components. Carob phenolic fraction consists mainly of soluble and insoluble tannins, flavonon glycosides and different forms of gallic acid – free, gallotannins and methyl-galat. In addition, carob´s water extracts also contain smaller amounts of catechin, myricetin rhamnoside, erioglictyol glycoside, quercetin glycoside and quercetin rhamnoside (Owen et al., 2003). These data indicate that carob fiber is rich in both amount and variety of phenolic

antioxidant substances, and its inclusion in the diet/foods may provide antioxidative and chemoprotective effects. For example, antiproliferative effects of carob extracts as well as protective effects on hemin induced modulation of colon cell growth parameters have been proven recently (Klenow and Glei, 2009; Corsi et al., 2002). Due to satisfying nutritional and sensory characteristics and mentioned high amount of phytochemicals exerting antioxidant properties, carob flour is starting to be acclaimed as an desirable ingredient to contemporary food industry in the area of developing healthy and functional food formulations or even nutritional supplements. One possible approach of carob utilization is the production of carob pod extract that might be used as the powerful natural antioxidant in the variety of (functional) food products. In order to be able to completely utilize the potential of carob as the source of polyphenolic compounds, efficient extraction procedures should be developed. Therefore the aim of this study was to maximize the extraction yield of polyphenols and achieve improved antioxidant activity of obtained extracts. In order to seek more environmentally friendly methods of extraction, only low toxic water-ethanol mixtures were investigated as possible extraction solvents.

MATERIALS AND METHODS

Carob pods

Carob pods were obtained from the private grower from Dalmatia (Primošten area). Prior to analysis 500 g of pods were washed in distilled water, dried, deseeded, chopped, air dried, ground to flour (particle size ≤ 2mm) and stored in plastic containers at 4°C until analysis.

Chemicals

All reagents used were of analytical grade. Folin-Ciocalteu reagent was purchased from Fluka (Switzerland); ABTS (2,2´-azinobis(3-ethylenebezoline-6-sulphonic acid) radical cation) and Trolox (6-hydroxy-tetramethylchroman-2-carboxylic acid) were from Sigma Aldrich (Germany).All other used chemicals were from Kemika (Croatia).

Total phenolic content determination

Total phenolic content in obtained carob pod extracts was assessed using Folin-Ciocalteu spectrophotometric method (Singleton and Rossi, 1965). The method is based on the ability of phenolic hydroxyl groups to reduce phosphomolibdates and phosphovolframates from the Folin- Cicoalteu reagent which results in development of the characteristic blue color. The absorbance was recorded spectrophotometrically at 725 nm and results were expressed as tannic acid equivalents (mg of TAE/100 g on dry matter basis).

Determination of tannin content

Determination of tannin content was conducted according to the indirect procedure by Makkar et al., (1993) with some modifications. Tannins were precipitated in obtained extracts using PVPP and the content of the remaining polyphenols was assessed in obtained supernatants using Folin-Ciocalteu method. The content of tannins was calculated as the difference between the total phenolic content of analyzed extracts prior and after the tannin precipitation and expressed as tannin acid equivalent.

Determination of radical scavenging activity

Radical scavenging activity of carob extracts was assessed using the Trolox Equivalent Antioxidant Aactivity Assay (TEAC) which is based on the ability of antioxidant molecules to quench the $ABTS^{\cdot+}$, a blue-green chromophore with the characteristic absorption at 734 nm. Estimation of ABTS radical scavenging activity was conducted according to the procedure of Re et al. (1999). Appropriate solvent blanks were run in each assay. The percentage of inhibition was calculated using the equation (1) and compared with standard calibration curve for Trolox. The results were expressed as mg Trolox equivalent per 100 g on dry matter basis.

$$\Delta A_{(sample)} = \frac{A_{t=0min(sample)} - A_{t=3min(sample)}}{A_{t=0min(sample)}} - \frac{A_{t=0min(ethanol)} - A_{t=3min(ethanol)}}{A_{t=0min(ethanol)}} \quad (1)$$

Statistical analysis

Extractions of polyphenols from milled carob pods were conducted in duplicate; total phenolic content, tannin content and TEAC in each obtained extract were determined in triplicate. Results are presented as means ± standard deviation. One way ANOVA with Bonferoni post hoc test were used to establish the significance of differences of yields obtained using different extraction procedures.

Pearson correlation was used to establish correlation coefficients and significance between polyphenolic content and antiradical activity of obtained extracts Statistical analysis was performed using Prism 3.0 software (Graph Pad Prism, version 3.02).

Experimental design

Extraction of phenolic compounds from different food matrixes is highly influenced by their chemical nature, food characteristics (sample particle size, presence of interfering substances) and extraction method applied. Therefore, there is no uniform procedure for the optimal extraction of all polyphenols from all food matrixes. In this work four extraction procedures often used for extraction of polyphenols from carob pods were evaluated (Table 1, procedures 1, 2, 3, 4). The most suitable extraction conditions were determined, using the recoveries of total phenolic content, tannin content and ABTS radical scavenging activity as the criteria. Procedures with best extraction yields were then additionally modified in order to try to additionally increase target yields in the final extracts. In order to remove soluble carbohydrates, prior to extraction procedures 1g of carob pod powder was extracted with 3 ml of cold water, allowed to stand for 12 hours at 4 °C and filtered. The residue obtained after filtration was air dried for 24 hours, and then dispersed in 30 mL of solvent (water, 50% ethanol or 80% ethanol). At the end of extraction samples were centrifuged at 3500 g for 10 minutes. Clear supernatants (diluted if necessary) were used for determination of total phenolic content, tannin content and TEAC values.

RESULTS AND DISCUSSION

Tabble 1. Carob's total phenol and tannin yields depending on the solvent type and applied extraction conditions

solvent	water		EtOH (50% v/v)		EtOH (80% v/v)	
extraction	total phenols	tannins	total phenols	tannins	total phenols	tannins
	mg tannic acid equivalents/100g dry matter					
EXTRACTION 1 *(37°C, 2h)*	44.41±2.72	27.94±0.37	129.98±5.99	90.51±9.54	79.38±8.99	48.16±10.25
EXTRACTION 2 *(100°C, 30min)*	538.89±4.2	385.08±1.95	283.81±1.89	273.95±2.21	132.86±1.31	123.85±2.85
EXTRACTION 3 *(21°C., over night)*	45.28±2.23	37.15±2.12	145.56±0.61	131.80±0.29	99.04±0.08	82.44±0.44
EXTRACTION 4 *(ultrasound, 5 min)*	51.61±5.84	32.48±6.81	217.60±21.45	168.15±16.82	96.77±2.94	60.64±5.70

Total phenol and tannin yields obtained by four different extracting procedures, often used for extraction of polyphenols from plant materials are presented in Table 1. Performing one way ANOVA showed the existence of significant differences of obtained extraction yields depending on both, type of extraction as well as the type of solvent. However, in the case of extraction procedures were moderate temperatures were used (procedures 1,3,4) extraction yield depended primarily on the type (polarity) of used extraction solvent. As presented in the table, the highest extraction yields were obtained using 50% ethanol as the extraction solvent. However, the combination of polar solvent (water) and high temperature (boiling with reflux) was shown to be the most efficient way for extracting both total phenols and tannins from carob pods. Obviously, the high temperature during the extraction process enabled the extraction of bound phenolic compounds that are not extracted when common extraction conditions are applied and are best soluble in polar solvents such as water.

Since one of the main goals of this work was to maximize antioxidant activity of obtained extract, the impact of applied extraction procedures on $ABTS^{\cdot+}$ scavenging activity was also tested. Obtained results are presented in Figure 1.

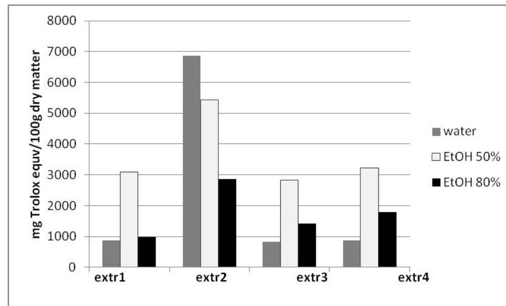

Figure 1.Obtained TEAC values depending on the solvent type and applied extraction conditions

As in the case of total phenolic content, the best antioxident yields were obtained after reflux cooking of carob pods with water extract obtained after boiling of milled carob pods for 30 min (extraction 2) showing the highest radical scavenging activity. Carob extracts obtained by other tested extraction procedures had significantly lower antiradical activity that was highly depended on the polarity of applied extraction solvent. Again, in extraction procedures where moderate temperatures were applied 50% ethanol was proven to be the most efficient solvent for extraction of antioxidants from carob. Ultrasound extraction was found to be the second most efficient way for extraction of phenolic/antioxidant compounds. In order to improve its efficiency, the extraction times were elongated to 15, 30 and 45 minutes (Table 2). As obvious, significant increase of total phenol and tannin content was observed in extracts obtained using the 30 minute ultra-sound extraction in relation to shorter procedures. However, further elongation of extraction duration did not result in significantly higher extraction yields. 50% ethanol was proven to be the most efficient solvent used extracting twice as more of phenolic compounds then 50% ethanol and as much as four times higher amounts of phenolics in comparison to pure water.

Table 2. Carob's total phenol and tannin yields depending on the solvent type and the length of theultrasound extraction

solvent	**water**		**EtOH (50% v/v)**		**EtOH (80% v/v)**	
	total phenols	tannins	total phenols	tannins	total phenols	tannins
time/min	*mg tannic acid equivalents/100g dry matter*					
5	51.61±5.84	32.48±6.81	217.60±21.45	168.15±16.82	96.77±2.94	60.64±5.70
15	54.84±2.12	38.27±2.00	381.81±1.01	352.32±13.00	207.17±4.13	152.91±2.34
30	78.34±3.79	60.06±6.39	506.93±16.00	412.75±34.00	243.75±1.79	198.56±2.06
45	85.37±2.66	69.12±3.98	561.78±9.88	421.99±29.15	271.66±3.74	202.15±13.55

Obtained TEAC values (Figure 2) correlated well with total phenolic ($r=0.9505; p<0.0001$) and tannin content ($r= 0.9770$; $p<0.0001$). Significant increase of antiradical activity was measured in extracts obtained after elongating the extraction time from 5 to 15 and 30 minutes while further elongation was proven to be unnecessary. Again among used extraction solvents, 50% ethanolic extracts possessed the highest radical scavenging activity.

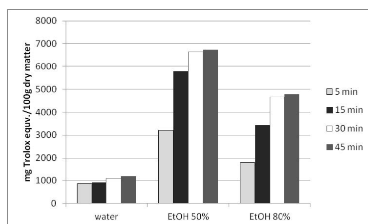

Figure 2.Obtained TEAC values, depending on the duration of applied ultrasound extraction

The elongation of reflux cooking from 30 to 45 and 60 minutes respectively, resulted in increased values of all observed parameters. As obvious from Table 3, further elongation of the process did not provide statistically significant improvement of extraction yields.

Table 3. Total phenol, tannin and TEAC yields depending on the duration of reflux cooking

EXTRACION	time/min	total phenols	tannins	TEAC
		mg tannic acid equv/100g dry matter		*mg TE/100g dry matter*
cooking in the boiling water-bath using water as extraction solvent	30	538.89±4.20	385.08±1.95	6866.6±45.27
	45	750.99±27.56	449.62±9.09	12852.34±15.68
	60	993.32±49.95	539.30±32.93	15631.62±369.10
	75	1087.23±33.17	542.78±22.07	16119.44±38.99

CONCLUSION

Presented results confirm the well known fact that carob pod can be considered as rich source of polyphenolic dietary antioxidants, the majority of which are tannins. They also suggest that different extraction conditions significantly influence polyphenolic content and antioxidant activity of obtained extracts. The major parameters that can modulate antioxidant yields are temperature, duration of extraction and the solvent used. The application of high temperature during extraction in combination with polar solvent (water) enables the extraction of bound phenolics that cannot be extracted when mild extraction conditions are applied. Among investigated extraction procedures (reflux cooking, shaking at room temperature and 37°C, and ultrasound extraction using water, EtOH 50% and EtOH 80% respectively) 60 minute long reflux cooking with water was shown to be the method of choice for producing carob pod extracts characterized with high polyphenolic content and potent radical scavenging activity.

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INFLUENCE OF TECHNOLOGICAL PROCESSING ON AVAILABLE METHIONINE STABILITY IN DIETETIC TEA BISCUITS

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ABSTRACT

Since, during food processing, the methionine may react with other food components causing a loss of its bioavailability, the impact of technological procedure on stability of available methionine during production (baking) of nine different types of dietetic tea biscuits has been determined. The investigations were carried out on dietetic tea biscuits based on wholegrain wheat flour additionally enriched with different raw materials (oat or barley wholegrain flour, buckwheat flour, amaranth flour or full fat soya flour) or different pure fibers (oat or apple fibers). A biscuit with added sucrose was also prepared to examine influence reducing sugars on methionine stability. The available methionine was determined by the McCarty and Sullivan spectrophotometric method at 513 nm following the papain hydrolysis of the samples. Obtained results of the analysis show that the content of available methionine in examined biscuits varied between 0.134 g/100g dry matter (biscuit prepared with sucrose) and 0.173 g/100g dry matter (biscuit prepared with soya flour).

After baking, the content of available methionine was lower in all investigated samples, with the highest reduction in biscuit prepared with sucrose. Greater stability of available methionine could be obtained by utilisation of sugar replacements and artificial sweeteners instead of reducing sugars for biscuits preparation. Taking into account recommended daily intake for adults, 100 g of examined biscuits could cover up to 19% daily requirements for methionine, thus representing a good source of this essential amino acid.

KEYWORDS: *available methionine; wholegrain cereal; dietetic biscuits*

INTRODUCTION

Today people consume products on whole grain basis (pasta, bread, biscuits) more frequently regarding such food can improve health and prevent or reduce a range of chronic diseases. Cereal and legume seeds represent main source of primary food proteins and also are source of resistant starch, fatty acids, antioxidants, dietary fiber etc (Slavin et al., 1997). It is known that during processing of food, enzymatic and non enzymatic browning reactions of amino acids and proteins, which are essential components of the diet needed for maintenance of normal function of organism, with carbohydrates, oxidized lipids and oxidized phenols can cause loss in nutritional quality and potentially in safety of food (Friedman, 1996). Methionine can be converted to S-adenosylmethionine which is universal methyl donor and is required in most or all methylation events occurring in the body. It also constitutes important antioxidant defence mechanism as an efficient oxidant scavenger (Levine et al., 1996). Methionine is very sensitive to oxygen and heating treatment and losses, which consequently may increase the deficiency of this amino acid in foodstuffs as a result of food processing operations such is drying, roasting or treatment with oxidizing agents (Pieniażek et al., 1975). Since methionine plays an important role in human metabolism and taking into account the fact that its stability can be affected by various factors, the content of available methionine during production (baking) of nine different types of dietetic tea biscuits has been determined.

MATERIALS AND METHODS

The investigation was carried out on nine types of experimental biscuits prepared in laboratory conditions by modification of original recipe for dietetic tea biscuit (reference sample) in order to achieve the optimal

recipe for fiber rich biscuits in regard to technological possibilities, i. e. without significant interrupt of the organoleptic properties of each product.

For biscuits preparation, whole grain- and white wheat flour were used as basic flours and all modifications of recipe were on account white wheat flour. Added flours (oat-, barley-, buckwheat- amaranth-, and soya full fat flour) were purchased at the local market and added pure fibers (oat fiber, type HF 600 and apple fiber, type AF 400-30) were provided from Vitacel , Germany. A biscuit with sucrose addition instead of sugar replacements was also prepared.

The resulting dough was manually shaped into circular form (diameter 50 mm and thickness 9 mm). The biscuits were baked at 175°C, cooled down and milled to 0.5 mm mesh. Differences in biscuits composition are shown in Table 1.

Table 1. Differences in composition of nine investigated laboratory prepared biscuits

Composition of biscuits				
Sample	Whole Grain Wheat Flour (%)	White Wheat Flour (%)	Raw Material (%)	Pure Fiber (%)
reference sample	60	40	/	/
with sucrose	60	40	/	
with oat flour	60	10	oat flour (30%)	/
with barley flour	60	10	barley flour (30%)	/
with buckwheat	60	10	buckwheat flour (30%)	/
with amaranth	60	10	amaranth flour (30%)	/
with soya flour	60	10	soya flour (30%)	/
with oat fiber	60	25	/	oat fiber (15%)
with apple fiber	60	25	/	apple fiber (15%)

The available methionine was determined by the McCarty and Sullivan specrophotometric method in defatted samples on wavelength of 513 nm after papain hydrolysis (Pieniażek et al., 1975) and was calculated according to the linear regression equation ($y=0.329x + 0.028$). Fat content was determined by Soxhlet method with five hours extraction time. Moisture content was determined on Microwave MLS-1200 Mega moisture analyser.

RESULTS AND DISCUSSION

The technological processes of production such as blending the ingredients, baking and cooling might influence the stability of methionine especially if they are occurring on high temperatures and in the presence of fat which might oxygenize. Biochemical, physical and chemical reactions which occur in the dough during the baking are very complex and involve evaporation of water, degradation of protein, melting fat, destruction of starch, darkening and Maillard's reaction as well as the expansion of dough caused by a thermal gas expansion. Therefore, in order to get an understanding the influence of the technological processes during the biscuit production on the stability of available methionine, its content was determined in the dough and the final product, the biscuit (Table 2). The amount of available methionine in examined dough samples ranged from 0.153 g/100g dry matter in sample prepared with pure oat fiber addition to 0.188 g/100 g dry matter in sample prepared with amaranth flour. Depending on the examined biscuit, the content of available methionine varied from 0.134 g/100g dry matter in biscuit prepared with sucrose to 0.173 g/100g dry matter in biscuit prepared with full fat soya flour. Regarding that all investigated samples (except sample with added sucrose) were prepared with exactly the same amounts of whole grain wheat flour, vegetable fat, sugar substitute (isomalt), artificial sweetener (sucralose), powder milk, salt and flavour to prevent their influence on the final results, all differences in available methionine content originated from addition different raw materials or pure fiber.

Table 2. The available methionine content in investigated biscuits

Sample	Dough	Biscuit
	g/100g dry matter	
reference sample	0.171±0.009	0.161±0.003
with sucrose	0.164±0.005	0.134±0.003
with oat flour	0.161±0.004	0.156±0.003
with barley flour	0.172±0.006	0.161±0.003
with buckwheat flour	0.178±0.000	0.170±0.004
with amaranth flour	0.188±0.014	0.171±0.007
with soya flour	0.184±0.005	0.173±0.002
with oat fiber	0.153±0.004	0.150±0.001
with apple fiber	0.155±0.004	0.148±0.002

The results are reported as mean ± SD of three parallel investigations

Although all nine types of biscuits were prepared and baked under the same conditions, it is visible from the analysis of the results, that technological processes during biscuit production did not have an equal impact on available methionine stability (Figure 1). The decline of this amino acid was observed in all investigated samples, with the lowest amount in biscuit prepared with pure oat fiber (1.86%), while substitution of sucrose instead of sugar replacements had the most significant effect on methionine content decrease (18.46%).

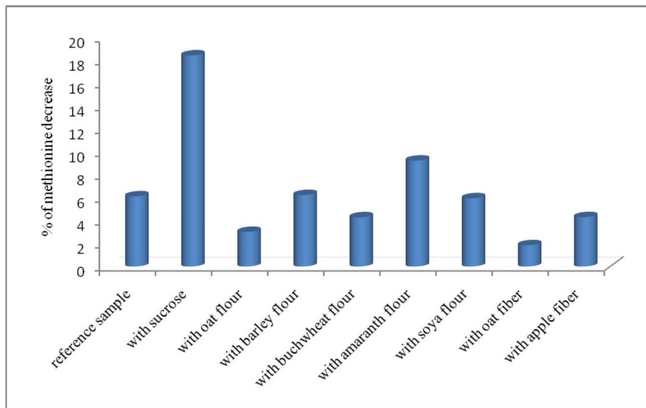

Figure 1. Percentage of available methionine decrease during biscuit production

Regarding oxidized form of lipids increase degradation of available methionine, the amount of total fats was also determined. Depending on the investigated biscuit, the fat content ranged from 18.401 g/100g dry matter in sample prepared with pure oat fiber to 22.558 g/100g dry matter in biscuit prepared with full fat soya flour. Considering mentioned we would expect the highest decline of available methionine content in biscuit prepared with soya full fat flour. However, soya flour is rich in antioxidants which neutralized free radicals thus reducing a loss of available methionine. The highest decline of available methionine observed in biscuit prepared with sucrose appears most probably due to a form of nonenzymatic browning that is Maillard reaction. This type of reaction is responsible for development of color and flavor during biscuit production which is important for acceptable organoleptic features but also leads to reduction of available methionine content due to nonenzymatic chemical reaction which involves condensation of an amino group (methionine) and a reducing group (sucrose).

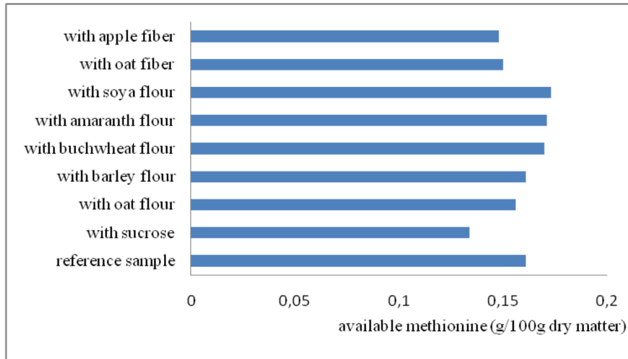

Figure 2.The influence of raw material composition on the available methionine content

The analysis of the results had shown that change in raw materials structure affects on the available methionine content (Figure 2). The highest proportion of available methione was found in biscuit prepared with full fat soya flour what was expected regarding high protein content in this flour (Kaić-Rak, Antoić,1990).

Comparing measured values of available methionine content with Recommended Daily Intake (FAO/WHO, 1991), 100 g of examined biscuits can satisfy 14.7 % up to 19.01 % of daily needs of adult person.

CONCLUSION

From the presented data it is visible that although, during the production, the level of available methionine were reduced in all experimentally prepared biscuit, examined products are still a good source of this essential amino acid for human organism.

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Food Engineering

NEW METHODOLOGY TO INVESTIGATE THE CRYSTALLINE STATE OF COCOA BUTTER IN CHOCOLATE USING DSC

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ABSTRACT

Cocoa butter is a vegetable fat that can exist under six different forms. Differential Scanning Calorimetry (DSC) is a tool that is often used to study cocoa butter crystallization. However, no systematic method has been established to analyze the resulting curves and therefore obtain clear interpretation of the tempering process and fat bloom. The aim of this work is to establish a method to identify the crystalline state of cocoa butter in chocolate during tempering and blooming. The presented methodology will be a tool for the industry to study the tempering process and the quality of the final product. This method will also be useful to enhance the comprehension of the still misunderstood blooming phenomena.

A standard procedure is developed to measure the thermal behavior of a chocolate sample using DSC. A mathematical model is used to fit the DSC curves and determine the thermal parameters as the melting temperature, the endset temperature and the enthalpy of reaction.

This method is applied to follow the tempering process of chocolate. Also, to study the fat bloom, a well-tempered chocolate is subjected to temperature variations to induce blooming. Thermal analyses of the crystalline state of cocoa butter are made throughout the all experience using DSC. The thermogram of a well-tempered chocolate shows a well defined thin peak. While the curve of a bloomed chocolate shows a wider peak with a shoulder and a peak temperature up to 1°C higher.

KEYWORDS: *DSC; Cocoa butter polymorphism; Crystallization; Heat transfer*

INTRODUCTION

Cocoa butter is a vegetable fat that is commonly assumed to exist under six different forms, named I to VI following the Wille and Lutton nomenclature (Wille, Lutton, 1966). This polymorphism has a central role in the techno-functional properties of chocolate, including its aspects and its long time conservation (resistance to crystalline changes such as fat bloom). Only two of these six forms (namely form V and VI) are desired. Obtaining them depends on processing conditions. Therefore, chocolate has to be tempered. Tempering is a temperature program applied to the liquid chocolate to induce the nucleation of the desired forms. It is a crucial step in the chocolate processing to obtain a good quality product.

Blooming is an often encountered problem in the chocolate production. Mostly due to temperature variation, a thin white film appears on the surface of chocolate. The external aspect and the taste are modified and the product cannot be commercialized anymore. The physical phenomena responsible of the blooming are still misunderstood (Lonchampt, Hartel, 2004).

Differential Scanning Calorimetry (DSC) is a tool that is often used to study cocoa butter crystallization (Fessas et al. 2005; Spingno et al. 2001). However no official protocol exists and, in the literature, a large variation of the main parameters of a DSC measurement as the temperature slope is founded. Moreover, no systematic method has been established to analyze the resulting curves and therefore obtain clear interpretation of crystallization processes and problems, such as the tempering process and fat bloom.

The Avrami model is often used to describe fat crystallization (Padar et al. 2008) but this is an empirical model with only a limited physical meaning. Le Révérend et al. (2009) applied this model to cocoa butter. They simplified the system of six crystalline forms considering only two crystalline forms, the stable (desired

forms V and VI) and the unstable (I to IV).

In this work, a protocol with the best experimental parameters is developed. The temperature slope, the start and end temperature and the sample mass are optimized to obtain a DSC curve with well defined peaks and a minimized noise.

To enhance the interpretation of the DSC curves a mathematical model based on the hypothesis that crystallization is limited by the heat transfer and considering two crystalline forms is developed. This model will be used to identify the crystalline state of cocoa butter in chocolate during the tempering process and blooming.

MATERIALS AND METHODS

Chocolate

Dark chocolate is provided by Puratos – Belcolade (Erembodegem, Belgium). This chocolate has the following composition (mass fractions): 48.5% sugar (saccharose), 28.5% cocoa butter and 22.5% cocoa (solid). The chocolate is stored at 18 °C.

DSC

Thermal analyses are realized with a DSC-1 from Mettler-Toledo (Belgium). Samples between 5 and 15 mg are loaded in standard 40 µ L aluminum pans with piercing lids. To obtain a curve with well separated peaks without noise and have a reasonable time of experiment a temperature slope of 0.2 °C/min is selected. The melting temperatures ranging from 13.0 to 36.3 °C, we define the start temperature at 8 °C and the end temperature at 45 °C. To ensure that nothing happens at the start and end temperature, a plateau of five minutes is imposed at the beginning and the end of the experiment.

The resulting curve show the heat provided to the sample (in mW) versus the sample temperature. To compare different DSC experiments, these results have to be standardized. Therefore the heat provided to the sample is divided by the sample mass and the temperature slope to obtain the specific heat expressed in $J.g^{-1}.°C^{-1}$.

To obtain a good reproducibility, resulting curves are corrected to have a flat base-line. Between 38 and 43 °C, the chocolate is liquid and the DSC signal is a straight line with a positive slope. This line is fitted by the function Polyfit in MATLAB and subtracted from the DSC signal at all temperatures. The resulting curve, which is called the *DSC curve* in the rest of the paper, is used for all our analysis.

Mathematical model

The objective of our model is to predict the crystalline state of cocoa butter in chocolate as a function of the operating temperatures.

As proposed by Le Réverend *et al.* (2009), only two crystal forms are considered. Three states for the chocolate are considered: liquid, stable and unstable crystals, respectively denoted with L, S and I subscripts. As similar equations are written for stable and unstable form, a generic N subscript is also used to describe stable and the unstable crystals.

The assumption is made that crystallization is limited by the heat transfer through the liquid chocolate. The crystallization rate R_N ($kg.s^{-1}$) is proportional to a driving for defined as the difference between the temperature T_{lsN} (°C) of the liquid surrounding the crystal seed and a reference melting temperature T_{fN} (°C).

$$\boldsymbol{R_N = k_{cinN}\left(T_{lsN} - T_{fN}\right) A_{cN}} \tag{1}$$

where k_{cinN} ($kg.s^{-1}.°C^{-1}.m^{-2}$) is the rate of crystallization constant and A_{cN} (m^2) the crystal surface. The local temperature in the liquid surrounding the crystal seed is correlated to the liquid bulk temperature T_{lb} (°C) assuming that a thermal boundary layer e is present around the crystal. Considering a local steady state, the heat flux Q_{chN} ($J.s^{-1}$) reaching the crystal is equal to the amount of heat used for melting. Thus equations

(2) and (3) can be written

$$\Delta H_{sN} R_N = Q_{chN} \tag{2}$$

$$Q_{chN} = k_{ch}(T_{lb} - T_{lsN}) \tag{3}$$

where ΔH_{sN} (J.kg^{-1}) is the enthalpy of solidification of the crystal forms, k_{ch} (W.m^{-2}.°C^{-1}) is the heat transfer coefficient and T_{lb} is the bulk temperature of the liquid chocolate. Rearranging equations (1), (2) and (3) leads to

$$Q_{chN} = k_{effN}(T_{lb} - T_{fN})A_{cN} \tag{4}$$

where k_{effN} is a global heat parameter containing the rate of crystallization constant and the heat transfer coefficient.

$$k_{effN} = \frac{1}{\frac{1}{k_{ch}} + \frac{1}{k_{cin}\Delta H_{sN}}} \tag{5}$$

Knowing that the crystallization rate describe the evolution of the mass of crystals of form N, we can express the evolution of the total mass of crystals of form N m_{TcN} (kg) as follow:

$$\frac{dm_{TcN}}{dt} = \frac{N_{cN}\, k_{effN}\, A_{cN}}{\Delta H_{sN}}(T_{lb} - T_{fN}) \tag{6}$$

where N_{cN} is the number of crystals of form N and t (s) is the time.
Assuming spherical crystals, the evolution of the crystal radius r_N (m) over time can be expressed by

$$\frac{dr_N}{dt} = \frac{k_{effN}}{\Delta H_{sN}\, \rho_{cN}}(T_{lb} - T_{fN}) \tag{7}$$

where ρ_{cN} is the density of the crystals.
The evolution of the crystal radius can be linked to the evolution of the N form cristal mass fraction

$$\frac{dX_N}{dt} = \frac{N_{cN}\, \rho_{cN}}{m_{TcN}} 4\,\pi\, r_N^2 \frac{dr_N}{dt} \tag{8}$$

The heat quantity of the system Q_{syst} (J) taking the melting temperature of the stable crystals as the temperature of reference is given by

$$\begin{aligned} Q_{syst} = {} & (1 - X_U - X_S) m_T C_{pL}(T_{lb} - T_{fS}) \\ & + X_U m_T \left(\Delta H_{sU} + C_{pU}(T_{lb} - T_{fU}) \right. \\ & \left. + C_{pL}(T_{fU} - T_{fS})\right) \\ & + X_S m_T \left(\Delta H_{sS} + C_{pS}(T_{lb} - T_{fS})\right) \end{aligned} \tag{9}$$

where C_{pN} (J.kg^{-1}.°C^{-1}) is the heat capacity for the different states (liquid, stable and unstable) and m_T is the total mass of the system.

Supposing that the system is adiabatic ($dQ_{syst}/dt = 0$), that the C_{pN} do not change as a function temperature, we can describe the evolution of the bulk temperature over time by

$$\frac{dT_{lb}}{dt} = \frac{\Delta H_{sU} + (C_{pU} - C_{pL})(T_{lb} - T_{fU})}{(1 - X_U - X_S)C_{pL} + X_I C_{pI} + X_S C_{pS}} \frac{dX_U}{dt} + \frac{\Delta H_{sS} + (C_{pS} - C_{pL})(T_{lb} - T_{fS})}{(1 - X_U - X_S)C_{pL} + X_I C_{pI} + X_S C_{pS}} \frac{dX_S}{dt} \tag{10}$$

Adimensionnalising by $z_N = \frac{r_N}{r_{0N}}$, $\theta = \frac{(T_{lb} - T_{fS})C_{pS}}{\Delta H_{sS}}$, $\theta_U = \frac{(T_{fU} - T_{fS})C_{pS}}{\Delta H_{sS}}$, $\tau = \frac{t\, k_{effS}}{r_{0S}\rho_{cS}C_{pS}}$, $\tau_U = \frac{\Delta H_{sU}\, \rho_{cU}\, r_{0U}\, k_{effS}}{\Delta H_{sS}\, \rho_{cS}\, r_{0S}\, k_{effU}}$, $\beta = \frac{\Delta H_{sU}}{\Delta H_{sS}}$, $\alpha_L = \frac{C_{pL}}{C_{pS}}$ and $\alpha_U = \frac{C_{pU}}{C_{pS}}$, equation (7) for stable and unstable crystals and the heat balance (equation (10)) become:

$$\frac{dT_{lb}}{dt} = \frac{\Delta H_{sU} + (C_{pU} - C_{pL})(T_{lb} - T_{fU})}{(1 - X_U - X_S)C_{pL} + X_I C_{pI} + X_S C_{pS}} \frac{dX_U}{dt} + \frac{\Delta H_{sS} + (C_{pS} - C_{pL})(T_{lb} - T_{fS})}{(1 - X_U - X_S)C_{pL} + X_I C_{pI} + X_S C_{pS}} \frac{dX_S}{dt}$$

(10)

(11)

(12)

This system of differential equations is solved by the function ode15s in MATLAB 7.2 (The MathWork, USA)

Accelerated blooming

To enhance the comprehension of the mechanisms leading to chocolate's fat bloom, experiments of accelerated blooming by submitting the chocolate to temperature variations are realized (Ali *et al.* 2001).

During ten days, well tempered chocolates are put alternatively six hours at 30 °C and eighteen hours at 18 °C. Before and after each passage at 30 °C, a DSC experiment is realized.

RESULTS AND DISCUSSION

Figure 1 shows DSC curves of three different samples of the same chocolate pellet. Peaks are well defined and a good reproducibility between the three measurements is observed.

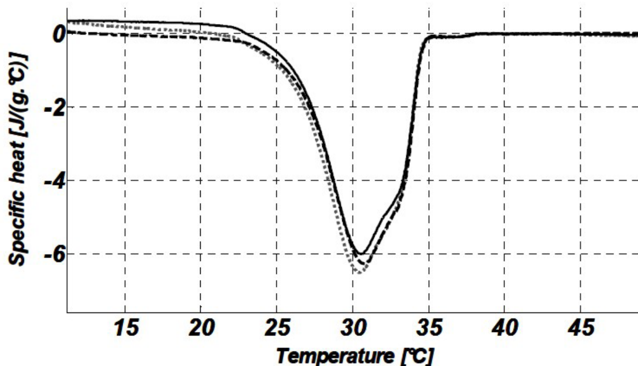

Figure 1. Standardized and corrected DSC curve for 3 different samples realized between 8 and 45 °C with a temperature slope of 0.2 °C/min.

Before the accelerated blooming experiment, each DSC curve of the chocolate in its initial state shows one peak with a peak temperature of 32.5 ± 0.3 °C and a temperature of endset of 33.7 ± 0.3°C. After at least 2 temperature cycles another peak appears and a migration of the peak that existed before is observed. The peak temperature of the new peak is at 33.7 ± 0.3 °C and the endset temperature is at 35.5 ± 0.5 °C. This peak is supposed to show the presence of form VI. Although a change appears in the DSC curve, no blooming is observed.

In our model, an adiabatic system is considered. To model a DSC experiment a term representing the heat source is added to equation (10). The results of the model for T_{lb} = 15 °C, X_U = 0.1 and X_S = 0.2 as initials conditions are shown in figure 2.

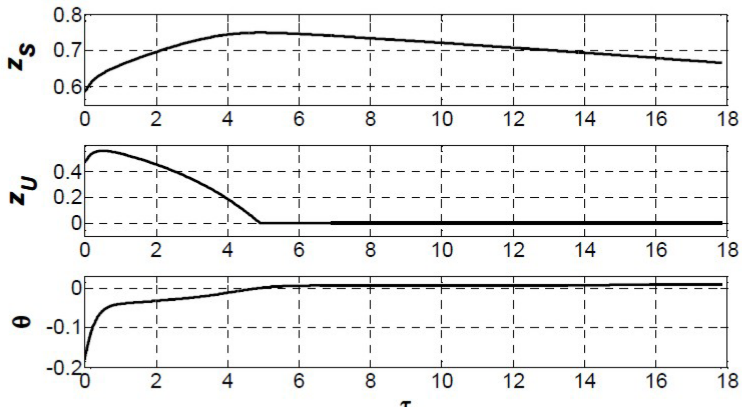

Figure 2. Evolution of zS, zU, and θ in function of τ predicted by the model.

In a first time, until τ = 0.55, the temperature increase rapidly due to the crystallization of stable and unstable crystals. In a second time, for τ between 0.55 and 5.00, the temperature continue to increase but more slowly. At this time, the temperature is higher than the melting temperature of the unstable crystals, so the unstable crystals melt while the crystallization of the stable crystals goes on. For τ higher than 5.00, the temperature of the system exceeds the melting temperature of the stable crystals. These crystals melt consuming the heat source until the mass fraction of stable crystals reaches 0 for τ = 52.52 (not shown on the figure). After the melting of all crystals, the temperature increases again more rapidly due to the constant heat source.

These results are coherent with the experimental observations but, as the heat source hasn't been evaluated yet, no comparison with experimental results is available.

CONCLUSION

The measurements parameters selected for a DSC measurement ensure to obtain a curve with well defined peaks. With the correction applied to the DSC signal, a DSC curve with a flat base line is obtained and the principal value as the peak temperature or the endset temperature can be determined.

Accelerated blooming experiments show that when chocolate is submitted to temperature variation, polymorphic transformations are induced even if no blooming is observed.

The developed model gives a coherent prediction of the evolution of the bulk temperature when the chocolate is melted. But some experimental parameters still need to be evaluated to experimentally validate the model. That will be done in a future work.

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THE EFFECT OF AGGLOMERATION ON PHYSICAL PROPERTIES AND BIOACTIVE COMPOUNDS OF COCOA DRINK MIXTURES PREPARED WITH VARIOUS SWEETENERS

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ABSTRACT

Agglomeration is a process used to improve reconstitution properties of cocoa powders in liquids. This process has been used for instantization of numerous food products, due to its ability to improve dispersion and solubility properties of the powders while reducing the fines in the final product. The process also enables production of more attractive powdered products (both in appearance and sensory properties) and decreases the apparent density and tendency to cake during storage.

In this study physical properties and bioactive content of powdered cocoa drink mixtures prepared from cocoa powders with two different fat contents and different sweeteners (sucrose, aspartame/acesulfame K and stevia), were investigated before and after agglomeration. Particle size and density characteristics of the formulated mixtures and agglomerates, as well as dispersibility and wettability were evaluated. Content and composition of polyphenols was determined using UV-vis spectrophotometry. Antioxidant capacity of cocoa mixtures and agglomerates was evaluated by using ABTS and FRAP assays.

Agglomeration significantly affected physical and bioactive properties of cocoa drink mixtures. Total phenol, flavonoid and flavan-3-ol content, as well as the antioxidant capacity decreased after agglomeration of all cocoa drink mixtures. Both cocoa powder mixtures and cocoa agglomerates prepared with the cocoa powder containing higher fat content (16-18%) generally provided lower bioactive content, compared to the mixtures prepared with low fat cocoa.

As expected, agglomeration resulted in agglomerates with higher median diameter and Hausner ratio values. Agglomerated cocoa mixtures also showed better reconstitution properties in comparison to non-agglomerated cocoa powder mixtures. Best results were obtained for cocoa agglomerates containing low fat cocoa.

Results indicate that agglomeration improves the physical properties of cocoa drink mixtures, regardless of their composition, but negatively affects the beneficial bioactive constituents and thus requires further studies in order to improve characteristics of the final product.

KEYWORDS: *agglomeration; cocoa; sweeteners, physical properties; polyphenols*

INTRODUCTION

Owing to the growing evidence that confirm the health benefits of cocoa antioxidants, especially polyphenols, and due to growing consumer demands, there is an increasing interest in improvement of cocoa drinks, aimed to reducing the sugar content and enhancing the content of beneficial bioactive constituents. As an alternative to sucrose, whoose consumption has been considered to be implicated in the development of dental caries, obesity, and metabolic diseases associated with obesity (Department of Health, 1994) the potential of using alternative sweeteners, especially naturally derived like diterpenic glycosides from the leaves of *Stevia rebaudiana* (Mogra, Dashora, 2009) is arousing much attention in the recent years. It has recently been established (Belščak-Cvitanović et al.,2010) that the application of a range of different sweteneers can be used for the production of powdered cocoa drink mixtures, which ensures both substantial amount of bioactive compounds and excellent sensory properties of the final product.

In the production of ready-to-drink beverages, manufacturers need to take into account the specific characteristics of cocoa powder and other ingredients, since they can have a major impact on the quality of final product. Especially some properties, like bulk density, compressibility (compaction), and flowability are essential to product handling, processing, and transportation; some others, such as particle size and instant properties, are related to quality and consumer acceptance (Aguilera, 2001).

It is well known that sole cocoa powders show poor reconstitution properties, such as dispersibility, wettability and solubility. This can be improved by adding other components to the powder (e.g. sugars, vitamin mixtures, milk powder) and instantizing the powder with the addition of lecithin. Another alternative for the improvement of cocoa dispersibility and instant characteristics is the agglomeration, during which the sugar crystals are moistened with water or steam, (lecithinated) cocoa and other solid particles adhere to the wet sugar crystals, and finally the agglomerated particles are dried. Agglomeration is the most effective method to improve the rehydration characteristics of dried food powders (Barletta, Barbosa-Carnovas, 1993). It adjusts the particle size distribution, bulk density, as well as improves wettability (or penetration of liquid into the porous system) due to faster capillary action. It provides mechanical stability and improves solubility (Schubert, 1980).

Various techniques can be used to agglomerate cocoa beverage powders: steam agglomeration (Visotto et al., 2010), fluid bed agglomeration (Kowalska, Lenart, 2005) and thermal agglomeration (Omobuwajo et al., 2000). High shear mixers can also be used for wet agglomeration of cocoa powder beverages (Vu et al., 2003). Due to the improvement of beneficial reconstitution properties upon agglomeration it is important that the degree of agglomeration and the compactness of the powder can be controlled during the production process (Benković, Bauman, 2009, 2011).

Since no literature data are available reagrding the effect of agglomeration on the bioactive costituents of powdered cocoa drink mixtures, the purpose of this study was to provide a cocoa drink mixture with good physical properties to enable good flow properties and, as well, to achieve a high content of polyphenolic compounds that exhibit positive health effects. Therefore the present study involves the comparison of physical properties and bioactive content of powdered cocoa drink mixtures comprising cocoa powders with two different fat contents and different sweeteners (sucrose, aspartame/acesulfame K and stevia), before and after agglomeration.

MATERIALS AND METHODS

Mixture formulation

The basic recipe for the mixtures was 30% (w/w) of cocoa powder and 70% (w/w) sucrose (mixture A). Two adittional mixtures were formulated consisting of 30% cocoa powder, 10% palatinose, 10% eritrythol, 30% maltodextrine, 19.3% inulin and 0.7% aspartame/acesulfameK (mixture B) and 30% cocoa powder, 19% palatinose, 10% trehalose, 20% oligofructose, 20% inulin and 1% stevia sweetener (mixture C). Sugars were added according to relative sweetness. The mixtures were prepared with cocoa powder containing two different fat content (10-12% fat and 16-18% fat). All the components were mixed together in a Turbula mixer (Willy A. Bachofen Maschienenfabrik, Muttenz, Switzerland) for 10 minutes to obtain a homogenous blend.

Agglomeration

Fluidized bed agglomeration of the mixtures was performed using STREA 1 fluidized bed agglomerator/ dryer/coater. Parameters kept constant during the agglomeration process were air temperatue (60 °C) and instantizer concentration (Metarin – water soluble lecitin was added using peristaltic pump at a concentration 0.8% w/w of a final product). Water was also added using a peristaltic pump based on visual inspection of the fluidisation chamber – if agglomerate formation was visible, additional water was not added to the process. After agglomeration, agglomerates were spray dried, cooled and their physical and bioactive properties were determined.

DETERMINATION OF PHYSICAL PROPERTIES

Particle size

Particle size of the mixtures before and after the agglomeration was measured using Mastersizer 2000 (Malvern Instruments, UK). Mastersizer measurements are based on laser diffraction technique and Mie light scattering theory. All the samples were analyzed on a Malvern 2000 particle size instrument equipped with Scirocco 2000 dry dispersion unit. All the measurements were done in triplicate and the results are expressed as median diameter (d_{50}) mean ± standard deviation.

Hausner ratio

Hausner ratio was calculated using equation 1:

$$Hr = \frac{Tapped\ bulk\ density\ [kgm^{-3}]}{Loose\ bulk\ density\ [kgm^{-3}]}$$

Loose and tapped bulk densities were determined using STAV 2003 jolting volumeter (J. Engelsmann AG, Ludwigshafen) based on a method described by Haugaard Sørensen et al. (1978).

Wettability

Wettability is the ability, expressed as time in seconds, necessary for a given amount of powder to penetrate the quiet surface of the water. It was determined using 100 mL distilled water at 20 °C ± 2 °C in a glass beaker and 10 grams of the mixture/agglomerates, as described by Haugaard Sørensen et al. (1978).

Dispersibility

Dispersibility of samples was determined by dissolving approximately 10 g of each sample in 100 ml of distilled water at 27 °C. The mixture was manually stirred continuously for 1 min and allowed to rest for 30 min for the suspended particles to settle down before the supernatant was carefully decanted. The density of the supernatant was then determined by filling an aliquot of the supernatant into a 50 ml density bottle. The mass of the filled bottle was noted. The weight of the dispersed solid was calculated as twice the difference in the mass of the supernatant and an equal volume (50 ml) of distilled water (Shittu, Lawal, 2007).

Determination of Quality Parameters of Cocoa Drinks

Preparation of Cocoa Drinks

To simulate household preparation conditions, cocoa drinks were prepared according to a procedure described by Lee et al. (2003): 7.3 g of powdered cocoa drink mixture before and after the agglomeration (equivalent to 2 tablespoons) was dissolved in 200mL of boiling, distilled water and stirred with a glass rod. The samples were cooled and centrifuged at 3500 rpm for 5 min, and the resulting supernatants were used as the final samples.

Determination of Total Phenol (TPC) and Flavonoid Contents (TFC)

TPC of cocoa drinks was determined spectrophotometrically according to a modified method of

Lachman et al. (1998). In order to determine flavonoids in the experimental samples, the formaldehyde precipitation was used (Kramling, Singleton, 1969). Flavonoid content was calculated as the difference between total phenol and nonflavonoid contents. All determinations were carried out in triplicates and the results were expressed as mg/L of gallic acid equivalents (GAE).

Determination of Flavan-3-ol Content using the Vanillin Assay

Cocoa drinks were analyzed for their flavan-3-ol content using a method described by Di Stefano et al. (1989). The content of flavan-3-ols was calculated according to the formula (+)-catechin = 290.8xΔE, and the results were expressed as milligrams of (+)-catechin per liter.

ABTS Radical Scavenging Assay

The Trolox equivalent antioxidant capacity (TEAC) of cocoa drinks was estimated by using the ABTS radical cation decolorization assay (Re et al., 1999). The results, obtained from triplicate analyses, were expressed as Trolox equivalents and derived from a calibration curve determined for Trolox

(100-1000 μM).

Ferric Reducing/Antioxidant Power

The ferric reducing/antioxidant power (FRAP) assay was carried out according to a standard procedure by Benzie and Strain (1996). All measurements were performed in triplicate. Aqueous solutions of $FeSO_4$ x $7H_2O$ (100-1000 μM) were used for the calibration curve, and the results are expressed as millimolar Fe^{II}.

RESULTS AND DISCUSSION

Hausner ratio, as a ratio between tapped and loose bulk density, represents a useful and easy parameter for powder characterisation. The usefulness of this ratio arises because it deals with properties relevant to moving, rather than static, powder (Barbosa – Canovas et al., 2005). As shown in Table 1, Hausner ratio of the agglomerates was lower than Hr of the mixtures, indicating that the agglomerate showed better flow stability. It could also be observed that the changes in Hr values of the mixtures and the agglomerates were higher for mixtures and agglomerates made with cocoa powder containing 10-12 % fat (changes in Hr values ranged from 0.15 to 0.20 for CP 10-12% fat, and 0.03 – 0.15 for CP 16-18% fat).

Particle size enlargement accomplished during the agglomeration process was more visible with mixtures containing sugars in powdered form, while the mixtures containing only crystalline sugar (mixture A, both percentages of fat) did not show such a high change in d_{50}. However, it is important to emphasize that agglomeration occurred sooner with the mixtures containing crystalline sugar and no extra water addition was necessary, while with mixtures containing fine particles extra water addition was required to promote agglomeration.

Table 1. Physical properties of cocoa powder drink mixtures before and after agglomeration

Cocoa fat content	*Mixture*	**Hausner ratio (tapped bulk density/loose bulk density) [/]**	**d_{50} [μm]**	**Dispersibility (%)**	**Wettability (s)**
		Before agglomeration			
10-12%	**A**	1.19	527.76±23.21	10.43 ± 0.68	57.00 ± 9.00
	B	1.18	112.88±2.63	20.18 ± 0.23	905.00 ± 23.00
	C	1.25	103.75±3.12	31.62 ± 1.10	1521.00 ± 64.00
16-18%	**A**	1.10	489.78±18.46	25.59 ± 0.76	435.00 ± 24.00
	B	1.13	108.29 ±5.57	22.75 ± 0.64	227.00 ± 29.00
	C	1.19	95.65±2.99	22.19 ± 0.32	166.00 ± 8.00
		After agglomeration			
10-12%	**A**	1.04	550.44±6.91	44.57±1.52	4.70±0.25
	B	1.03	360.46±12.50	47.43±1.77	9.07±0.90
	C	1.05	279.33±9.96	39.71±2.01	9.54±1.00
16-18%	**A**	1.07	501.73±28.19	24.19±0.99	7.30±0.50
	B	1.04	410.92±17.90	61.52±3.20	18.24±1.00
	C	1.04	308.40±25.91	33.40±1.07	25.92±2.00

Beside particle size enlargement, agglomeration resulted in porous agglomerates which exhibited better reconstitution properties – better dispersibility and wettability. Percentage of particles dispersed in water increased after agglomeration, while the wettability time decreased significantly, indicating that porous structures (agglomerates) show markedly better reconstitution behaviour. Statistical analysis showed a significant correlation between Hr, dispersibility ($r_s = -0.80$, $p < 0.05$) and wettability values ($r_s = 0.75$, $p < 0.05$). These results showed that higher Hr values negatively affected dispersibility and positively affected wettability. A significant correlation was also detected between d_{50} and wettability ($r_s = -0.62$, $p < 0.05$).

Table 2 provides an overview of the polyphenolic compounds content and antioxidant capacity of powdered cocoa drink mixtures affected by the agglomeration process. As can be seen, the agglomeration significantly ($p<0.05$) decreased the content of total polyphenols and flavonoids, as well as the antioxidant capacity of powdered cocoa drink mixtures, with the exception of flavan-3-ol compounds whoose content was decreased after the gglomeration but in a much lower ratio compared to total polyphenols and flavonoids. On average a 45% decrease in TPC was observed after agglomeration of powdered cocoa mixtures prepared with cocoa containing lower fat content (10-12%), and a 42% decrease in TPC of powdered cocoa mixtures prepared with cocoa containing higher fat content (16-18%).

However, it was observed that in comparison to non-agglomerated cocoa mixtures, where no significant differences were established between the mixtures prepared with different sweeteners (A, B, C), upon agglomeration much higher variability occured between the cocoa mixtures (A, B, C) composed of cocoa powder with equal fat content. This was also observed for TFC of experimental cocoa drink mixtures, indicating that the content of these beneficial compounds depends on the agglomeration process, and the composition of employed ingredients (in this case sweeteners).

According to the obtained results, mixture B (containing aspartame/acesulfame sweeteener) prepared with cocoa powder containing lower fat content (10-12%) exhibited the highest content of TPC and TFC both before and after agglomeration, while among mixtures prepared with cocoa powder of higher fat content (16-18%) mixture C (containing stevia sweeteener) was characterized with the highest content of TPC and TFC both before and after agglomeration.

Table 2. Comparison of the total polyphenols, flavonoids and flavan-3-ols content and antioxidant capacity of experimental cocoa drink mixtures before and after agglomeration

Cocoa fat content	*Mixture*	**TPC** (mg GAE/L)	**TFC** (mg GAE/L)	**Flavan-3-ols** (mg catechin/L)	**ABTS** mM Trolox	**FRAP** mM Trolox
		Before agglomeration*				
10-12%	**A**	445.00 ± 12.21	308.18 ± 19.3	50.56 ± 0.23	1.83 ± 0.13	3.15 ± 0.18
	B	467.73 ± 9.64	335.45 ± 5.14	73.67 ± 2.04	1.36 ± 0.09	2.67 ± 0.39
	C	410.91 ± 23.14	255.91 ± 20.78	57.62 ± 1.13	1.49 ± 0.02	2.92 ± 0.22
16-18%	**A**	350.00 ± 19.28	215.91 ± 4.50	54.81 ± 1.25	1.62 ± 0.05	2.48 ± 0.46
	B	349.55 ± 7.07	175.91 ± 10.29	69.82 ± 3.40	1.34 ± 0.09	3.11 ± 0.17
	C	380.45 ± 1.93	199.09 ± 7.23	50.24 ± 3.40	1.34 ± 0.09	3.22 ± 0.09
		After agglomeration				
10-12%	**A**	156.82 ± 12.21	82.73 ± 15.43	46.38 ± 1.50	1.45 ± 0.00	0.69 ± 0.03
	B	300.91 ± 50.14	209.55 ± 59.78	72.63 ± 2.84	1.15 ± 0.04	1.10 ± 0.02
	C	271.36 ± 19.93	190.00 ± 43.71	53.54 ± 2.81	1.22 ± 0.03	0.98 ± 0.08
16-18%	**A**	185.91 ± 14.78	108.18 ± 19.28	50.18 ± 1.27	1.42 ± 0.03	1.17 ± 0.13
	B	193.64 ± 70.71	128.18 ± 60.43	63.40 ± 0.91	0.95 ± 0.12	1.42 ± 0.06
	C	244.55 ± 16.71	165.45 ± 7.71	47.18 ± 0.32	1.26 ± 0.07	1.15 ± 0.07

* - excerpt from Belščak et al. (2010)

Antioxidant capacity of powdered cocoa mixtures detremined by both ABTS and FRAP assays was decreasing after the agglomeration process. Although according to the results of FRAP assay a higher decrease rate could be observed, the consistent decrease of antioxidant capacities between both methods indicated that the decrease of polyphenolic compounds after agglomeration coincided with the decrease of the corresponding antioxidant capacity.

CONCLUSIONS

Agglomeration results in porous agglomerates, which show markedly better physical properties. On the other hand, it has a negative influence on the amount of bioactive compounds present in the cocoa drink powders. The agglomeration process results with a decrease of all polyphenolic compounds, as well as the antioxidant capacity of powdered cocoa drink mixtures. The bioactive compounds content of agglomerated cocoa drink mixtures depends on the type of sweetener used for the preparation, so further analyses are required to provide more information regarding this observation.

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INFLUENCE OF HULL AND IMPURITIES IN THE SEED ON SENSORY QUALITY OF COLD-PRESSED OLEIC TYPE SUNFLOWER OIL

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ABSTRACT

Cold-pressed oils are characterized by specific sensory characteristics, especially the odour and taste of the original raw material, making it completely different from the refined one. Beside the type of materials, the quality of oil obtained by cold pressing is influenced by many other factors, such as quality of raw materials, processing technology, the hull, or impurities (foreign matter) content in the material for pressing, oil storage conditions, etc. In order to prolong the expiry date of the oil, breeders make constant efforts to create hybrids with modified fatty acid composition, i.e. with high oleic acid content.

Different portions of the hull (0-20%) and impurities of organic matter (0-10%) in the material for pressing influence the sensory quality of cold-pressed oleic type sunflower oil, in which the content of oleic acid was 83.32%. Sensory evaluation was carried out by three member panel of experienced tasters. Effect of the hull and impurities on the colour of oil has been investigated by spectrophotometric measurement at 455 nm transparency in relation to carbon tetrachloride and the determination of pigment content: total carotenoids and total chlorophylls.

The total score for the sensory quality of oils ranged from 14 to 23 (maximum score was 25). The carotenoids content in tested samples ranged from 2.86 to 9.48 mg kg^{-1}, and chlorophylls content from 0.00 to 3.15 mg kg^{-1}. Between the values of transparency and total carotenoids content, there was a negative correlation with a high correlation coefficient ($R^2>0.9$).

The presence of high quantities of hull and impurities in the material for pressing has a very negative influence on sensory quality of oils obtained by pressing with the screw press. This influence is, in a far greater extent expressed in the aroma of oils (odour and taste) than in his appearance and colour. The presence of hull and impurities increase the content of total carotenoids and chlorophylls in the oil, and thus the reduction of transparency.

KEYWORDS: *high-oleic sunflower cold-pressed oil, hull, impurities, sensory characteristics, pigments*

INTRODUCTION

With the increased use of plant oils, more knowledge about their quality is required (Kamal-Eldin, 2006). Although there are a lot of indicators, which can be objectively measured and which can express chemical, biological, and nutritive and health quality, a quality evaluation of edible nonrefined oils is very important, and the first place is given to sensory quality. Over the last ten to twenty years, in our country, there has been an expansion and establishments of small factories for the production of edible unrefined oils, the so called „mini-refineries". These factories produce mostly sunflower oils, both linoleic and, increasingly, the oleic type (Dimić, 2005).

In comparison to refined oils, cold-pressed oils have more prominent sensory quality, therefore these oils contribute to creating a specific aroma of food and provide special gastronomic pleasure (Dimić, 2000, Šmit et al., 2005). It is well known that choice and quality of raw material are one of the most important quality parameters of oil made by cold pressing, apart from the importance of other parameters. Some of the numerous quality factors of cold-pressed oils are: technology of processing, storage conditions of seeds, presence of impurities, premises, as well as hull in the material for pressing, etc. (Dimić et al., 1997; Dimić, 2005). The

oleic type is especially important in sunflowers (Miklič et al., 2008; Raβ et al., 2008; Garcés et al., 2009), for which the accepted criteria for the amount of oleic acid in oil is more then 80% (Regulations, 2006).

The high content of monounsaturated oleic acid, as well as the possibly altered content and composition of some minor components, ensures that this oil has a much higher oxidative stability, in other words better shelf life (Kamal-Eldin, 2006; Miklič et al., 2008; Raβ at al., 2008). Due to this fact, oil with the higher content of oleic acid is recommended in nutrition as a partial or complete substitute for the standard linoleic type oil and/or saturated fats. On the other hand, due to its high oxidative stability at high temperatures, it can be used as a frying medium for thermal methods of food preparation (Marmesat et al., 2005; Kamal-Eldin, 2006; Raβ at al., 2008; Garcés et al., 2009). In France, U.S.A. and Spain, the high-oleic type of sunflower became the prevalent type, and in Hungary, Ukraine, Romania and Argentina its production is significantly increasing (Dozet and Vuković, 2007). In our country, amongst the first ones in Europe, hybrids with the high content of oleic acid were created, of which best known are NS-Olivko and NS-Oliva (Škorić et al., 1994; Miklič et al., 2008). Taking into consideration the fact that cold-pressed oil is widely used on the market, and since sensory quality and content of pigments, which influence the colour, are extremely important, both from the aspect of an oil producer, as well as consumers, this paper analyzes sensory parameters (characteristics) and colour of cold-pressed high-oleic sunflower oil, depending on the different presence of hull and impurities in the starting material for pressing.

MATERIALS AND METHODS

Within the scope of this paper, eleven samples of cold-pressed high-oleic sunflower oil were prepared. Oils were made from six-month old seeds of domestic hybrid NS-H-6326 from experimental growing in 2009. The content of the oil has a high amount of the oleic fatty acid C 18:1, 83.32%w/w, linoleic, C 18:2 and palmitic acid C 16:0, in an amount of 7.46%w/w and 4.32%w/w, respectively.

Cold-pressed oils were produced in the mini oil plant by pressing of sunflower seeds with the given content of hull and impurities, with the use of a screw press „Anton Fries", Germany, with the average capacity of 8.6 kg h^{-1} and 35-40 min^{-1} rotations of the screw. Oil temperature immediately after its been taken out of the press was 55-60°C. Pressed oils were kept for 24 hours at a room temperature (20-25°C) for sedimentation of residue, after which the top layer of oil was decanted and filtrated through an ordinary laboratory filter paper. Identification of analyzed samples is given in the following form: X%Hu+Y%Im. X marks the hull (Hu) share in %w/w, and Y marks the impurities (Im) in %w/w, in the starting pressing material.

Sensory evaluation of samples was done by a three-member expert committee, who evaluated the following quality parameters: appearance, odour, taste, aroma and colour (Dimić and Turkulov, 2000). For evaluation of individual sensory parameters (characteristics), the system of analytical-descriptive tests was used with points ranging from 0 (unacceptable quality) to 5 (optimal quality) (Radovanović and Popov-Raljić, 2001). Apart from the sensory analysis, oil colour was determined also by spectrophotometric methods as transparence measuring at 455 nm in relation to carbon-tetrachloride (Dimić and Turkulov, 2000), as well as by determining of total carotenoids (as β-caroten) and content of total chlorophylls (Wolff, 1968). Results of determining of transparence and content of total carotenoids and chlorophylls are expressed as a mean value of three replication and their standard deviations (X±SD). Furthermore, there is statistically significant difference between samples, obtained by independent t-test (95%; $p<0.05$). Statistical analysis of experimental data was performed by the statistical packages MS Excel 2007 and Statistica 9.0 (StatSoft, Tulsa, USA).

RESULTS AND DISCUSSION

Results which were obtained by sensory evaluation of samples are shown in Figure 1. The results show that samples 10%Hu+0%Im may be characterized as oils with pleasant taste and aroma with maximal total sensory score, but seemingly brighter then the optimal greasy yellow and with a somewhat mildly expressed characteristic odour. Taste (aroma) of this oil is characteristic, optimally defined as the aroma of dried and healthy raw kernels of sunflower. The overall sensory rating is high for the samples 5%Hu+2.5%Im, 15%Hu+2.5%Im and 10%Hu+5%Im, with certain variance of the taste, which is insufficiently distinguishing

sunflower. Rather low overall sensory ratings (14-17 scores) are for the samples 0%Hu+0%Im, 0%Hu+5%Im, 5%Hu+7.5%Im, 15%Hu+7.5%Im, 10%Hu+10%Im and 20%Hu+10%Im. Sample 0%Hu+0%Im has a bit weaker aroma due to its aroma being less characteristic of the raw material, due to the fact that this oil was made by pressing totally cleaned and shelled seeds and raw kernel.

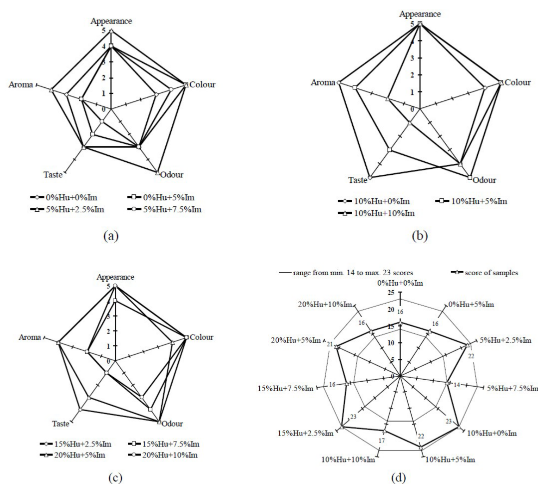

Figure 1. Scores of sensory quality of cold-pressed high-oleic sunflower oil:
a) samples with 0-5%Hu and 0-10%Im, (b) samples with 10%Hu and 0-10%Im,
(c) samples with 15-20%Hu and 0-10%Im and (d) total sensory scores of all samples

Other samples have aroma far worse than the characteristic one and have a characteristic odour and taste of the raw material, but with weak strange smell and prominent strange taste. These samples have the worst aroma of all examined samples. These oils have uncharacteristic taste with very prominent strange taste, and at the same time, uncharacteristic odour, with the presence of prominent strange smell. With these sensory parameters (characteristics), these oils could not be approved for direct consumption, pursuant to valid regulations pertaining to the quality of edible oils. According to the evaluation results of aromas of all samples it can be concluded that presence of impurities in the pressing material has the most unfavorable influence on the oil aroma. Comparing further results of aromas of all samples simultaneous presence of impurities and hull in the pressing material has unfavorable effect on oil aroma. On the other hand, however, the presence of hull has favorable effect on oil aroma (eg. sample 15%Hu+2.5%Im). Samples 15%Hu+7.5%Im, 10%Hu+10%Im and 20%Hu+10%Im have the weakest aroma, especially taste, which can be explained by the fact that presence of a certain part of impurities (in this case 7.5 and 10%), that is impurities and hull (10-20%) in the pressing material lead to the occurrence of unpleasant bitter taste of cold-pressed oil.

Results of this research about favorable effect of hull on aroma of cold-pressed sunflower oil are in accordance with available reference information. Premović et al. (2010) in previous research obtained similar results about effects of hull and impurities in the starting material for pressing on aroma of cold-pressed sunflower oil (linoleic type). Figure 1 shows that most samples have maximal number of points when appearance, as a parameter of sensory quality (5%Hu+2.5%Im, 10%Hu+0%Im, 10%Hu+5%Im, 10%Hu+10%Im, 15%Hu+2.5%, 20%Hu+5%Im and 20%Hu+10%). Contrary to these samples, which are „brilliantly" clear and transparent, samples 0%Hu+0%Im, 0%Hu+5%Im, 5%Hu+7.5%Im and 15%Hu+7.5%Im have noticeable mild turbidity without a visible residue, therefore receiving fewer points for appearance. By comparing appearances of all samples, it can be concluded that the presence of hull (10-20%), does not have a negative effect on the appearance, in the case when the presence of impurities amounts to less or to 5%. In samples 0%Hu+5%Im, 5%Hu+7.5%Im and 15%Hu+7.5%Im quantities of impurities (≥5%) present in oils have

equal negative effects on the appearance, causing reduced clarity, i.e. occurrence of mild turbidity. Also, the appearance of the sample 0%Hu+0%Im is similar because it is used only for pressing the kernel. Figure 1 shows of all analyzed oil samples, seven have maximal number of points when colour, as a parameter of sensory quality is concerned. These oil samples have a characteristic, attractive and completely specific yellow colour, while others samples (0%Hu+5%Im and samples with lower hull and highest impurities content) have a characteristic colour with certain deviations in shade, therefore receiving fewer points. This fact may also be explained by the negative effects of impurities, that is by simultaneous presence of impurities and hull in the starting pressing material on oil colour. The results of this research show that impurities present in the starting material for pressing have negative effects on oil colour. Differences in oil colour of samples, determined visually, are also confirmed both by values of transparence, as well as content of total carotenoids and content of total chlorophyll, shown in Table 1.

Table 1. Transparence and content of pigments (total carotenoids and total chlorophylls) of cold-pressed high-oleic sunflower oil

Identification	Transparence (% at 455 nm with CCl_4)	Content of total carotenoids, as β-carotene, mg kg^{-1}	Content of total chlorophylls, as chlorophyll A+B, mg kg^{-1}
0%Hu+0%Im	71.96 ± 0.14^{aA}	2.86 ± 0.02^{aA}	0.00 ± 0.00^{aA}
0%Hu+5%Im	54.26 ± 0.03^{bC}	4.89 ± 0.02^{bB}	0.93 ± 0.01^{bB}
5%Hu+2.5%Im	50.52 ± 0.03^{cD}	5.38 ± 0.02^{cC}	0.66 ± 0.01^{cC}
5%Hu+7.5%Im	44.00 ± 0.09^{dE}	6.46 ± 0.02^{dDF}	1.98 ± 0.01^{dD}
10%Hu+0%Im	60.30 ± 0.08^{eB}	3.99 ± 0.02^{eE}	0.00 ± 0.00^{aA}
10%Hu+5%Im	43.59 ± 0.06^{fE}	6.22 ± 0.02^{fF}	0.63 ± 0.01^{cC}
10%Hu+10%Im	33.10 ± 0.05^{gG}	9.08 ± 0.02^{gG}	3.09 ± 0.02^{eE}
15%Hu+2.5%Im	43.34 ± 0.06^{hE}	5.81 ± 0.02^{hBCF}	0.00 ± 0.00^{aA}
15%Hu+7.5%Im	41.00 ± 0.27^{iEF}	7.31 ± 0.02^{iD}	1.32 ± 0.01^{bB}
20%Hu+5%Im	36.71 ± 0.02^{jF}	8.34 ± 0.02^{jG}	1.84 ± 0.01^{fD}
20%Hu+10%Im	32.00 ± 0.05^{kG}	9.48 ± 0.02^{kH}	3.15 ± 0.02^{eE}

The results are presented as the mean value ± standard deviation (n=3). Different superscript letters present significant differences among oil samples. Superscript letters $^{a, b, c...}$ (p<0.05) and $^{A, B, C...}$ (p<0.001).

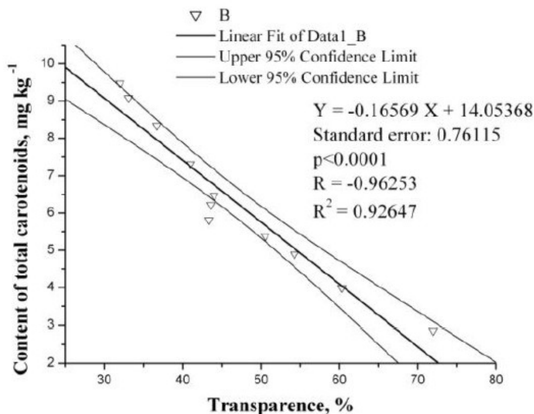

Figure 2. Correlation between content of total carotenoids and transparence of cold-pressed high-oleic sunflower oil

Higher amounts of total carotenoids are present in palm oil, oil of pumpkin seed, corn germs, sunflower, etc (Dimić, 2005). According to the research results of Tuberoso et al. (2007), the content of β-carotene in sunflower oil is 0.1 mg/kg, while the content of total carotenoids is 2-4 mg kg^{-1}. In soybean oil and rapeseed oil, the content of total carotenoids is 20-35 mg kg^{-1} and 25-100 mg kg^{-1}, respectively (Dimić i Turkulov, 2000).

The content of chlorophyll in the analyzed samples ranges from traces to 3.15 mg kg^{-1} (Table 1). Samples 0%Hu+0%Im, 10%Hu+0%Im and 15%Hu+2.5%Im contain total chlorophylls in traces (less than detection limit), samples 10%Hu+5%Im, 5%Hu+2.5%Im and 0%Hu+5%Im have a bit higher amount of total chlorophylls, respectively: 0.63±0.01, 0.66±0.01 and 0.93±0.01 mg kg^{-1}. Then, according to the increasing amount of chlorophyll, follow samples 15%Hu+7.5%Im (1.32±0.01 mg kg^{-1}), 20%Hu+5%Im (1.84±0.01 mg kg^{-1}) and 5%Hu+7.5%Im (1.98±0.01 mg kg^{-1}). Their highest content is in samples 10%Hu+10%Im (3.09±0.02 mg kg^{-1}) and 20%Hu+5%Im (3.15±0.02 mg kg^{-1}). Comparing the values of content of total chlorophylls in the analyzed samples, it was concluded that the presence of impurities in the pressing material gives higher content of total chlorophylls in oil than the presence of hull, and that simultaneous presences of impurities and hull in the pressing material cause the highest content of total chlorophylls.

By a statistical analysis of the results, it was determined that there are statistically significant differences in the content of total chlorophylls in the most analyzed oils ($p<0.05$ and $p<0.001$). There are no statistically significant differences in the total content of chlorophyll ($p<0.05$ and $p<0.001$) for the oil samples, which didn't contain more than 2.5% impurities in the pressing material during the preparation, regardless of the hull percentage. Also, there are no statistically significant differences ($p<0.05$ and $p<0.001$) in the total chlorophylls content for the samples with the highest total amount of chlorophylls (more than 10% hull and 10% impurities in the pressing material), Table 1.

By further comparison of these results with the colour results obtained by sensory analysis, it was determined that oil samples with lower and minimal content of total carotenoids and chlorophylls have the minimal points for colour as a parameter of sensory quality. Higher amounts of total chlorophylls are mainly present in unrefined olive oil and grape seed oil (Dimić, 2005). According to the information taken from the cited works, (Dimić and Romanić, 2004), content of total chlorophylls in virgin olive oil from the Mediterranean countries ranged from 7.73-15.08 mg kg^{-1}. Content of total chlorophylls in sunflower oil ranges from 0.5-0.8 mg kg^{-1} (Dimić and Turkulov, 2000). Content of total chlorophylls in cold-pressed sunflower oil ranges from traces to 0.99 mg kg^{-1} (Premović et al., 2010), while content of total chlorophylls in cold-pressed sunflower oil of oleic type is 5.15 mg/kg (Dimić and Romanić, 2004).

CONCLUSION

It has been concluded that both presence of impurities, as well as simultaneous presence of impurities and hull in the pressing material, have negative effects on sensory parameters of cold-pressed high-oleic sunflower oil; the presence of impurities causes lower sensory quality of oils. By comparing results of sensory analysis of oil, it can be argued that aroma, i.e. odour and taste of oil, depend more heavily on the presence of impurities, and simultaneous presence of impurities and hull than appearance and colour of oil. Apart from this, presences of impurities and hull in the starting material for pressing have almost identical effects on appearance and colour of oil.

Summing up the results of determining sensory quality, it can be concluded that by pressing of seed with the presence of hull (at the amount of 20%), the highest-quality cold-pressed oil is produced in relation to the aroma, i.e. odour and taste. Furthermore, presence of impurities and hull in the starting material for pressing in higher amounts causes higher content of total carotenoids and chlorophylls, as well as lower value of transparence in cold-pressed high-oleic sunflower oils.

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DEHULLING EFFICIENCY OF HIGH-OLEIC SUNFLOWER SEED ON THE LABORATORY \"AIR-JET\" IMPACT DEHULLER

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ABSTRACT

Based on market demands and nutrition, sunflower breeding has been focused on development of hybrids with high content of oleic acid in oil. Because the dehulling is extremely important for obtaining high-quality oil, high-oleic sunflower seed is small, the hull is thin and fitting to the kernel, it is necessary to optimize the process of dehulling. Successful dehulling requires the appropriate moisture in the seed and the appropriate pressure of air depending on type of dehuller.

In laboratory „air-jet" dehuller investigates the effectiveness of dehulling hybrid Olivko of high-oleic sunflower seed. Using three-level full factorial experimental design (3^2) the effect of seed moisture and pressure in the dehuller has been explored for dehulling quality. Prepared samples with a moisture content of 6, 8 and 10%, are being dehulled at a pressure 400, 600 and 800 kPa. The dehulled material is determined by the percentage of the following fractions: whole kernels, particles of kernel having size ≥ 3 mm, particles of kernel having size < 2 mm, „kernel powder", hull and undehulled seeds. The dehulling performance is evaluated as dehulling efficiency (E criteria) and quality of dehulling (K1 and K2 criteria).

The best dehulling of the high-oleic sunflower seed, or highest ratio of the whole kernels and particles of kernel having size ≥ 3 mm (53.46%), is performed at 800 kPa pressure and 6% - moisture content. Dehulling efficiency of oleic type of seed is 48.91%. Dehulling seeds of high-oleic sunflower hybrids has been very problematic. The content of moisture in the seed and the pressure of air in the dehuller have a significantly influence on the dehulling efficiency and dehulling quality of seeds.

KEYWORDS: *high-oleic sunflower seed; dehulling; moisture; pressure of air; dehulling efficiency*

INTRODUCTION

With the sunflower, the oleic type has specific importance (Miklič et al., 2008; Raβ et al., 2008; Garcés et al., 2009), where according to accepted criteria oleic acid content in oil, must be greater than 80% (Regulations, 2006). Regarding the total world production, the standard sunflower hybrids are still dominating. In France, U.S.A. and Spain, the high-oleic type of sunflower became the prevalent type, and in Hungary, Ukraine, Romania and Argentina its production is significantly increasing (Dozet and Vuković, 2007). In our country, amongst the first ones in Europe, hybrids with the high content of oleic acid are created, where the best known are NS-Olivko and NS-Oliva (Škorić et al., 1994; Miklič et al., 2008).

The seed of sunflower hybrids which produces high amounts of oil, contains 75-80% kernel and 20-25% hull (Dimić, 2005). The sunflower's hull contains very small amounts of lipids and is mainly consisted out of cellulose and hemicellulose matters. During the seed's preparation for further processing, partial or total removal of the hull is done to improve the quality of the gained products (refined or unrefined oils, kernel and kernel products, crushed oilseed, mill cake etc.) (Dimić, 2005; Grompone, 2005). Hybrids produce high amounts of oil, both linoleic and high-oleic, the hull is thin (0.10-0.40 mm) and firmly attached to the kernel, so the dehulling is more difficult. The hull is removed by dehulling, usually mechanically with dehullers and includes phases of crushing the hull and releasing the seed and the separation of the hull from the kernel. Dehullers work on different principles, like mills, rotating rollers, or on the principle of centrifugal force, the principle of pneumatic shock and the like (Dimić, 2005, Sharma et al., 2009).

Next to the size and shape of the seed, the quality of the hull (thickness, firmness) as well as its resistance to

cracking as very important parameters for seed dehulling, the moisture content of a seed is one of the most relevant parameters for the successful dehulling (Dimić, 2005; Grompone, 2005).

Having in mind that the dehulling of sunflower seeds is extremely important for obtaining high-quality crude, especially non-refined edible oil, this paper's aim was to investigate and determine the optimal conditions for the process of dehulling of seeds with the laboratory „air-jet" dehuller.

MATERIALS AND METHODS

In laboratory „air-jet" dehuller is tested on Olivko dehulling hybrid's effectiveness of high-oleic sunflower seed (HOSS). The content of the oleic acid (C 18:1) in HOSS oil is 78.00%w/w and the content of linoleic acid C 18:2 is 11,06%w/w. Sunflower grows in the region of Vojvodina. Seed is cleaned and dried to moisture content of 4.5-5.0%, immediately after the harvest, and it is stored until the preparation in PE bags. Moisturising the seed has been done using laboratory sprayer. To achieve uniform distribution of moisture the seeds are well mixed before the dehulling has been left in sealed containers for three days. Three portions of 250 g of sample is dosed in the injection unit of the laboratory pneumatic „air-jet" dehuller, and the pressure of the air flow in the „pneumatic cannon" is adjusted to 400, 600 and 800 kPa. After dehulling, the obtained samples at the exit of the dehuller are quantitatively collected, classified by manual separation of five fractions as follows: whole kernels (A), particles of kernel having size ≥ 3 mm (B), particles of kernel having size < 2 mm and „kernel powder" (F), hull (H) and undehulled seeds (U). Mass of certain fractions are measuring, and the mass fraction is expressed in %w/w on the basis of the undehulled seed mass (250 g).

Response surface methodology is used to determine the effect of seed moisture and pressure of air in the dehuller for the effectiveness of prepared dehulling sunflower seed using different seed moisture content and pressure of air in the dehuller levels, as it is reported to be an effective tool for optimizing a process. A 3^2 three-level full factorial experimental design has been employed to collect the data necessary for optimization of the dehulling procedure. The coded and uncoded parameter values are presented in Table 1. The dehulling performance (Z is dependent variables) is evaluated as dehulling efficiency (E criteria), quality of dehulling (K1 and K2 criteria) and calculated as follows: E=H×r, K1=F+U and K2=A+B. r being the ratio of seed mass to hull mass in the whole seed. This value is obtained experimentally by measuring the mass of hull separated after manually dehulling of the three portions of about 10 g of seeds (r is 4.88±0.19 for HOSS).

Table 1. Values of coded levels of seed moisture and pressure of air in the dehuller for the samples preparation

Independent variables	Symbols		Levels		
	Coded	Actual	0	1	2
Seed moisture, %	X	w	6	8	10
Pressure of air in the dehuller, kPa	Y	p	400	600	800

Moisture content of the seeds, after the moisturising is 6.11, 7.98 and 10.12%.

Having statistically processed the experimental values of the dependent variables using the chosen regressive equation: $Z = b_0 + b_1X + b_2Y + b_{11}X^2 + b_{22}Y^2 + b_{12}XY$. In this equation, b_0, b_1, b_2, b_{12}, b_{11} and b_{22} represent regression coefficients. The response surface and contour plots for these models are plotted as function of two variables.

Results of determining are expressed as a mean value of three replication and their standard deviations (X±SD). Furthermore, there is statistically significant difference between samples, obtained by independent t-test (95%; $p<0.05$). Statistical analysis of experimental data is performed by the statistical packages MS Excel 2007 and Statistica 9.0 (StatSoft, Tulsa, USA).

RESULTS AND DISCUSSION

Table 2 and Figure 1 show the changes in dehulling efficiency and dehulling quality of high-oleic sunflower seed (HOSS), depending on the moisture content of seeds at dehulling and pressure of air applied in the dehuller.

Table 2. The results of dehulling performance of high-oleic sunflower seed (HOSS) for different values of seed moisture and of pressure of air in the dehuller

Dehulling performance	Seed moisture (w), %	Pressure of air in the dehuller (p), kPa		
		400	600	800
E criteria	6	30.67±0.42aA	45.65±1.35bA	48.91±2.76cA
	8	24.67±1.51aB	23.09±2.30aB	41.04±5.28bB
	10	6.62±0.28aC	14.54±2.21bC	21.79±0.08cC
K1 criteria	6	68.91±3.52aA	45.83±2.56bA	36.35±2.97cA
	8	80.09±4.13aB	71.28±5.04bB	47.47±2.46cB
	10	92.60±0.82aC	83.40±3.12bC	65.06±4.12cC
K2 criteria	6	24.71±3.34aA	44.66±2.09bA	53.46±3.68cA
	8	14.77±1.06aB	23.88±2.20bB	43.99±4.37cB
	10	6.01±0.69aC	13.56±2.84bC	30.29±3.86cC

E criteria (%): E=H×r (H is the share of hull and r being the ratio of seed weight to hull weight in the whole seed), K1 criteria (%): K1=F+U (F is the share of particles of kernel having size < 2 mm and „kernel powder" and U is the share of undehulled seeds) and K2 criteria (%): K2=A+B (A is the share of whole kernels and B is the share of particles of kernel having size ≥ 3 mm)
The results are presented as the mean value ± standard deviation (n=3)
[a, b, c] - different lower case superscripts represented significant difference (p<0.05) of dehulling performance with different pressures of air in the dehuller (rows)
[A, B, C] - different upper case superscripts represented significant difference (p<0.05) of dehulling performance with different seed moisture contents (columns)

With the seed moisture content of 6-10% and the pressure of air in the dehuller of 400-800 kPa, the dehulling efficiency (E criteria) is in the range of 6.62-48.91%. Considering that E criteria directly indicates the dehulling efficiency of sunflower seeds, it is desirable that its value is as high as possible. Maximum efficiency HOSS dehulling (48.91%) has been observed at moisture content of seeds of 6% as for the pressure of air in the dehuller of 800 kPa.

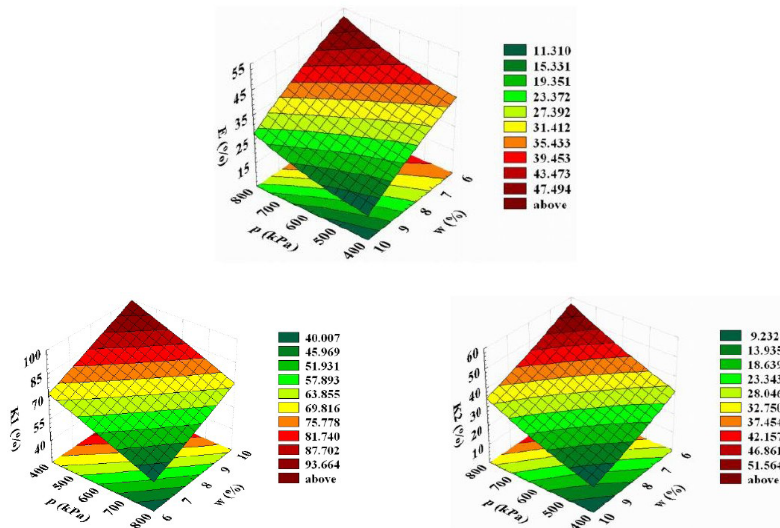
At the pressure of 800 kPa, and at moisture content above 6% the HOSS dehulling efficiency significantly decreases (p<0.05). If the moisture content does not change with 6% and the pressure is not reduced below 800 kPa, the dehulling efficiency of high-oleic type of sunflower is also significantly reduced (p<0.05). Therefore, reducing the moisture content of seeds and increasing the pressure in the dehuller, a significant increase (p<0.05) in dehulling efficiency of the HOSS can be observed. The smallest dehulling efficiency of seed (6.62%) were at the maximum seed moisture content (10%), and minimum pressure of air in the dehuller of 400 kPa. According to the results of Sharma et al. (2009), by dehulling the seeds of the different hybrids with high oil production using the centrifugal dehuller (with a peripheral speed of 39.5 m s^{-1}) the maximum dehulling efficiency is determined at 61.89-72.15%, with the moisture content of 8.5%.

With the seed moisture content of 6-10% and the pressure of air in the dehuller of 400-800 kPa, the proportion of fraction particles of kernel having size < 2 mm, „kernel powder" and undehulled seeds (K1 criteria), is in the range 36.35-92.60%. Bearing in mind that the presence of this fraction is undesirable, the aim is that its share should be smaller. The lowest value of K1 criteria (36.35%) is obtained in the HOSS, with 6% - seed moisture content and pressure of air in the dehuller of 800 kPa. With HOSS K1 criteria, it is significantly increased (p<0.05), at a pressure of 800 kPa, and the moisture content higher than 6%, as well as with the pressure values of less than 800 kPa and 6% moisture content. Significantly (p<0.05) higher values of K1 criteria is obtained if the moisture content does not change, and the pressure is reduced of less than 800 kPa (400 and 600 kPa). In this case, by observing the share of fraction particles of kernel having size < 2 mm, „kernel powder" and undehulled seeds, it can be concluded that reducing seed moisture and increasing pressure in the dehuller, the quality of dehulling of high-oleic sunflower seed significantly increases (p<0.05).

With the 6-10% - seed moisture content and the pressure of air in the dehuller of 400-800 kPa, the proportion of fraction whole kernels and particles of kernel having size ≥ 3 mm (K2 criteria), is in the range 6.01-

53.46%. Keeping in mind that the proportion of this fractions' fraction directly points at the quality of seed dehulling, the aim is that it should have higher value. The maximum value of K2 criteria (53.46%) has been obtained with the seed moisture content of 6% and pressure of air in the dehuller of 800 kPa. The smallest share of the aforementioned fractions (6.01%) has been obtained with the 10% - seed moisture content and the pressure of air in the dehuller of 400 kPa. K2 criteria for HOSS statistically significantly decreases ($p<0.05$), at the pressure of 800 kPa, the moisture content of more than 6%, the pressure values of less than 800 kPa and an moisture content (6, 8 and 10%). In this case as well, by observing the share of fraction whole kernels and particles of kernel having size ≥ 3 mm, it can be concluded that the quality of reducing the moisture content of seeds and increasing the pressure in the dehuller significantly increases ($p<0.05$).

The obtained results indicate well-known fact that the dehulling of the high-oleic hybrids is quite problematic (Tranchino et al., 1984). The morphological characteristics of seeds significantly contribute to it, ie., extremely thin hull, which firmly adheres to the kernel, and a small proportion of the hull in the seed. According to the results of Karlovic et al. (1992), the best dehulling of the sunflower seeds Olivko has been achieved with the seed moisture content of 5% and pressure of 620 kPa. Dehulling efficiency of the consuming sunflower ranges from 60.00 to 79.00%. This difference is precisely due to the fact that the hull of consuming sunflower seeds is thicker (0.24 to 0.28 mm), and its share in the seed higher (30%). With the sunflower seeds of the high-oleic (both high-oleic and linoleic types) hull thickness varies in the range 0.10 to 0.23 mm, and the proportion of the hull is 20-25% (Dimić, 2005). Dehulling of the seed is particularly important in the processing of sunflower, keeping in mind that the hull substantially impairs the quality of oil (Dimić et al., 1999). This becomes even more important in the production of cold-pressed oils with which no additional processing or refining is applied (Dimić, 2005). According to the results of Šmit et al. (2005), cold-pressed oil made from dehulled sunflower seed has much better quality and shelf life than oil made from non-dehulled seeds.



E - E criteria (%), E=H×r, H is the share of hull and r being the ratio of seed weight to hull weight in the whole seed, K1- K1 criteria (%), K1=F+U, F is the share of particles of kernel having size < 2 mm and „kernel powder" and U is the share of undehulled seeds (%), K2 - K2 criteria (%), K2=A+B, A is the share of whole kernels and B is the share of particles of kernel having size ≥ 3 mm
w - seed moisture (%) and p - pressure of air in the dehuller (kPa)

Figure 1. 3D graphics surface plots of dehulling performance changes of high-oleic sunflower seed (HOSS)

Figure 1 shows the 3D graphics of dehulling performance changes (E criteria), the share fraction of particles of kernel having size < 2 mm, „kernel powder", undehulled seeds (K1 criteria) and share fraction of whole kernels and particles of kernel having size ≥ 3 mm (K2 criteria) depending on the moisture content of seeds at dehulling and applied pressure of air in pneumatic dehuller.

Equations 1-3, obtained by using response surface methodology (Figure 1), represent the dependence of E, K1 and K2 criteria of seed moisture content and pressure of air in the dehuller.

$$E = 37.246 + 1.574\,w + 0.001\,p - 0.446\,w^2 + 4.881\cdot10^{-5}\,p^2 - 0.002\,wp \quad (1)$$

$$K1 = 44.580 + 7.838\,w - 0.056\,p + 0.062\,w^2 - 1.464\cdot10^{-5}\,p^2 - 0.001\,wp \quad (2)$$

$$K2 = 54.549 - 9.440\,w + 0.045\,p + 0.313\,w^2 + 3.804\cdot10^{-5}\,p^2 - 0.003\,wp \quad (3)$$

These equations which were derived and based on experimental data, can be applied to calculate the (response) values depending on variables (E, K1 and K2 criteria) at the desired value of moisture content of seeds and the pressure of air in the dehuller. Linear dependence (y=ax+b) between experimental and obtained theoretical values depends on variables with high coefficient of determination $R^2 \geq 0.95$, confirming the application's possibility of experimental model for that purpose. The above are marked with x for experimental values, with y for the calculated (predicted) values, and a and b are coefficients (Table 3).

Table 3. The values of coefficients a and b and statistics of linear dependence between computational and experimental obtained values of the dependent variables

Dehulling performance	Coefficients of linear equation	Coefficient of correlation, R	Coefficient of determination, R^2	Standard error	p - value
E criteria	a = 0.9536	0.9733	0.9473	0.08502	p<0.0001
	b = 2.7686	(0.97)	(0.95)	2.68447	
K1 criteria	a = 1.0413	0.9833	0.9668	0.07297	p<0.0001
	b = –1.0210	(0.98)	(0.97)	4.96578	
K2 criteria	a = 0.9703	0.9868	0.9738	0.06016	p<0.0001
	b = –0.3336	(0.99)	(0.97)	1.93706	

E criteria (%): E=H×r (H is the share of hull and r being the ratio of seed weight to hull weight in the whole seed), K1 criteria (%): K1=F+U (F is the share of particles of kernel having size < 2 mm and „kernel powder" and U is the share of undehulled seeds) and K2 criteria (%): K2=A+B (A is the share of whole kernels and B is the share of particles of kernel having size ≥ 3 mm)

CONCLUSION

Dehulling seeds of high-oleic sunflower hybrids has been very problematic. In terms of dehulling there are extent differences due to different conditions of dehulling - the moisture content of seeds, while dehulling and pressure of air in the dehuller are marked. Maximum dehulling efficiency and the best dehulling quality of the high-oleic sunflower seeds is achieved when the seed moisture content is 6% and the pressure of air in the dehuller is 800 kPa. This type of sunflower seed in these conditions is found to have maximum dehulling efficiency and highest share of the whole kernel and the large particles of kernel (size ≥ 3 mm). The share of small particles of kernel (size < 2 mm), „kernel powder" and undehulled seed with the above mentioned values of seed moisture content and pressure of air in the dehuller, is minimal. Obtained equations using response surface methodology, with a high coefficient of determination ($R^2 \geq 0.95$) can be applied to calculate the values of E, K1 and K2 criteria, with various values of seed moisture content and pressure of air in the dehuller.

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ANALYSIS OF THE SENSITIVITY OF FLAVONOÏDS UNDER HEAT PROCESS ACCORDING TO THEIR STRUCTURES

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ABSTRACT

Solutions of Rutin, Esculin and Naringin were heated at different temperatures (70-130 °C) during two hours in a water/oil bath. Residual concentrations were followed by HPLC and antioxidant capacity was measured by the ABTS method. It appears that according to their structures, flavonoids have different sensitivity to heat. Esculin is not affected by a heat treatment at 130 °C during 2 hours; low degradations (20 %) occur for Naringin above 110 °C; Rutin is highly degraded from 90 °C. The evolution of residual concentrations were established and modelled for degraded molecules (Rutin, Naringin) by an Arrhenius approach. For Rutin, an increase of temperature accelerates the reaction of degradation. The rate of degradation *k* ranges between $6 \cdot 10^{-4}$ and $1.59 \cdot 10^{-1}$ min^{-1} for temperature between 70 and 130°C, whereas Naringin is less sensitive, reaction rate constant ranges between $4 \cdot 10^{-4}$ and $1.9 \cdot 10^{-3}$ min^{-1} for temperatures above 100 °C. The Rutin half-live time ranged from 19.25 to 0.07 h when temperature increased from 70 to 130 °C; for Naringin, calculated half-live times at 110, 120 and 130 °C were 28.9, 7.2 and 6.1 h respectively. Activation energy for Rutin and Naringin are in the same order of magnitude: 104 ±3 kJ/mol. Solutions of Rutin and Naringin heated remains an antioxidant activity despite the degradation of the native molecules. Final antioxidant activity is equal to 54 % of the initial antioxidant activity for Rutin and 164 % for Naringin. Indeed, degraded products have an antioxidant activity sometimes higher than the native molecule; degraded products from Naringin have an antioxidant activity of 0.37 µmol/mg instead of 0.24 µmol/mg for Naringin.

KEYWORDS: *heat process; flavonoid; antioxidant capacity; modelling*

INTRODUCTION

Over the last few decades, links between diet and general well-being have been investigated. Numerous studies have provided evidence of a potential role for flavonoid-rich foods in the lowering the risk of specific diseases (cancers, coronary diseases) (Tomas-Barberán et al., 2000). This benefit effect is attributed to antioxidant activity of flavonoids (Cao et al., 1997; Heim et al., 2002). Most of the studies on flavonoids and their antioxidant activity focused on raw foods, whereas the human diet includes mainly cooked and processed foods. These units operations and the food matrix of the diet formulation affect the biodisponibility and the antioxidant activity of flavonoids (Nicoli et al., 1999). So, to understand the role of flavonoids in human diet the effect of the process and the food matrix need to be investigated deeply. In fact, few studies have examined the effect of thermal processing on flavonoid behaviour in solution. The degradation of flavonoids is not only a function of temperature and magnitude of heating; it may depend also on other parameters such as pH, phytochemicals, structure and even the presence or absence of oxygen (Buchner et al., 2006; Friedman, 1997; Takahama et al., 1986).

The objective of this paper is to study and model the effect of heat treatment, on the flavonoid content and their antioxidant activity. To attempt this goal, three molecules with different structures were chosen to establish their sensitivity to heat. Relevant studies on effect of processes should be realized independently of food matrix effect to avoid interactions between flavonoids and food matrix (Freeman et al., 2010; Hidalgo et al., 2010). Thus, this study was led on aqueous solutions of flavonoids, to mimetic behaviour of flavonoids in food without having effect of the food matrix. The study of the effects of a heat treatment leads to the establishment of flavonoid degradation kinetics. Thus, modelling these kinetics is relevant to predict the influence of processing on the food product quality, particularly nutrients losses. Modelling of degradation kinetics includes calculating the reaction order, the rate constant and activation energy, which are essential data to minimize undesired changes and optimize process design and food formulation.

MATERIALS AND METHODS

Substrates

Three molecules were studied. Table1 summarizes their molecule structures and their solubility in water

Table 1. Characteristics of studied molecules

Molecules	Structure	Solubility	Concentration in water (g/l)
Rutin Flavonoid (flavonol)		In water : 125 mg.l^{-1}	0.03
Esculin Glucose heterosid		In water: 1.9g. l^{-1}	0.5
Naringin Flavonoid heterosid		In water (40°C): 1 000 mg.l^{-1}	0.2

Reagents

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were purchased from Sigma -Aldrich, Trolox, esculin sesquihydrate (≥97.5%) and potassium persulfate were purchased from Fulka. Methanol and ethanol were from Carlo Erba and VWR respectively. All reagents and solvents were of analytical grade

Preparation of model aqueous solutions

For the heat treatment experiments, aqueous solutions were prepared (Esculin 1,36mM; Naringin 0.34mM and Rutin 0.049mM, respectively). The substances were solubilized for 4h in the dark at 50°C. Every solution was prepared in the same way for the different time-temperature heating combinations used.

Thermal treatment of model solutions

The different model solutions were filtered through a syringe filter (0.20μm) then 10ml samples of each solution were put in a screw cap pyrex tubes and heated from 70 to 130°C for a total duration of 120min. Samples were taken every 15min, cooled in an ice (or water) bath and analyzed by HPLC/DAD.

Thermal treatments were conducted on a water bath (Memmert, WNE 10) for temperatures below 100°C while they took place in an oil-based bath (Hubert W8518D) for others temperatures.

HPLC/DAD analyses

Phenolic compounds were analyzed in an Elite Lachrom HPLC system (VWR HITACHI) consisted of a quaternary pump (L-2130), an autosampler (L-2200) and a diode-array detector (L-2455). The analyses were carried on a C18 (150x4.6 mm) column (Grace) with a water-methanol mobile phase (50:50 (v/v)) at a flow rate of 1mL/min in an isocratic mode (4min). The injection volume was of 50μL and the column temperature being set at 50°C. The detection was performed simultaneously at 200, 280, 245 and 350nm. Triplicate measurements were taken for all samples.

Antioxidant capacity, using the ABTS assay

ABTS assays were performed with a spectrofluorimeter SAFAS (Xenius, France) equipped with a 96-well polystyrene plate. Scavenging free radical potentials were tested in an aqueous solution of ABTS according to the method described by Re et al. (1999) with modification for use in microplates. ABTS was dissolved in water to a 7mM concentration. The radical generation was obtained by adding potassium persulfate to attend a final concentration of 4.25mM. The solution was kept in the dark at room temperature for 12h-16h. The same solution was then diluted with ethanol to have an absorbance of 0.70±0.02.

To determine the scavenging activity of different model solutions, each well of the spectrophotometer plate was filled with 220μL of the ABTS solution to which a volume of 80μL of standard (Trolox) or a tested sample dilution (1, 0.8125, 0.625, 0.4375, 0.25, 0.125, 0) was added. The blank well was filled with 300μL of methanol. Results were expressed as micromoles of Trolox equivalents TEAC values (Trolox Equivalent Antioxidant Capacity). The percentage of inhibition was calculated by the equation: Inhibition percentage where A_C and A_S are the absorbance of the control and of the test sample, respectively.

Statistical aalysis

Statistical analysis was performed by using the freeware R 2.11.0. Student's tests for two samples and ANOVA for more samples were used, criterion for statistical significance was $P<0.05$.

RESULTS AND DISCUSSION

Degradation kinetics

Solutions of Rutin, Esculin and Naringin were heated between 70 and 130°C during 2 hours. Evolution of flavonoid residual concentrations are shown Figure 1. Standard deviations are too low to be seen on the graphs, thus we can consider differences between curves for different temperatures as significant. Figure 1a highlights the degradation of Rutin in aqueous system under heating. Rutin degradation increased with heating magnitude and duration. At low temperatures (70°C) less than 10% of Rutin content was degraded. At 100°C, less than 15% of the flavonoid content remained in the model solution. After 45min at 130°C Rutin was no more detectable by the HPLC/DAD system. Naringin showed a higher stability than Rutin when heated at 100,110, 120 and 130°C. In fact, as illustrated in Figure 1c, no degradation was found for temperatures below 100°C. Less than 2% decrease was reported at 100°C and only 19.58% of Naringin content degradation were reported when the model solution was heated at 130°C for 120min. Esculin (Figure 1b) was found to be heat stable until 130°C during 2 hours.

The three molecules show different sensitivity to heat. Some authors have already suggested that this difference of sensitivity could be due to difference of structures. Rutin shows a higher stability compared to its aglycon form (Quercitin) (Buchner et al., 2006; Friedman, 1997; Makris, Rossiter, 2000; Takahama, 1986).

These findings are attributed to the prevention of carbanion formation because of the glycosylation of the 3-hydroxyl group in the C-ring (Buchner et al., 2006; Friedman, 1997; Takahama, 1986). Authors reported also that Luteolin was more stable to heat than rutin and luteolin-7-glucoside when heated at 180°C for 180min (Murakami et al., 2004).

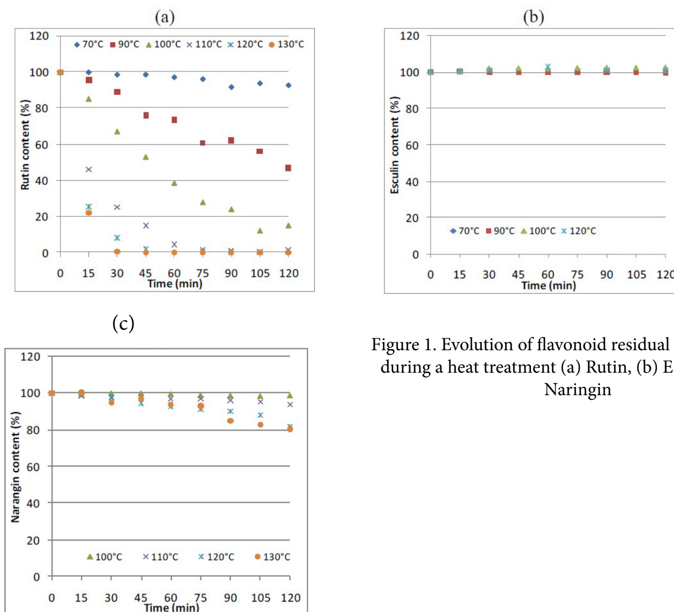

Figure 1. Evolution of flavonoid residual concentrations during a heat treatment (a) Rutin, (b) Esculin and (c) Naringin

Evolution of antioxidant activity during heat process

To investigate whether phenolic compounds degradation is associated with antioxidant activity changes, the antioxidant capacity of the model solution was also studied. On Table 2, the antioxidant activity for Rutin, Naringin and Esculin are indicated for different temperatures. Antioxidant activity is expressed in Trolox Equivalent Antioxidant Capacity in % and initial values of TEAC as considered as 100 %. In the control phenolic samples (0min of reaction time), rutin showed greater antioxidant ability when compared with naringin, the TEAC values was of 2.36 and 0.26 mM respectively. These results are very close to those reported by Heim et al. (2002), 2.4 and 0.24 mM respectively. The higher TEAC value of rutin can be attributed to its 3',4'-catechol (dihydroxy benzene) structure and the 2-3 double bond of the ring C (Table 1) which increases flavonoid antioxidant ability as suggested by Rice-Evans et al., (1996,1995). Naringin structure being lacking of these two features have a lower activity as radical scavenger.

Both a decrease (rutin) and an increase (naringin) in antioxidant activity were observed upon the two flavonoids degradation however no antioxidant activity changes were found in the esculin model solution with a constant value of 0.34mM which is slightly higher then narangin TEAC value. As showed in Table 2, a significant decrease of the antioxidant capacity of the rutin model solution was observed when samples were heated for 120min at temperatures ranging from 70 to 130°C. The minimum antioxidant capacity was observed at 130°C after a 120min heating duration. In fact, the rutin solution TEAC value dropped to 2.06µmol/mg compared to an initial value of 1.9µmol/mg being then reduced by nearly 50%. In opposition to these findings, naringin antioxidant capacity was found to increase with heating time and magnitude (Table 2). Antioxidant capacity increased by 55.24% at 130°C with a corresponding TEAC value of 0.37µmol/mg compared with a starting value of 0.24µmol/mg when naringin model solution was not yet heated. For Esculin the

antioxidant activity remains constant (100 %) conforming the absence of the degradation of this compound.

Table2. Evolution of the antioxidant power for Rutin, Naringin and Esculin with heat treatment

	70°C		90°C		110°C		130°C	
TEAC (Rutin) (%) ± 2%	0 min	120 min	0 min	120 min	0 min	120 min	0 min	120 min
	100	98.9	100	99.7	100	59.6	100	53.1
	100°C		110°C		120°C		130°C	
TEAC (Naringin) (%) ± 2%	0 min	120 min	0 min	120 min	0 min	120 min	0 min	120 min
	100	102.5	100	103.9	100	101.9	100	163.9
	70°C		90°C		100°C		120°C	
TEAC (Esculin) (%) ± 2%	0 min	120 min	0 min	120 min	0 min	120 min	0 min	120 min
	100	101.1	100	100.8	100	101.5	100	98.4

Modelling of degradation kinetics

Degradation kinetics of Rutin and Naringin were modeled. These kinetics follow a first-order reaction model according to (1).

$$\ln(C_t/Co) = -kt \quad (1)$$

Where C_0 is the initial flavonoid content and C_t is the flavonoid content after t minutes of heating at a given temperature, k is the reaction rate constant (min^{-1}).

The reaction rate constants were determined (Table 3). Good determination coefficients were obtained.

Table 3. Kinetic parameters of Rutin and Naringin during thermal degradation

Model solution	Temperature (°C)	k (min^{-1})	$t_{1/2}$ (h)	Ea(kJ/mol)
Rutin	70	$6\cdot10^{-4}$ (0.8364)[a]	19.25	107.4 (0.9887)
	90	$5.8\cdot10^{-3}$ (0.9676)	1.99	
	100	$1.7\cdot10^{-3}$ (0.9590)	0.68	
	110	$5.09\cdot10^{-2}$ (0.9889)	0.23	
	120	$7.22\cdot10^{-2}$ (0.9690)	0.17	
	130	$1.59\cdot10^{-1}$ (0.9659)	0.07	
Naringin	110	$4\cdot10^{-4}$ (0.9209)	28.88	100.6 (0.8425)
	120	$1.6\cdot10^{-3}$ (0.9564)	7.22	
	130	$1.9\cdot10^{-3}$ (0.9284)	6.08	

[a] Numbers in parentheses are the determination coefficients.

For Rutin, an increase of temperature accelerates the reaction of degradation. k ranges between $6\cdot10^{-4}$ and $1.59\cdot10^{-1}$ min^{-1}. Whereas Naringin is less sensitive, reaction rate constant ranges between $4\cdot10^{-4}$ and $1.9\cdot10^{-3}$ min^{-1} for temperatures above 100°C.

The temperature dependence of the degradation rate constants can be fitted with the Arrhenius model ac-

cording (2). The high values of R^2 reported in Table 3 confirm this finding.

$$\ln k = \ln k_0 - \frac{Ea}{RT} \quad (2)$$

k is the rate constant (min^{-1}), Ea is the activation energy ($kJmol^{-1}$), R the gas constant 8.314J/mol K, and T the heating temperature (K).

Half-live times $t_{1/2}$ are also calculated (3):

$$t_{1/2} = \frac{\ln 2}{k} \quad (3)$$

where $t_{1/2}$ is the heating time required to degrade 50% of the flavonoid (min),

According to Table 3, the degradation rate of Rutin increased with heating temperature and time. The $t_{1/2}$ ranged from 19.25 to 0.07h when temperature increased from 70 to 130°C. Rutin stability decreased at high temperatures as indicated by low $t_{1/2}$ values found.

Naringin is gradually degraded with increasing temperatures. The calculated $t_{1/2}$ values at 110, 120 and 130°C were 28.9, 7.2 and 6.1h respectively. More than 6 hours at a heating temperature of 130°C are needed to degrade 50% of the initial Naringin content while only a 4.32min heating duration at the same temperature induces a 50% loss of the initial Rutin content in model solution. Activation energy for Narangin is relatively high as regard the reaction rate constants (100.62 kJ/mol). By taking account uncertainty, we can consider Activation energy for Rutin and Naringin in the same order of magnitude. Ea values highlight that Rutin is more susceptible to heat than Naringin.

CONCLUSION

The effect of heat treatment of the three flavonoids indicated that in one hand that the sensitivity of these compounds depends upon their structure and in the other hand the products of the degradation exhibit an antioxidant power. This antioxidant activity is more pronounced for Naringin than for Rutin. This behaviour could be attributed to the nature of the formed products. To illustrate this behaviour RMN analyses will be done to identify the degraded products. In this study we focused only on the effect of heat treatment but these effects can be modified by the presence of other food ingredients depending on the composition of the food matrix. Others studies will deal with the impact of food matrix on the flavonoid degradation.

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EXTRACTION OF LYCOPENE TOMATO OLEORESIN

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ABSTRACT

The interest for components extracted from plant matrices, like lycopene, is due to their pharmacological features. Also, the application of these components is mostly related to the nutraceutical food. Lycopene is a red carotenoid pigment, a phytochemical found in tomato and other red fruits and vegetables. It is a powerful carotenoid antioxidant that provides protection against damage from free radicals, thus reducing risk of various cancers.

The aim of this study was to establish appropriate conditions for extraction of tomato oleoresin with high lycopene content. Extraction of oleoresin from dry tomatoes was performed by maceration technique, applying organic solvents: acetone, methanol and *n*-hexane. The influence of the temperature (30-50 ^{o}C), time (30-300 min), particle size (0.1-1mm) and ratio of solid and liquid phase (0.02-0.1% w/v) was studied using acetone at maceration of dry tomatoes fruits. Extraction efficiency was compared in terms of the oleoresin yield and the quantity of obtained lycopene.

The methanol gave the highest oleoresin yield compared to the acetone and *n*-hexane. From the aspect of lycopene content, acetone and *n*-hexane showed the same extraction efficiency. At extraction with acetone, the contact between solid and liquid phase of 5h enabled obtaining satisfactory oleoresin quantity and lycopene content. The ratio of solid and liquid phase of 0.02%w/v was more favorable in comparison to the other investigated ratios. Oleoresin quantity increased by raising the extraction temperature to 50 ^{o}C. However, the highest lycopene content in tomato oleoresin was measured in the extract obtained at 40 ^{o}C. Related to the lycopene content, it is better to perform extraction at lower time contact of solid and liquid phase using smaller particle size, and *vice versa*.

KEYWORDS: *dry red tomato; oleoresin yield; lycopene content*

INTRODUCTION

Lycopene is major carotenoid pigment principally responsible for the characteristic red colour of tomatoes and other red fruits (Brandt et al. 2006; Collins et al. 2006). It is one of the popular pigments highly accepted by food industry as a food colorant (Mortense 2006; **Calvo et al. 2008**). Also, lycopene has attracted attention due to its biological and physiochemical properties. The health benefits of lycopene as a micronutrient is based on its apparent ability to provide protection against many chronic diseases such as cancer and cardiovascular diseases due, in most cases, to its antioxidant properties (Basu and Imrhan, 2007; Jian et al., 2007; Kavanaugh et al., 2007; Rao and Rao, 2007; Story et al., 2010).

Lycopene oleoresin from tomato is a lycopene-rich extract from tomato ripe fruits. It consists of tomato oil in which lycopene, together with a number of other constituents that occur naturally in tomato, is dissolved. These constituents include fatty acids and acylglycerols, unsaponifiable matter, water soluble matter, phosphorous compounds and phospholipids (EFSA, 2008; FAO JECFA, 2009; Rath et al., 2009). Lycopene can be extracted from tomato using organic solvents (Breithaupt, 2004; Periago et al., 2006; Calvo et al. 2007; Choksi and Joshi, 2007; Naviglio et al. 2008) or supercritical CO_2 (**Vasapollo** et al., 2004; Topal et al., 2006; Lenucci et al., 2010) obtaining a lipid extract rich in carotenoids; the concentration of lycopene obtained depends on factors like the source of lycopene and the extraction conditions. Extracts obtained with supercritical CO_2 do not contain residual solvent, but this technology is expensive.

The objective of this work was to study the influence of solvent type, extraction temperature, time, ratio of solid and liquid phase, and particle size on red dried tomato fruits extraction efficiency, expressed by the yield of oleoresin and lycopene content in it, and to establish mathematical models to predict system responses.

MATERIALS AND METHODS

Plant material

Red dried tomato fruits, product of AD Agrova from Skopje, R. Macedonia. The content of dry matter in plant material is determined by drying in oven at 105°C till constant mass achievement (AOAC, 925.10) was 7.14%. The red dried tomato fruits were grounded using Retsch ZM1 mill (Germany) and after that sieved through sieves system with mesh size from 0.1mm to 1mm. The weight of each fraction is gravimetrically determined (Table 1).

Table 1. Distribution of particles size

Particle size (mm)	0.1	0.125	0.2	0.315	0.5	0.63	0.8	1	< 1
Fraction yield (%)	2.1	0.6	6.6	8.7	19.1	30.1	0.2	22.2	11.87

Solvents

Methanol, acetone and *n*-hexane were used for extraction of plant material. Pro-analysis-grade solvents were purchased from A. D. Alkaloid (Skopje, R. Macedonia) and Merck (Haan, Germany).

Extraction procedure

The samples of 1 g (0.0001 g accurate weight) were extracted in thermostatic water bath by maceration technique using solvents with different polarity degree (methanol, acetone and *n*-hexane), at different temperatures (30, 40, 50 and 60°C), time of extraction (30-300 min), solid/liquid ratio (0.02%w/v to 0.1%w/v) and different sample particle size (0.1mm to 1mm). After extraction, the solvent was removed under vacuum (rotary vacuum evaporator, type Devarot, Slovenia, 35 °C, atm. pressure). Solvent traces from the extracts i.e. oleoresins were discharged by drying the sample at 40 °C, 105 mPa (vacuum drier, Heraeus Vacutherm VT 6025, Langenselbold, Germany). Obtained oleoresins were cooled in a dessicator and weighed.

Determination of oleoresin quantity

The steps of drying, cooling and weighting were repeated until the difference between two consecutive weighs was smaller than 2 mg. The extraction procedure was performed in duplicate. The yield of oleoresin was estimated according to dry matter weight in extracted quantity of sample. The oleoresin was transferred into a 50 mL volumetric flask and filled to 100 mL with *n*-hexane (1st dissolution).

Determination of lycopene content

The content of lycopene in the oleoresins was determined by reading of the absorbance on 503 nm on the spectrophotometer (Varian Cary Scan 50 spectrophotometer, Switzerland) in 1cm quartz cells, at 25°C. 1 mL of the 1st dissolution was dissolved and filled up to 10 mL with *n*-hexane and absorbance was measured. The lycopene content was calculated using the extinction coefficient of lycopene ($^{1\%}E_{503nm}$= 310) in *n*-hexane (Davis et al., 2003). The content of lycopene in the extract was expressed in g/kg.

Statistical analysis

The statistical analysis and evaluation of the data were performed using STATISTICA 8 (StaSoft, Inc., Tulsa, USA) software.

RESULTS AND DISCUSSION

Selection of the most appropriate solvent for extracting the analytes of interest from the plant matrix is a basic step in the development of solvent extraction methods. The influence of the solvent type on the extract yield and lycopene content in the extract is presented on Figure 1. For methanol, acetone and n-hexane, the values of density and dielectric constant are 0.7598 g/cm3 and 32.3, 0.7613 g/cm3 and 20.7, 0.6184 g/cm3 and 2.02, respectively. By density and dielectric constant increasing, higher extract quantities were obtained (Figure 1a). In the case of lycopene, due to its non polar characteristics, higher lycopene content in extract was obtained when n-hexane was applied at extraction of red dried grinded tomatoes (Figure 1b).

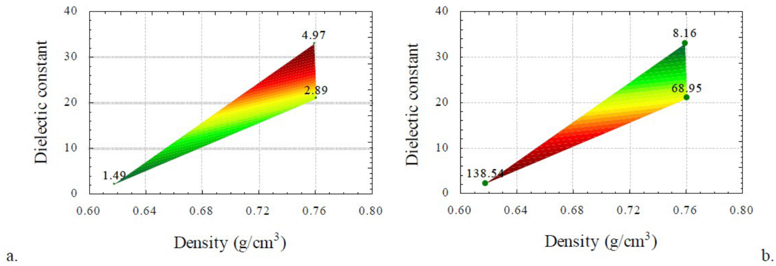

Figure 1. Yield of oleoresin (%) - a and lycopene content in the extract (g/kg) - b obtained at different density and dielectric constant of the solvent (30oC, 1h, 0.63 mm particle size, 0.04 g/mL solid and liquid phase ratio)

Figure 2 represents the influence of the extraction time on the oleoresins quantity and content of lycopene in oleoresins, obtained at 30oC, 0.63 mm particle size of pant material and 0.04 g/mL ratio of solid and liquid phase. By increasing the time, higher quantity of oleoresin was obtained. The highest lycopene content in the investigated extraction parameters was reached with 180 min contact of solid and liquid phase.

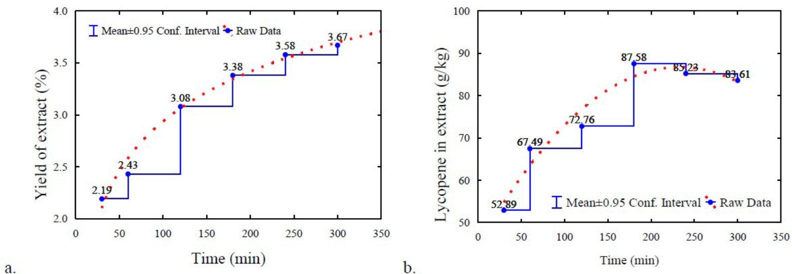

Figure 2. Yield of the extract (a) and lycopene content in the extract (b) obtained at different times of extraction (30oC, 0.63 mm particle size, 0.04 g/mL solid and liquid phase ratio, acetone)

Table 2. Regression coefficients, R2, adjusted R2 and p for yield of the extract and lycopene in the extract obtained at different times of extraction (30oC, 0.63 mm particle size, 0.04 g/mL solid and liquid phase ratio, acetone)

	Yield of extract (%)	Lycopene in extract (g/kg)
b_o (intercept)	1.80036	44.65076
b_1	0.01282**	0.36065*
b_1^2	- 0.00002**	- 0.00077*
R^2	0.9954	0.9467
adjusted R^2	0.9924	0.9112
p or probability	0.0003	0.0123

Subscripts: 1 = time (min)

*Significant at 0.05 level; **Significant at 0.01 level; ***Significant at 0.001 level;

The effect of the extraction time as independent variable upon the dependent variables i.e. yields of the extract and lycopene content in extract by using response surface analysis was estimated.

Table 2 presents the liner and quadratic coefficients of the time of extraction in the models for yield of extract and lycopene content in extract, and their corresponding R^2 when acetone was used as extraction solvent of red dried tomatoes at 30°C, 0.63 mm particle size, and 0.04 g/mL of solid and liquid phase ratio. As it can be observed, the R^2 values for the response variables were higher than 0.94 when oleoresin quantity and lycopene content in oleoresin are concerned. This indicates that the regression models adequately explained the process. The probability (*p*) values of regression models show no lack-of-fit ($p < 0.01$). As it can be observed from Table 2, yield of the extract was significantly affected by the positive linear effect ($p < 0.01$) and negative quadratic effect of ($p < 0.01$) of the time of extraction. In the case of lycopene content in the extract, the same effects of the time of extraction were confirmed at $p < 0.05$.

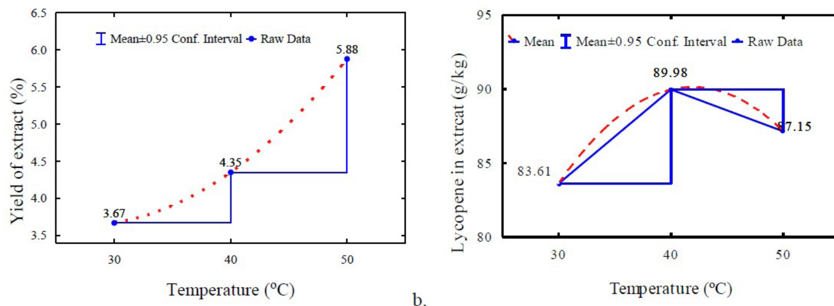

Figure 3. Yield of the extract (a) and lycopene content in the extract (b) obtained at different temperatures (5h, 0.63 mm particle size, 0.04 g/mL solid and liquid phase ratio, acetone)

As can be seen from Figure 3a, change of the extraction temperature from 30 to 50°C positively affects the quantity of the extract. In accordance to the lycopene content, at 40°C the highest quantity was obtained, compared to the quantity of lycopene determined in the extract obtained at 30 and 50°C (Figure 3b).

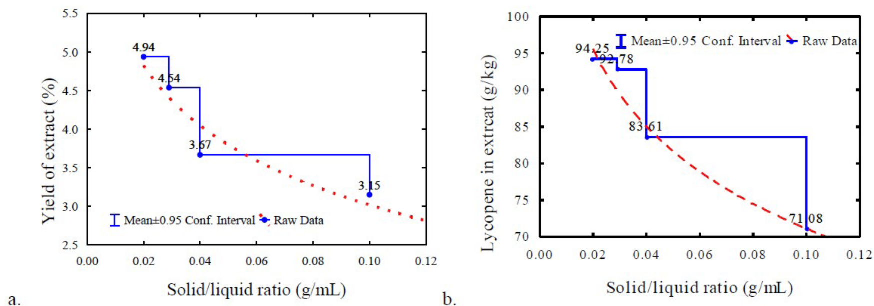

Figure 4. Yield of the extract (a) and lycopene content in the extract (b) obtained at different solid/liquid phase ratio (30oC, 5h, 0.63 mm particle size, acetone)

Decreasing of the raw material quantity in the quantity of solvent used from 0.1 to 0.02 g/mL enabled obtaining higher quantity of oleoresins, and also, higher quantity of lycopene in oleoresins (Figure 4b).

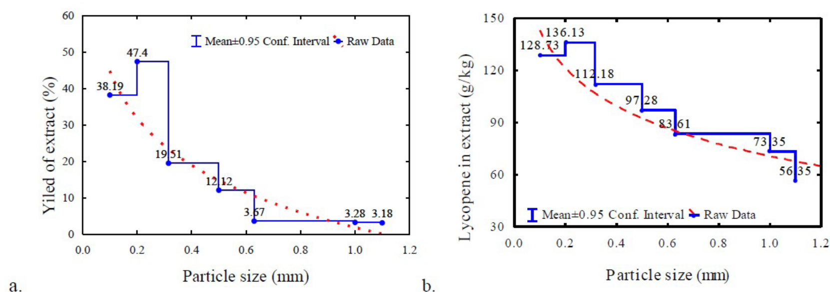

Figure 5. Yield of the extract (a) and content of lycopene in the exract (b) obtained at different particle size of raw material (30oC, 5h, 0.04 g/mL solid and liquid phase ratio)

According to Figure 5, the yield of the extract and lycopene content in the extract increased as the particle size decreased, caused by increasing of the contact surface between solid and liquid phase.

The influence of particle size on the quantity of oleoresin and lycopene is presented by the coefficients of the proposed model in Table 3. As indicated by *p* value, negative linear effect ($p < 0.05$) of particle size as independent variable is confirmed to be significant for oleoresin quantity and lycopene quantity in it, as investigated dependent variable.

Table 3. Regression coefficients, R^2, adjusted R^2 and p for yield of extract and lycopene in extract obtained at different particle size of raw material (30°C, 5h, 0.04 g/mL ratio of solid and liquid phase, acetone)

	Yield of extract (%)	Lycopene in extract (g/kg)
b_o (intercept)	51.93200	146.0990
b_1	-125.21600*	- 111.4130*
b_1^2	- 0.00002	- 31.0533
R^2	0.9361	0.9472
adjusted R^2	0.8762	0.9208
p or probability	0.1532	0.0028

Subscripts: 1 = particle size (mm)
*Significant at 0.05 level; **Significant at 0.01 level; ***Significant at 0.001 level;

CONCLUSION

Under the studied process conditions, methanol gave the highest oleoresin yield in comparison to the acetone and n-hexane as used extraction solvents for red dried tomatoes. The obtained results recommended extraction of red dried tomatoes with acetone, at temperature of 40oC, during 180 min, 0.02% w/v solid and liquid phase ratio, and 0.2 mm particles size for obtaining tomato oleoresin with the satisfactory lycopene content.

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RHEOLOGICAL CHANGES AND SENSORY PROPERTIES OF CHOCOLATE DURING CONCHING PROCESS

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ABSTRACT

The primary object of this research was to determine changes during conching process of rheological and sensory properties of chocolate masses with different composition. Total duration of conching was 24 hours (4 hours of dry conching and 20 hours of wet conching). Emulsifier soya lechitin was added after 18 hours of wet conching. All samples were conched under same conditions and taken out during wet conching period every two hours. Device for measurements of casson plastic viscosity and casson yield value was rotation viscosimeter. Sensory valuation of each sample was also done.

The obtained results showed intensive changes of rheological and sensory properties after 6 hours of conching. It was also shown that chocolate with highest amount of milk fat formed rheological properties after 16 hours of conching and after that, changes of plastic viscosity and casson yield value were irrelevant. All samples showed biggest changes of sensory properties within 10 hours of conching, especially change of odor.

Chocolates with higher content of milk fat required shorter period of conching in order to accomplish desirable changes of rheological properties. On the other hand, chocolates with higher amount of cocoa parts, without milk fat, required longer period of conching. These chocolates showed significant changes of rheological properties after 22 hours of conching. All the way until the end of conching process, highest changes of aromatic properties showed chocolate with higher content of cocoa parts.

KEYWORDS: *chocolate, rheology, conching, sensory analysis*

INTRODUCTION

Melted chocolate is a suspension of sugar, solid parts of the cocoa bean and milk powder (in case of milk chocolate) in continuous phase of cocoa butter. In fact, continuous phase is a mixture of cocoa butter and other types of added fats such are milk fat, fat substitutes etc. In order to achieve stable suspension, complicated multistage process is applied. It is necessary to take special care of technological process, because any mistakes done during each stage can not be reclaimed. Quality of raw material and processing parameters affect physical and sensory properties of chocolate. Conching implies long lasting and strong stirring of chocolate mass during which chocolate mass is heated. Higher proportion of cocoa butter require longer conching period. Rheological properties of chocolate mass are affected mostly by free milk fat from milk powder because it is well-united with cocoa butter (Scherun, 2001). If free milk fat is well incorporated in continuous phase of milk chocolate, it has beneficial effect on flowing properties of chocolate mass. Liang and Hartel (2004) studied influence of different types of milk powder on rheological properties of chocolate. Higher amount of milk fat lowers viscosity and shear stress values, and has beneficial effect on sensory properties. Emulsifier also affects rheological properties of chocolate. Schantz and Rohm (2005) reported that increase of lecithin concentration to 0,4 % caused decrease of shear stress values, while lecithin amount from 0,4 % to 0,7 % caused slight increase of shear stress. Through the action of heat during conching, evaporate undesirable volatile compounds and characteristic properties of chocolate are obtained. Most intensive evaporation of undesirable aromatic compounds (aldehydes) is in the initial phase of conching while chocolate mass is still grainy and loose. Studies have shown that smaller proportion of aldehyde in chocolate give more expressed and rounded aroma (Ziegleder, 1997). During so called dry conching, chocolate mass becomes thick and doughy. During first few hours of wet conching plastic properties of chocolate mass are

formed. After 12 to 13 hours of conching, shear stress values decrease and changes in rheological properties become more evident. At the end of conching rheological properties of chocolate mass can be described by plastic viscosity and Casson yield value, it becomes homogenous mixture which is a function of raw materials, temperature, time and mechanical processing (Afakova et al. 2007). Acerbity of chocolate mass decreases during conching, but changes in polyhydroxyphenols need to be studied. Chocolate mass gets a slightly bitter taste, so it can be assumed that influence of heat in slightly acidic environment causes changes of theobromine and other bitter peptide derivates in which amino acids proline and valine are present (Boler and Braun, 2001). At the end of conching emulsifier lechitin is added. Addition of lecithin in the amount of 0.4 % affects Casson yield value in the same way as increase of cocoa butter for 3 to 8%. Lecithin also stabilizes dispersion system, makes viscosity at the high temperatures more stable and improves product texture (Schantz et al. 2006).

In this research changes of rheological parameters and sensory properties of chocolate mass during conching period were studied. Each sample of chocolate mass contained different milk components or didn't have milk components at all.

MATERIALS AND METHODS

Sample preparation

Samples were manufactured by standard procedure in confectionary factory „Zvečevo". Five samples of chocolate mass were done. Three contained different milk components, and two were done without milk, but had different shares of cocoa mass.

MC-1 sample contained 34 % of cocoa parts (27% of fat from cocoa parts), 15 % of milk parts (4% of milk fat). Whole milk powder produced by spray drying was used. MC-2 sample had the same composition as MC-1 sample, but milk powder used to prepare sample was produced by rollers drying. MC-3 sample contained 31 % of cocoa parts (27% of fat from cocoa parts), 20% of milk parts (8% of milk fat). Sweetened condensed milk was used. MC-4 sample contained 28% of fat from cocoa parts, 31% of cocoa mass (without milk components). MC-5 sample contained 31% of fat from cocoa parts, 43% of cocoa mass (without milk components). Two hours before the end of conching process, emulsifier soya lecithin in amount of 0.4% was added.

Technological parameters

Chocolate masses were mixed in the automatic machine for 360 s. Afterwards, the technological process continuously for the refining of the roll refining. Temperatures of rollers were 28˚C, 30˚C, 35˚C, 55˚C and 30˚C. MC-1, MC-2 and MC-3 samples were subjected to dry conching for 4 hours at temperature 52-56˚C, and wet conching for 20 hours at 55-59˚C. MC-4 and MC-5 samples were processed by dry conching for 4 hours at 52-56˚C and wet conching for 20 hours at 65 to 70˚C. In order to make analysis of rheological and sensory properties, each sample was exempted every two hours, first one only after 6 hours of conching.

Rheological properties measurement and results processing

The rheological properties were measured by rotation rheometer model DV-III+, Brookfield Engineering Laboratories (SAD), with concentric cilinders SC4-14. The rheometer was connected with computer equipped with software Rheocalc V3.2., leading the measurements and doing data analysis. Samples were thermostated 20 minutes at 40˚C.The measurements were carried out at 40˚C at 0-50 rpm. Each measurement was conducted in two parallel.

Calculation of plastic viscosity and Casson yield value was done by NCA/CMA Casson model

$$(1+a)\sqrt{\tau} = 2\sqrt{\tau_0} + (1+a)\sqrt{\mu D} \qquad (1)$$

a = ratio between the outer radius of the inner cylinder and inner radius of the outer cylinder;

τ = shear stress (Pa); τ_0 = yield value (Pa); μ = plastic viscosity (Pa·s); D = shear rate (s^{-1})

This is a standard model of National Confectioners Association (NCA) and Chocolate Manufacturers Association (CMA) (1973).

Mean and standard deviation of all parameters were calculated. Data were analysed by Fisher LSD test ($p<0.05$), Statistica 7.0 software.

Sensory analysis

Sensory evaluation included color change, solubility, odor and flavor of each sample. In the evaluation participated seven trained analyzers. From 0 to 5 points could be assigned to each property. Higher grade indicated a greater degree of acceptability of required property. Scores obtained for each sample were summed and devided by 7 (number of analyzers) and were used as summary rating in the evaluation of samples.

RESULTS AND DISCUSSION

Generally, results obtained in this research showed changes of rheological properties during conching. Doughy chocolate mass in the beginning of conching resisted stirring, what manifested as increase, decrease and increase again of plastic viscosity and yield value. In the initial phase of measurements, all samples had high values of shear stress which represented as flowing resistance. Chocolates also expressed plastic properties. It can be seen (Table 1) that the beginning of rheological properties forming, in case of milk chocolate, was after 16 hours of conching. These changes resulted from long lasting and strong stirring, acting simultaneously with high temperature and air ventilation. Stirring causes homogenization i.e. each solid particle becomes covered with fat (Becket, 1999; Ziegleder, 2005). Chocolate masses (MC-4 and MC-5) which did not contain milk components, only after 22 hours of conching achieved approximately equal value of Casson yield value as milk chocolates (Table 1).

Table 1. Influence of conching on Casson plastic viscosity (μ_{CA}) and Casson yield value (τ_{CA})

	MC – 1	MC – 2	MC – 3	MC – 4	MC – 5
	μ_{CA} (Pa·s)				
6 hours	5.79 ±0.02	7.69±0.01	8.36±0.04	25.36±0.02	39.25±0.07
8 hours	6.98±0.07	8.98±0.01	6.26±0.04	21.38±0.15	27.36±0.02
10 hours	9.40±0,04	7.56±0,05	6.01±0.10	18.69±0.01	24.65±0.02
12 hours	6.54±0.11	6.98±0.09	5.98±0.07	17.31±0.02	21.36±0.01
14 hours	12.3±0.08	5.36±0.02	7.31±0.14	19.29±0.06	19.36±0.00
16 hours	6.82±0,02	4.36±0.02	8.26±0.02	11.57±0.04	15.21±0.04
18 hours	4.69±0.02	4.39±0.11	6.31±0.02	11.59±0.02	14.30±0.05
20 hours	4.85±0.03	3.55±0.07	6.31±0.01	10.35±0.06	12.00±0.12
22 hours	4.02±0.14	3.85±0.02	4.31±0.01	9.25±0.05	8.31±0.01
24 hours	1.89±0.03	1.59±0.01	1.32±0.08	6.30±0.02	7.30±0.08

	MC – 1	MC – 2	MC – 3	MC – 4	MC – 5
	τ_{CA} (Pa)				
6 hours	195.90±0.18	201.31±0.02	210.31±0.01	256.11±0.01	244.15±0.09
8 hours	184.60±0.02	196.34±0.12	200.9±0.10	239.54±0.04	239.90±0.07
10 hours	147.70±0.02	156.51±0.16	149.6±0.02	201.81±0.07	211.30±0.13
12 hours	129.40±0.04	123.55±0.01	85.3±0.12	187.94±0.02	198.70±0.11
14 hours	123.70±0.01	99.69±0.02	89.66±0.15	156.72±0.05	201.30±0.02
16 hours	101.70±0.17	64.36±0.04	79.35±0.02	154.33±0.06	210.50±0.02
18 hours	53.78±0.02	38.31±0.07	38.55±0.06	131.54±0.09	187.80±0.08
20 hours	28.60±0.06	24.38±0.14	37.33±0.03	89.61±0.02	105.60±0.07
22 hours	27.30±0.09	23.59±0.02	29.36±0.08	41.65±0.08	64.30±0.09
24 hours	18.60±0.03	16.56±0.05	14.65±0.09	23.59±0.09	38.60±0.01

Usage of milk powder dried on rollers as ingredient of milk chocolate, at the end of conching caused lowest values of yield value and plastic viscosity (samples MC-2 and MC-3). Most probable reason was bigger share of free fat in this type of milk than in milk powder produced by spray drying. Free milk fat is well mixed with cocoa butter. If it's well incorporated in the continuous phase of milk chocolate, it affects beneficially flowing properties of chocolate mass. Powder milk manufactured by rollers drying contains more than 80 % fat in the free form, while powder milk manufactured by spray drying has only 10 % of free fat. Results obtained in this research are in accordance with research done by Keogh et al 2001. They showed that increase of free milk fat share from 40 to 96g/100g of total milk fat, causes decrease of plastic viscosity values about 50 %. Liang and Hartel (2004) also showed that increase of proportion of free fat causes viscosity and shear stress reduction, and affects beneficially sensory properties of chocolate.

After 16 hours of wet conching, sample MC-2 had approximately the same value of plastic viscosity as sample MC-1 after 18 hours. The eventual addition of emulsifier to MC-2 sample after 16 hours of conching, would probably cause decrease of rheological parameters values, same as it did to MC-1 sample at the end of conching.

Using milk with more free milk fat could significantly shorten duration of conching and achieve savings in energy consumption. Lowest value of plastic viscosity and yield value among all samples at the end of conching had MC-3 sample. This can be explained by the fact that MC-3 sample contained the biggest share of milk fat. Figure 1 shows relation between shear rate and shear stress at the end of conching. It can be seen that the highest values of shear stress had sample with the biggest amount of cocoa mass. Higher share of milk fat affects flowing properties and resistance to solidification of chocolate mass.

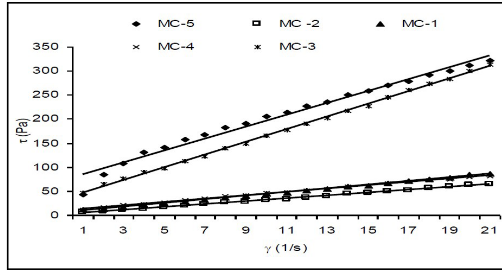


Figure 1. Relation between shear rate and shear stress at the end of conching

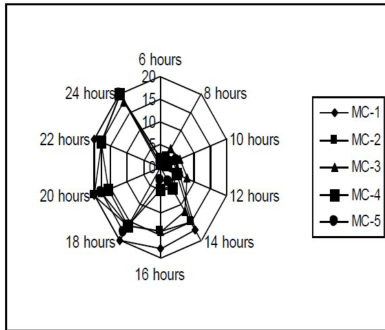


Figure 2. Change of sensory properties solubility

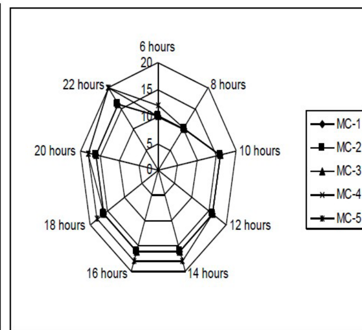
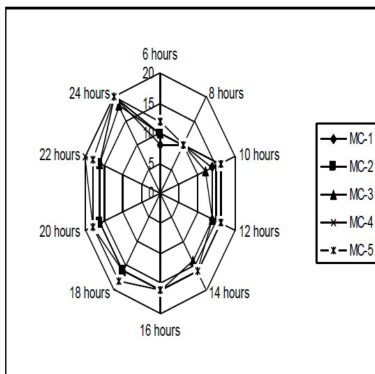


Figure 3. Change of ensory properties odor during during conching conching



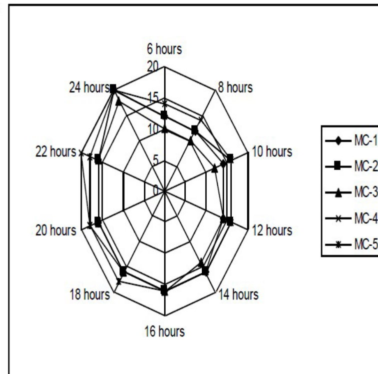


Figure 4. Change of sensory properties taste Figure 5. Change of sensory properties color during during conching conching

Properties of odor (Fig. 3) in all samples improved significantly after 10 hours of conching, and remained constant afterwards, with the exception of MC-5 sample, which contained more cocoa mass. Heat causes Maillard reactions and creation of aromatic compounds: esters, aldehydes, ketones and alcohols. Amino acids with thiol group from solid cocoa parts react with scented aldehydes and produce compounds which are responsible for characteristic chocolate aroma (Boler and Braun, 2001). These changes were more pronounced in samples which contained more cocoa parts (MC-4 and MC-5). Samples MC-2 and MC-3 had well defined property of solubility (Fig.2) after 20 hours of conching. These samples had bigger share of milk fat and cocoa butter, which wrapped all unfat particles of chocolate mass, rounded by heat and friction. Harmonious taste (Fig. 4) of chocolate mass was fully accomplished only after emulsifier was added (after 24 hours of conching). Emulsifiers in chocolate mass act as surfactants placing themselves on border between solid particles and fat. This causes decrease of surface tension on solid-fat border that manifests as decrease of plastic viscosity and yield value. This also causes forming of harmonious sensory properties and consistent color (Fig. 5) of chocolate. Sample MC-3 had the lowest grades of sensory properties although it had lowest values of plastic viscosity and yield value among all samples. MC-3 sample got lower marks of odor and taste because it didn't have expressed caramel aroma, which is normally achieved by the extended time of conching and with risen of temperature at the end of conching (Ziegleder, 2000). MC-4 got satisfactory marks after 20 hours of conching, although its plastic viscosity was relatively high. On the other hand, samples MC-1 and MC-2 had similar values of plastic viscosity after 18 hours (MC-2) and 22 hours (MC-1), but their sensory evaluation equalized after 20 (MC-2) and 22 (MC-1) hours of conching.

CONCLUSION

Properly done conching process affects most important properties of chocolate: structure, shine, flowing properties and aroma. There is a connection between rheological changes of chocolate mass and type of used milk component. Usage of whole milk powder produced by rollers drying shortens time of conching, but sensory evaluation should be also included. Chocolate masses without milk components, at the end of conching have higher values of plastic viscosity and Casson yield value, regardless they have higher amount of total fat. Higher share of cocoa mass in chocolate strongly affects sensory properties and makes bigger resistance to flowing of chocolate mass.

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APPLE CHIPS PRODUCED BY COMBINED DRYING TECHNIQUES

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ABSTRACT

Apples are perceived as healthy food and besides fresh can be consumed as dried fruits or as an ingredient in fruit-based snacks. The production of crisp apple chips with low fat and high fiber content are expected to be highly desirable by consumers. Apples before each experiment were washed and cut in form of discs with a diameter of 18 mm and 2-3 mm thickness. The apple discs were osmotically treated with 40 % glucose solutions and then dried at 105 °C in a convective dryer. Textural properties were measured on texture analyzer using compression/penetration tests, while L*, a* and b* color values were recorded by colorimeter and used to calculate the browning index. Sensory analysis of the chips was done with a group of 50 untrained panelists. The Hedonic scale from the sensory analysis showed high percent of acceptance among the panel group. The different osmotic solutions resulted in 53% water loss for the apple chips and less than 5% solute uptake. The penetration force of the control samples was around 3N. However, after the drying step the final samples were much harder and crispier than the fresh apples. There was not any statistically significant difference in the browning index of the fresh apples and the apple chips.

KEYWORDS: *apple chips; osmotic dehydration; browning index, crispy texture*

INTRODUCTION

The demand for healthy, natural and tasty processed fruits continuously increases, not only for finished products, but also for fruit-based snacks (Stepien, 2008). Most of the fruit and vegetable-based snacks currently available in the market are processed by frying in cooking oil. Another favored trend in the apple consumption is the dried apple. Dehydration process has been used for centuries as a way to preserve foods. The production of crisp apple chips with low fat and high fiber content are expected to be highly desirable by consumers. Dried fat free apple chips should be distinguished by their tender crisp texture, light attractive color and fruit flavor, so that they could be accepted for direct consumption (Konopacka, Plocharski, 2001). It is very important that the preservation process of the fruits improves the quality, stability, sensory and nutritional properties of the end product by combining the traditional drying methods with alternative drying methods. Therefore, the conventional method of convective drying can be coupled with an osmotic treatment of the apples. During the osmotic, partial dehydration and solute intake can be achieved by immersing the apples in concentrated aqueous solutions. Osmotic dehydration preserves attributes like color, firmness and flavor and it reduces water activity, providing high moisture products with extended shelf-life (Fernandes et al, 2008). Using the combined effect of the osmotic dehydration and conventional drying, the texture of the apples changes from elastic-visco-plastic to rigid, fragile and brittle, resulting in crispy apple chips (Farris et al, 2008). The texture of the apple chips can be evaluated by both sensory and instrumental methods.

The purpose of this investigation was to obtain dry and crispy, fat-free, apple chips, by using osmotic and conventional drying. The changes of the mechanical properties of the apple tissue were investigated by texture analysis before and after the drying process. The quality of the apple chips, in terms of color, texture and water activity, was evaluated during 60 days of storage. The overall acceptance of the new product was evaluated by means of sensory analysis.

MATERIALS AND METHODS

Raw material and sample preparation

The apple variety Idared was selected due to its availability in the region. Fruits were purchased from the local market and they were examined within maximum duration of 1 week storage in a cold chamber at 4 °C, to minimize development of chilling injury. Fifty apples were washed and manually cut with a specially designed sharp stainless steel blade into very thin sheets of approximately 2 mm. Then the apple sheets were cut by using a cork borer in form of discs with 18 mm diameter.

Apple chips production

The apple discs, 3 pieces per replicate, were subjected to osmotic dehydration in 40 % aqueous glucose solution. The osmotic treatment was carried out at temperature of 25 °C. Apple discs were placed in 200 mL glass jars where the material to solution ratio was 1:20 (m/m). The dewatering process was performed for 150 min and three samples were collected at different time intervals for the analysis of moisture and solute contents in the apple discs. After the osmotic dehydration the apple chips were dried at 105 °C in a drier for 120 min until, reaching stable water activity between 0.2-0.3. After the drying, the chips was cooled down to room temperature for 20 min and packed in multi-layered packaging bags. The packaging material was metalized polypropylene and the chips were packed under the nitrogen atmosphere to avoid moisture absorption, discoloration and spoilage during storage. The chips were stored for 60 days.

Measurement of moisture loss and solute uptake

Moisture content was measured gravimetrically by drying the samples at 105 °C. The moisture loss and solute uptake of the samples during osmotic process was calculated using the following equations:

$$Moisture\ loss = \frac{M_{w_o} - M_w}{M_o}$$

$$Solute\ uptake = \frac{M_s - M_{s_o}}{M_o}$$

where is mass of sample, is mass of water in sample at time t_o, is mass of water in sample at time t, is mass of solid content of sample at t and is mass of solid content of sample at t_o.

Measurement of color

To measure the color of all apple samples during the osmotic dehydration and at the end of the drying process, Dr. Lange spectro-color colorimeter was used. After collecting the three parameters that is L* (lightness), a* (greenness) and b* (yellowness) they were used to calculate the browning index. Browning index, BI, represents the purity of brown color and is considered as an important parameter associated with the browning of the samples (Jeong et al, 2008). The experiments were replicated five times.

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

Texture profile analysis

Texture measurements were conducted by means of a texture analyzer (TA-XT2i of Stable Micro Systems, Godalming, England). To determine the texture of the fresh apples and apple chips two types of mechanicals test were applied. For the puncture test apple discs were placed on the platform and the 2 mm probe was used to puncture them in the center. The probe moved with cross-head speed of 1 mm/s, trigger force 1 g and travel distance of the probe 5 mm, penetrating trough the whole height of the tissue. The pre- and post-test speed was set at 10 mm/s. The compression test was conducted using a 5 mm cylindrical probe. The probe descended at speed of 10 mm/s and compressed the sample at a speed of 1 mm/s up to a distance making 50% deformation in two repetitive cycles. The force-time curves were recorded by the instrument.

Sensory analysis

A sensory evaluation was also conducted to evaluate the overall acceptability of the product by using 9-point

Hedonic scale. The group consisted of 53 persons with age from 13 to 67, 33 woman and 20 men. All subjects declared themselves as consumers of potato chips and other crispy products. From the group 85 % stated frequency of chips consumption more than five times per month, while only 15 % consumed chips rarely. Testing was carried out in a sensory laboratory. The samples were served in random order, each in a separate packaging of 1.5 g.

Statistical analysis

Descriptive statistical analysis and one-way analysis of variance (ANOVA) were performed on the instrumental and sensory parameters to evaluate significant differences among the samples at 95 % confidence interval according to Tukey's test using Minitab 15 statistical software.

RESULTS AND DISCUSSION

Macedonian region is rich with different apple varieties, among which the Idared variety is the most abundant and available throughout the year, and therefore it was chosen for these investigations. The apples used in this study were selected at the same commercial maturity with 88.7 ± 3.9 % moisture content. The changes of the moisture and solid content during the osmotic process are presented in Figure 1. It was evident that the moisture loss increased with time of immersion. Solute uptake was not following the same pattern. The initial period of osmosis, during the first 90 min, showed no solid uptake within the tissue, meaning that the amount of sugar leaching out of the sample was greater than the amount of sugar penetrating into the apples from the osmotic medium. The final concentration of the solute uptake at the end of the treatment was less than 5 % or 0.05 $g_{glucose}/g_{sample\ mass}$. Similar results were reported by Khin et al, (2007), when working with Fuji apples. After the osmotic dehydration apples were further dried by convection drying to obtain the apple chips. The apple chips were dried until reaching stable water activity of the product of 0.3 resulting in total processing time of 4.5 h.

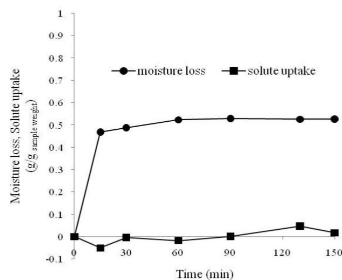

Figure 1. The kinetic profiles of moisture loss and solute uptake in the apple tissue during the osmotic process in 40 % glucose solution at 25 °C.

Changes in the surface color of apple samples in different stages of processing are presented in Table 1. The color of fresh apples was used as control. Browning can cause deleterious changes in the appearance and organoleptic properties of the food during post-harvest handling and processing. Therefore, browning index, as an indicator of the concentration of pure brown color in the apple flesh due to enzyme activity is very important parameter. The results proved that there were no statistically significant changes at the confidence level of $p > 0.05$ of the browning index among the fresh apples, the apple chips produced with the combined dehydration techniques and the apple chips stored for 60 days. The osmotic dehydration decreases enzymic browning and can retain or even improve food product color (Dermesonlouoglou et al, 2008), which was proven by the browning index values. Browning index is usually correlated with the content of oxidized phenols in apples. The increase of chroma values is also correlated with an increase in overall pigmentation, especially the level of phenolic compounds (Jeong et al, 2008). Besides enzymatic browning a non-enzymatic

browning can also occur during the air-drying of the sample. The appearance of brown spots and the development of taste are related to the chemical composition. Reducing sugars are usually involved in the non-enzymatic browning reactions, known as the Maillard reaction. Due to the leaching out of reducing sugars during the osmotic pre-treatment prior to drying the non-enzymatic browning reaction was minimized and enabled production of better looking chips, which was also reported by Leeratanarak et al, (2006). It was evident that the modified atmosphere packaging inhibited the development of flesh browning in apple chips during storage, because there were no statistically significant changes among the chips samples after preparation and after 60 days of storage.

Table 1. Browning index of different apple samples

Apple sample	Browning index
fresh apple	18.03 ± 2.32 [a]
apple chips	25.48 ± 4.64 [a]
packed apple chips during storage	29.63 ± 5.38 [a]

[abc] Values with different letter in a given column are significantly different (p<0.05)

The characteristic force-displacement curves for the penetration and compression tests from the texture profile analysis are presented in Figure 2a and 2b respectively. The curves from the penetration test presented the differences in the mechanical pattern of each sample. The fresh apple sample showed low penetration force and flatter peak, the work needed to penetrate trough the flesh was 0.02 MPa. The behavior of the apple chips was represented with a very sharp curve where the stress was 1.07 MPa. The initial quick, steep and linear tract (after just 0.05 mm deformation) was followed by two subsequent spiky peaks, which were related to violent ruptures and are typical for the crispy products. The similar behavior was reported for apple rings osmotically treated in maltose solution and dried at 90 °C by Farris et al, (2008).

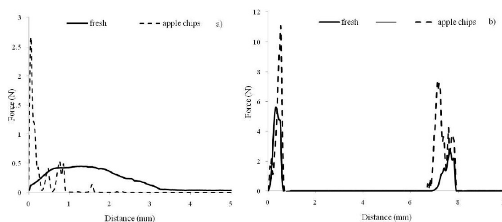

Figure 2. Typical force-displacement texture curves from the a) puncture and b) compression test.

The total sensory quality is a complex concept, which consists of several attributes perceived through the senses, such as appearance, odour, flavor and texture. Their quality, intensity and mutual relations are very important for product identification and acceptance by the consumers. Since 85 % of panelists confirmed themselves as potato chips consumers, there was a possibility for them to stick to the image kept in their mind about the appearance of a chip. The overall acceptance of the product, or the liking of the apple chips, was evaluated by 9-point Hedonic scale, presented in Figure 3. As results showed, most of the consumers expressed different level of liking the apple chips. The part of the panel group that did not like the product was less than 5 %. One should always remember that consumer liking of the product is not constant and may fluctuate together with the development of new habits and new products (Jaworska et al, 2008). Therefore, at the end the consumers were asked if they would consume, or even substitute the potato chips, with this healthy, fat-free apple chips. More than 95 % of them said that they would consume the apple chips if offered on the, market, 40 % would replace the potato chips with the apple chips, while 55 % would continue consuming both chips.

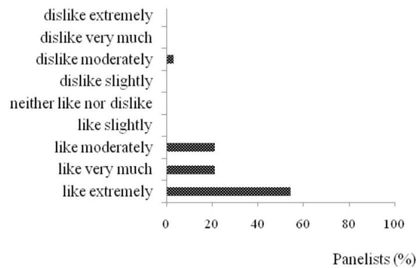

Figure 3. 9-point Hedonic scale

CONCLUSION

In this study the osmotic pre-treatment in combination with conventional drying was successfully used to formulate a new product, apple chips, with good color and texture and extended shelf-life due to the reduced water activity level. These dried apples having common characteristics of crispy products were evaluated by the consumers as very healthy and satisfying for consumption. During the process there was low solute uptake, which rendered the chips suitable for consumption even for diabetics.

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PRESERVATION OF DIOSPYROS KAKI WITH EDIBLE FILMS

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ABSTRACT

The persimmon, *Diospyros kaki,* which is also known as kaki, is a very popular fruit. Its widespread in Europe is now also increasing due to the growing consumption rate. The persimmon is nutritionally important crop since it has increased sugar content and at the same time is good source of biologically active compounds such as ascorbic acid and condensed tannins. Therefore exploring the ways of its preservation is highly important. Persimmon fruits of Macedonian variety were selected according to their ripeness. The fruits before each experiment were washed, peeled and cut in form of discs with a diameter of 18 mm and 2-3 mm thickness. The discs were treated either directly with different edible films or first osmotically dehydrated and then coated, before being air-dried and packed. Textural properties for fresh and coated fruits were measured on texture analyzer using compression/penetration tests. The color parameters of persimmon such as L*, a* and b* values were accessed by colorimeter. The edible films were characterized for strength by compression test. The initial penetration force of the control samples of 15.5 N, decreased in the range between 10 and 13.5 N for the different edible films, while the osmotic treatment +coating did not cause any changes in the tissue hardness. Hardness, cohesiveness and stickiness were calculated from the compression test profile. There was a statistically significant difference between the fresh and coated fruits in all color parameters. The use of edible coatings, alone and in combination with osmotic drying, proved to be suitable methods for persimmon preservation.

KEYWORDS: *persimmon; edible coatings; osmotic dehydration;*

INTRODUCTION

The persimmon fruit, Diospyros, is of Japanese origin and is commonly grown in warm regions of the world. There are four species of Diospyros, namely *Diospyros kaki, Diospyros virginiana, Diospyros oleifera* and *Diospyros lotus*. Persimmon, *Diospyros kaki*, is one of the most important species from nutritional point of view (Bibi et al, 2007). Its relatively high content of dietary fibers, total phenolics, main minerals and trace elements make persimmon preferable for a healthy diet (Luo, 2006). The persimmon is also a good source of biologically active compounds such as ascorbic acid and condensed tannins which are related to various physiological functions including a protective role against oxidative stress-related diseases, and antimutagenic and anticarcinogenic capacities (Del Bubba et al, 2009). The high level of persimmon production observed in the last several years makes it necessary to provide the consumers with new persimmon products, besides the fresh fruit, that will have prolonged storage life.

Minimally processed products are one of the most growing segments in the food retail chains. However, fresh-cut fruits are intensively studied because of the difficulties in preserving their fresh qualities for prolonged periods. The demand for lightly processed products with the same guarantees of safety as fruits treated by traditional methods of preservation has encouraged researchers to focus their efforts on studying new ways of extending shelf life of fresh-cut products (Igual et al, 2008). One of the possibilities is to coat the fruit with an artificial, edible barrier. The continuous consumer interest in high quality and food safety, combined with environmental concern, has induced the development of edible coatings that avoid the use of synthetic materials (Moreira et al, 2009). The edible coatings have potential to reduce weight loss, respiration rate and improve food appearance and integrity. It is one of the most effective ways to maintain the food quality. Among the most commonly used coating materials are the chitosan, due to its antifungal activity, and gelatine due to its availability and cost (Moldao-Martins et al, 2003; Park et al, 2004).

The aim in this investigation was to evaluate the effect of the different coating materials on certain

physico-chemical properties of the fresh-cut persimmon during one week storage at room temperature.

MATERIALS AND METHODS

Plant material and sample preparation

The persimmon variety, *Diospyros kaki*, was grown in an orchard in the south region of R. Macedonia. Mature/unripe fruits were harvested and divided in four lots. The first lot consisted of fresh-cut pieces of persimmon with no treatment, which were used as control samples. The second and the third lot were coated with edible coatings. The fourth lot represented the samples that were osmotically treated prior the coating. The fruits were washed and manually sliced with a specially designed sharp stainless steel blade into slices with 4-5 mm thickness. Discs with 18 mm diameter were cut out of the slices, using cork borer.

Coating and storage of persimmons

The following solutions were used: 1 % chitosan and 3 % gelatin for coating and 50 % sucrose for osmotic treatment. Chitosan solution was prepared by dissolving the chitosan in 1 % acetic acid. For complete dispersion of the chitosan, the solution was stirred overnight at room temperature and centrifuged to remove impurities before use. The gelatin and sucrose were prepared in distilled water. Solutions were heated to 70 °C to speed up the dissolving and then cooled to 30 °C before use.

The fruit pieces were immersed in the coating solutions of chitosan and gelatin for 5 min and then drained. The fruit pieces osmotically treated prior coating were immersed in the sucrose solution for 30 min, then removed from the osmotic solution, blotted with tissue paper and coated with chitosan according to the previously mentioned procedure. All treated fruit pieces were dried in air for 2 h to set a coat of the film on their surface. Afterwards they were stored for a week together with the control samples at 20 °C in a plastic tray vacuum sealed with plastic wrap.

Measurement of color

Flesh color was evaluated by Dr. Lange spectro-color colorimeter on 5 fruit pieces. After collecting the three parameters, L* (lightness), a* (greenness) and b* (yellowness) they were used to calculate the flesh color index:

$$color\ index = \frac{1000a}{Lb}$$

Texture measurements were conducted by means of a texture analyzer (TA-XT2i of Stable Micro Systems, Godalming, England). To determine the texture of the fresh and treated fruit samples compression test was applied. This test was conducted using a 5 mm cylindrical probe. The probe descended at speed of 10 mm/s and compressed the sample at a speed of 1 mm/s up to a distance making 50% deformation in two repetitive cycles. The force-time curves were recorded by the instrument.

Analytical methods

The changes in the soluble solids, titratable acidity and vitamin C content of the fresh and coated samples were evaluated on the fruit pulp. The fruit pulp was obtained by homogenization of 25 g of fruit flesh or approximately 10 fruit pieces by using grinder and centrifugation at 3500 rpm for 20 min. The soluble solids were determined using Abbe refractometer. Titratable acidity was determined by titration with 0.1 M NaOH until the pH change to 8.1 and expressed as g citric acid/ g fresh weight. Vitamin C (mg/100 g fresh weight) was determined by titrating fruit extract against standardized 2,6-dichlorophenol indophenols dye to light pink end point which persisted for 15 s. Water activity of the samples was measured with Testo-650 water activity meter. All experiments were done in 3 repetitions.

Statistical analysis

One-way analysis of variance (ANOVA) was performed on the physico-chemical parameters to evaluate significant differences among the samples at 95 % confidence interval according to Tukey's test using Minitab 15 statistical software.

RESULTS AND DISCUSSION

The physico-chemical properties of the fresh cut, control and coated persimmon pieces, after preparation and one week of storage at 20°C are presented in Table 1 and Figure 1. The data revealed that there were no significant changes in the solid content, titratable acidity, vitamin C content, color index and water activity between control and coated samples. The only treatment that showed significant changes in most of the properties was the osmotic treatment+chitosan coating. Due to the immersion in concentrated sucrose solution a solute uptake occurred which resulted in 11.3% increase of the solid content. Mass transfer during the osmotic treatment consists of simultaneous solute uptake and water removal, in and out of the plant tissue (Torreggiani, Bertolo, 2001). Besides the solute uptake, the water loss of the fruit samples was also determined and it was around 20%. The water loss was accompanied with some leaching out of the vitamin C and the citric acid present in the fresh plant tissue (Table 1).

After one week of storage at 20 °C, the coated samples exhibited better preservation of the nutritional values when compared to the control samples. The control fruit samples lost 45.5% of their titratable acidity and 55% of the vitamin C. The chitosan and gelatine coated fruits did not lose their titratable acidity value and had lower vitamin C loss. The samples coated with chitosan after the osmotic treatment showed 70% preservation of their vitamin C content. Persimmons, as fruits, are characterized by a bright orange-red colour and the coatings did not cause any significant changes in the color index immediately after preparation. During the one week of storage at room temperature, the chitosan and gelatine coating delayed the color evolution and therefore a slowdown of the flesh color change was observed.

Table 1. The effect of the different treatments on the solid content, titratable acidity, vitamin C content, color index and water activity immediately after preparation and after one week storage at 20 °C

Sample	Soluble solids (°Brix)	Weight loss (%)	TA (g/g)	Vitamin C (mg/100 g)	Color index	a_w
fresh cut	20.0 ^{a}	/	0.33 ^{a}	7.04 ^{a}	1.51 ^{a}	0.907
chitosan coated	20.0 ^{a}	/	0.33 ^{a}	7.04 ^{a}	1.63 ^{a}	0.905
gelatine coated	20.0 ^{a}	/	0.33 ^{a}	7.04 ^{a}	1.48 ^{a}	0.900
osmotic treated + chitosan coated	23.0 ^{b}	/	0.22 ^{b}	4.81 ^{b}	1.73 ^{a}	0.929
after one week storage						
fresh cut	17.5 ^{c}	15.3	0.18 ^{c}	3.20 ^{c}	9.32 ^{b}	0.883
chitosan coated	17.0 ^{c}	17.2	0.33 ^{a}	3.93 ^{c}	4.05 ^{c}	0.891
gelatine coated	20.0 ^{a}	3.4	0.33 ^{a}	3.33 ^{c}	3.87 ^{c}	0.895
osmotic treated + chitosan coated	20.4 ^{b}	11.5	0.13 ^{d}	3.38 ^{c}	35.47 ^{d}	0.882

[abcd] Values with different letter in a given column are significantly different ($p<0.05$)

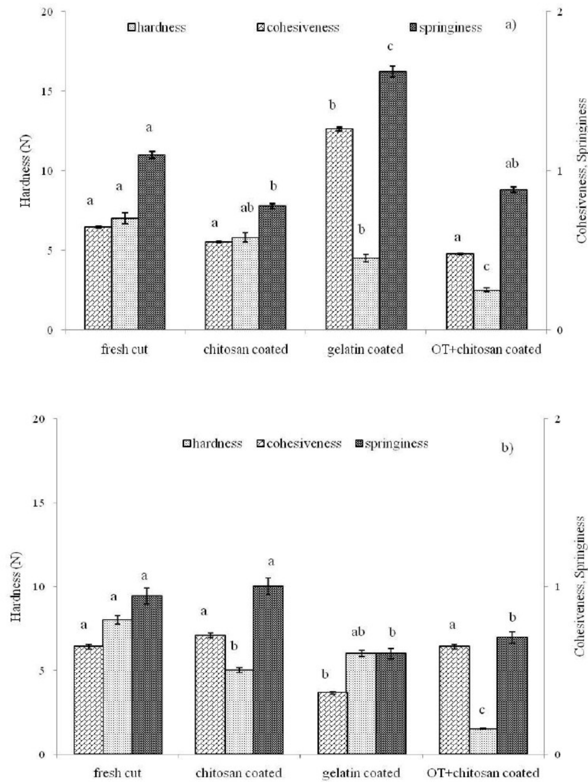

Figure 1. Hardness, cohesiveness and springiness of control and coated persimmon pieces a) immediately after preparation and b) after one week storage at 20 °C. Bars with different letter for a specific attribute are significantly different ($p<0.05$).

The preservation of the turgor pressure and therefore the maintenance of the structural integrity of the tissue is represented by fruits' hardness, while the springiness gives the cells' ability to regain their original form (Khin et al., 2007). From the texture analysis it was evident that the coated samples exhibited lower hardness than the control, but they preserved well the cohesiveness and springiness of the tissue (Figure 1). The most severe loss of the fruit texture immediately after coating was noticed in the samples osmotically treated prior the chitosan coating. In persimmon fruit, the maintenance of fruit quality during storage mainly depends on the rate of flesh softening. Therefore delaying softening during storage is an important aim in the postharvest persimmon research. The positive effect on delaying fruit softening was proved by the textural properties of the samples. The samples coated with edible films preserved well their hardness, cohesiveness and springiness which might be due to decreased activity of the cell wall degrading enzymes that are responsible for the fruit softening.

CONCLUSION

In this study three different coating treatments for minimal processing of fresh cut fruits were tested for better preservation of persimmon during storage at room temperature. The chitosan and gelatine coated fruits had the better preservation of the nutritional properties, color and texture, when compared to the control samples or samples osmotically dehydrated prior coating. Edible coatings could be considered as useful alternative for storage of fruits with fresh cut characteristics.

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THE EFFECT OF SELECTED CONDITIONS ON DEMINERALIZATION OF WHEY USING ULTRA- AND NANOFILTRATION

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ABSTRACT

Whey is a product rising in a diary technology during cheese manufacturing and it is often considered as a waste, however it contains a lot of utilisable components like whey proteins or lactose. Whey manufacturing using membrane separation processes represents one of examples of waste and intermediate products´ processing in food technology. The main aim of application of techniques such as nanofiltration and ultrafiltration is to obtain a material which could be further used in agricultural or food industry and reduce amount of waste products. These membrane processes are recently tested for whey purification and fractionation.

The separation conditions are one of the most important factors influencing the efficiency of nano- and ultrafiltration. The effect of pH, pressure, media properties, pre-treatment and membrane used on a quality of final product and filtration kinetics were tested. Cross-flow Ultrafiltrations on ceramic tubular membranes (Membralox, Pall) with different cut-off (100nm and 500nm) were used for pre-filtration. Flat polymeric membranes NF-245 (Dow Chemicals) and TFC-100 (Koch) and spiral wound module (NTR-7450-S2F, Nitto Denko) were used for nanofiltration.

Results showed that the single-step ultrafiltration on 500nm membrane was a sufficient purification before following nanofiltration regarding the NF filtration kinetics. Mass concentration factors for ultrafiltration were between 1.2 and 16.5. This value suggests potential industrial application. Rejection of lactose during UF was between 0 – 0.13 %. Different NF membranes were compared. MCFs on flat polymeric membranes were in range 1.4 -1.5. MCFs achieved on the spiral wound membrane were between 2.89 and 3.14. Steady state fluxes on spiral wound module were 0.42 - 0.82 l/h.m^2.

The ultrafiltration on 500nm ceramic membrane provides sufficient separation of proteins from whey (rejection of whey proteins were between 71 - 100 %) and improves properties of feed before following nanofiltration. Membrane properties, pH, temperature and pressure play an important role in nanofiltration separation. The rejection of lactose is lower on spiral wound NTR-7450-S2F (Nitto Denko) module than on flat polymeric membranes.

KEYWORDS: *whey, ultrafiltration, naofiltration, separation, lactose*

INTRODUCTION

Whey is a liquid product rinsing during cheese producing. It contains a number of next usable components like proteins and lactose. A separation of these substances can be provided by different methods and equipment. The evaporator with electrodialysis is usually used industrially for demineralization of whey (Rektor A., Vatai G. 2004). Nowadays, membrane separation technology is another possibility of whey processing. Nanofiltration (NF) is a good alternative for these processes (Rektor A., Vatai G. 2004). Connection of ultrafiltration and nanofiltration could produce whey protein concentrate and demineralised lactose.

The main problem of natural whey utilisation is high content of salts (especially NaCl) and that is why whey is often treated as a waste in waste water processing without further utilization. Therefore, the main objective of whey processing is a demineralization. Nanofiltration membranes are highly permeable for low molecular compounds like monovalent salts and guarantee a retention of components with a size bigger than 300 Da (Pan K. *et al.* 2011). These properties correspond with a demand of simultaneous concentration and demin-

eralization of whey during whey processing.

Pan K. *et al.* studied a nanofiltration of whey on the membrane TFC 2540 – SR2 (Koch). They showed the linear dependence of permeate flux on the pressure in between 5 and 13 bars. Higher pressure meant a decrease in permeate flux. The spiral wound polyamide membrane RA55 by Millipore was used for nanofiltration of ultrafiltrated whey permeate in Atra R. *et al.* (2005) study. It was found linear permeate flux´s dependence on pressure in an investigated range of 10-20 bars. Also with increasing concentration factor, the permeate flux decreased. Differences in ion rejection were shown in the work of Suárez et al. 2009. The monovalent ion rejection was much lower than the one of bivalents ions. The permeability of membrane was highest for K^+, Na^+ and P^+ followed by bivalents ions like Mg^{2+} and Ca^{2+}.

This paper describes a demineralization of whey on various NF membranes. The ultrafiltration was used as a pre-treatment process before nanofiltration. UF pemeates were used for two types of nanofiltation membranes: flat polymeric membranes and on spiral wound module. Nanofiltration conditions were 35°C and pressure 15 bars. The aim of this study was to show differences during nanofiltration on flat polymeric membranes and on spiral wound module and to find optimal conditions for nanofiltration of UF permeates. The effect of pH and used membranes were mainly observed.

MATERIAL & METHODS

Filtration equipment and conditions

All Ultrafiltrations were carried out on ultrafitration station (TIA, Bollene, France) at the pressure difference 2 bars and temperature 20°C.

Nanofiltration station ARNO 600 (Mikropur, Czech Republic) equipped with a dynamic cross flow cell DC-76 was used for experiments with flat polymeric membranes. Filtration conditions were: pressure 15 bar and temperature 35 °C. Nanofiltration station RO/NF system (TIA, Bollene, France) was used for spiral wound modules. Applied pressure and temperature were: temperature 20 and 35°C and pressure 15 bars.

Membranes

The ceramic tubular membranes with cut-off 500nm, 100nm and 5 kDa (Membralox Pall) were used for ultrafiltrations. Filtration area of these membranes was $0.24 m^2$.

Flat polymeric membranes NF-245 (Dow Chemicals, USA) and TFC-100 (Koch, France) and spiral wound module NTR-7450-S2F (Nitto Denko, Japan) were used for nanofiltration experiments. The filtration area of flat membranes was $0.0044 m^2$ and the spiral wound module area was $2.5 m^2$.

Analytical equipment

Lactose content was determined using anion-exchange chromatography with pulsed amperometric detection (Dionex, USA). Whey protein content was determined on HPLC system (Agilent Technologies, Germany) with UV/VIS detector (TSP Spectra System UV 200, Germany). pH was measured by THERM 2290-3 (ALMEMO, Germany). Total solid content (% w/w) was determined by drying at 105 °C during 4 hours.

Filtration medium

The natural salty whey from Czech dairies was used for all filtration experiments. Whey solution was used as a feed for ultrafiltration and obtained permeate was separated on nanofiltration membranes. The basic characteristic of whey used is shown in Table 1.

Table 1: The overview of conditions (pH, lactose content and total solid content of whey) used during ultrafiltration experiments.

Ultrafiltration	Total solid content [%]	pH	Lacotse content [g/l]	Total whey protein content [g/l]
UF1	8.39	6.52	69.90	6.97
UF2	10.46	6.34	73.44	5.93
UF3	10.50	6.47	74.57	5.93
UF4	9.40	6.10	75.72	6.04
UF5	8.47	6.07	88.33	6.17

Calculation

Apparent rejection R_i [%], mass concentration factor (MCF) [1], steady state permeate flux J_i [l/h.m^2], average permeate flux J [l/h.m^2] and pure water flux J_v [l/h.m^2] were calculated in this work. Apparent rejection R_i was calculated from *(1)* where $c_{i(downstream)}$ and $c_{i(upstream)}$ is the concentration [g/l] of component *i* in downstream (permeate) and in upstream (feed), respectively. Mass concentration factor (MCF) is expressed as a ratio of feed weight [kg] to the weight of retentate. All filtrations were carried out in duplicate thus the rejections are the average values from both measurements. Steady state permeate fluxes J_s [l/h.m^2] were calculated according to Cheryan (1998) from the formula *(2)* where J_i is a permeate flux at the time *t* [min], *a* and *b* are constants calculated from the equation using Microsoft Solver. Averages permeate fluxes *J* [l/h.m^2] were calculated for nanofiltration on flat membranes where steady state has not been achieved. *J* is expressed by the formula *(3)* where V_p is total volume of permeate, *S* is membrane area and τ is total time of filtration.

$$R_i = 1 - \left(c_{i(downstream)} / c_{i(upstream)}\right) \qquad (1)$$

$$J_i = J_s + a \cdot e^{(-b.t)} \qquad (2)$$

$$J = \frac{V_p}{\tau . S} \qquad (3)$$

Pure water flux was measured before every filtration under conditions similar to those of filtration.

Cleaning process

Cleaning of membrane was one of most important task in this work. The efficiency of cleaning procedure was determined before and after every filtration process. The filtration kinetics was strongly affected when the differences in pure water fluxes were bigger than 1%. The cleaning procedure of ultrafiltration membranes was carried out with 1% nitric acid, 1% sodium hydroxide and Ultrasill 115 at 60 °C. Ultrasill 115 and Ultrasil 73 at 30-40 °C were used for cleaning of nanofiltration membranes.

RESULTS & DISCUSSION

Ultrafiltration results

There were carried out eight ultrafiltration experiments of the natural whey on 500nm, 100 nm and 5 kDa ceramic tubular membrane (Membralox, Pall). Table 2 show results from five nanofiltration experiments where filtration kinetics was observed. Before ultrafiltration, these five samples were purified on a sieve with an aperture of 0.2 mm. The steady state permeate fluxes calculated from the equation *(2)* were in range 6.9 - 44.5 l/h.m^2. The fluxes were mostly affected by previous membrane cleaning expressed by the pure water flux. It is also evident that the lower the initial water flux was, the smaller was the steady state flux during filtration. From the data it is also obvious that the flux decline was very fast (compare initial and steady state

fluxes in Table 2, and also see Fig. 1) and membrane fouling was very severe. Therefore, a further study to improve permeate fluxes and reduce membrane fouling will be necessary.

Table 2: An overview of the kinetic results from UF experiments of natural salty whey on the 500nm membrane; MCF-mass concentration factor.

Ultrafiltration	Pure water flux [l/h.m^2.bar]	Initial permeate flux [l/h.m^2.bar]	Steady state flux [l/h.m^2.bar]	MCF [1]	Total time of UF [min]
UF1	697.7	92.2	6.9	5.1	310
UF2	135.3	9.24	8.2	1.2	102
UF3	1266.7	77.2	32.6	7.5	170
UF4	1443.1	197.4	31.2	16.5	230
UF5	1747.0	151.1	44.51	13.6	480

Table 3: Results of ultrafiltration kinetics and lactose rejection measured during ultrafiltration of natural salty whey. MCF – mass concentration factor, β-LgA – β-lactoglobulin A, β-LgB – β- lactoglobulin B, α-La – α-lactalalbumin.

Series No.		UF6		UF7	UF8
Stage UF pore size		100 nm	5kDa	5 kDa	500 nm
Pure water flux before UF (l/h.m^2.bar)		987	916	1015	1096
MCF (1)		4.43	2.3	-	-
Rejection of lactose (%)		0	0.08	0.13	0
Rejection of whey proteins (%)	$R_{\beta\text{-Lg A}}$	100	100	100	-
	$R_{\beta\text{-Lg B}}$	84.5	90.3	100	-
	$R_{\alpha\text{-La}}$	68.2	100	100	-

Table 3 shows the results from separation efficiency of next three ultrafiltrations. In this case the sieve has not been used before UF. Rejection of lactose was between 0 – 0.13 %. It confirms the fact, that ultrafiltration can serve for prefiltration and separation of whey proteins with minimum lactose losses.

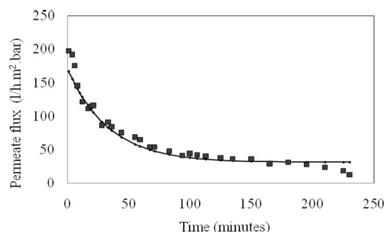

Figure1. The dependence the permeate flux of Ultrafiltration (UF4) on time; temperature 20°C and pressure diference 2 bars.

Nanofiltration results

Seven nanofiltration experiments were carried out on three different types of polymeric membranes. pH of feed in selected experiments was adjusted by addition of 16% HCl or 25% NaOH.

Table 4 shows conditions and results of filtration kinetic. MCFs on flat polymeric membranes were in range 1.4 -1.5. MCFs achieved on the spiral wound membrane were between 2.89 and 3.14. Steady state fluxes on spiral wound module were 0.42 - 0.82 l/h.m^2. Similarly with UF, the drop of permeate flux was close to 90 %. Due to the small filtration area of flat NF membranes, the steady state has not been achieved. Thus we calculated average permeate fluxes, which were 2.70 - 4.15 l/h.m^2.

Table 4. The kinetic result of nanofiltration experiments; MCF – mass concentration factor. * values of steady state fluxes *Js*, ** average permeate fluxes *J*.

Membrane		pH	T [°C]	Permeate flux [l/h.m2]	Pure water flux [l/h.m2]	MCF	NF total time [min]
NTR-7450-S2F	Spiral wound membrane	6.47	35	0.65*	14.97	2.93	521
NTR-7450-S2F		5.00	35	0.82*	12.29	3.14	514
NTR-7450-S2F		6.50	20	0.42*	11.75	2.89	525
TFC-100	Flat membrane	6.50	35	3.10**	5.92	1.37	440
TFC-100		5.01	35	2.70**	5.70	1.47	523
NF-245		6.40	35	4.30**	9.32	1.52	335
NF-245		4.98	35	4.15**	7.87	1.49	342

The rejection of lactose in dependence on pH is shown in Table 5. At the natural pH of whey (6.5) the rejection was higher on the membranes TFC-100 and NF-245 (nearly 100 %), whereas on the spiral wound membrane it decreased to 95.3 % and higher rejection was achieved at pH 5.

Table 5. Rejection of lactose, sodium, potassium, calcium and magnesium on single nanofiltration membranes at pressure 15 bars and temperature 35°C.

Membrane	NTR-7450-S2F		TFC-100		NF-245	
pH	6.5	5.0	6.5	5.0	6.5	5.0
Rlactose [%]	95.3	97.4	99.8	97.2	99.8	98.8
R Cl -[%]	11.4	9.7	66.1	56.9	47.3	37.2
R K+ [%]	38.1	–	67.4	60.8	40.8	33.8
R Na+[%]	38.9	–	60.8	62.9	44.8	8.9
R Ca2+[%]	41.7	45.9	–	85.6	76.0	–

CONCLUSION

Ultrafiltration serves as a pretreatment process for nanofiltration. It separates whey proteins with minimum lactose losses. Steady state permeate fluxes during ultrafiltration were 6.88 - 44.51 $l/hod.m^2.bar$. Large differences in pure water flux in ultrafiltration experiments were caused by insufficient membrane cleaning.

According to our presumption, higher values of MCF (2.89 - 3.14) were achieved on the spiral wound nanofiltration membrane in 505 - 521 minutes. The steady state fluxes for nanofiltration on spiral wound membranes were 0.42 - 0.82 $l/hod.m^2$.

MCF on flat sheet membranes was low (1.37-1.52). Calculated average permeate fluxes on flat sheet membranes were 2.7 - 4.3 $l/hod.m^2$.The membrane NF-245 provides higher fluxes parameter than the membrane TFC-100.

The rejections of lactose were in range 95.3 - 99.8% and only slightly affected by pH. The rejection of salts was higher on flat sheet membranes than on spiral wound module. Rejections of chlorides were in range 9.1- 66.1%. The rejections of cations were between 8.9 and 85.6%. The rejection of bivalents ions is higher than rejection monovalents ions on all membranes. Higher influence of pH on salts rejection was observed on the membrane NF-245 whereas rejection on the membrane TFC-100 was less affected by the pH. For good lactose/salt separation, low ion rejections are required. The lowest rejections of monovalent ions were achieved on the membrane NF-245 at pH 5 and on the spiral wound membrane NTR-7450-S2F. Values of bivalent ions rejections are comparable with those of lactose, which means they are difficult to separate from lactose using these membranes.

ACKNOWLEDGMENTS

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ANALYSIS OF MUSCAT GRAPES AND WINES FROM KUTJEVO REGION DURING THE THREE CONSECUTIVE YEARS

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ABSTRACT

Muscat is a new variety of grapes in Kutjevo vineyards, since 1998 it was included in the register of permitted varieties, the wine is allowed for sale under that name. Since it's a word about new variety, in three years, during the ripening there are few observed parameters like number of bunches per vine, cluster weight, the amount of sugar and the amount of acid. In all three years of harvesting regular harvest took off about 60% of the yield, and the remainder was harvested later as election vintage when grapes have a minimum of 105 Oechsle. Fermentation took place in 1000 litres size stainless steel tanks with addition of selected yeast, with the provisio that the temperature did not exceed 18 °C. The wine is at the end of fermentation cleared with pentagel, filtered on a plate filter and bottled over microfilter. The conclusion is that the variety Muscat as a highly aromatic variety very demanding and if not properly restrict yield, in apart from a few days when the relation between acid and sugar in the grapes in a specific relationship does not make harvest and made only one wrong manipulation, in the later treatment of wine it will absent a specific aromatic impression characteristic of the variety Muscat. In unfavourable climate years, as it was 2010, it should invest additional efforts, pay attention on yields in order to obtain success in wine quality.

KEYWORDS: *Muscat; grape; fermentation; wine; Kutjevo*

INTRODUCTION

Since the Muscat is a new variety, in Kutjevo vineyards it's needed to look at specifics that variety have for themselves when it comes to vineyard cultivation, with special emphasis on the training system, yield per vine and number of bunches per vine, and it is especially important to follow the course of ripening, increasing of sugar and reducing of acids. We talk about variety with a pronounced aromatic components mostly located in the skins of grapes, which are derived from terpenes compounds bound to L-ramnos (1).

MATERIALS AND METHODS

Plantation of Muscat varieties was planted in 2005 in K.O. Podgorje in Vineyard Kutjevo, approximately about 0.5 ha, 2500 vines, 25 lines per 100 vine in spacing of 0.9 m from the vine in the vine row, row spacing of the order of 2.60 m. The first yield was in 2008, and then two more harvest in 2009 and in 2010 that's in this work was taken into consideration. Based on an assessment of the optimal ratio of sugar and acid it's made a decision to start harvesting (3), in relation that the lower part of the healthiest grapes will left to harvest election. Election harvest took place just after the grapes are accumulated at 105 Oechsl. After pressing into deposited grape must selected yeast produced type Lallamand (4) will be added. Fermentation took place in stainless steel containers the size of 1000 litters, while maintaining the temperature to a maximum od 18°C. Fermentation lasted until the evaluation of the optimal relationship between alcohol and residual unfermented sugar, and cleared with pentagel 30 g/hl one month after filtration on Seitz filter plates K 700 and another one at K 100. The wine was stabilized before bottling by cooling to -7 °C and adding an appropriate amount of SO_2 and then filled into glass bottles over microfilters and manual filling.

They were made physical-chemical analysis according to article 37, paragraph 2 of Act on wine and organo-

leptic evaluation (3). The amount of sugar was measured with Oechsle scale and titrable acidity with 0.75 N NaOH with indicator bromthymol blue, on three occasions before the regular harvest, as well as continued monitoring of the relationship of sugars and acids in grape left for the late harvest. Organoleptic evaluation of wines was conducted in accordance with the ordinance on organoleptic evaluation of grape must and wine (4).

RESULTS AND DISCUSSION

Table 1 shows that three consecutive years were significantly different in the number of sunny days and the rainfall during vegetation year, so we could tell that 2008 was an average year, 2009 was extremely dry and sunny year and 2010 was year with a lot of precipitation, rain and cold weather.

Table 1. Climatic factors during the growing season for Vineyard Kutjevo, in the 2008, 2009, and 2010.

2008	Temperature (°C)	Sunshine hours (h)	Rainfall (mm)
April	12.5	173.6	81.6
May	17.6	286.3	29.2
June	20.8	251.7	141.3
Jully	21.4	270.9	131.9
August	21.8	335.9	42.0
September	15.8	155.7	108.2
Average / sum	18.3	1474.1	534.2
2009	Temperature (°C)	Sunshine hours (h)	Rainfall (mm)
April	14.7	203.9	20.6
May	18.1	269.0	61.1
June	18.9	217.4	88.4
Jully	22.7	339.7	38.8
August	23.0	296.4	55.2
September	20.0	226.8	24.1
Average / sum	19.6	1553.2	288.2
2010	Temperature (°C)	Sunshine hours (h)	Rainfall (mm)
April	12.4	203.1	105.8
May	16.2	169.7	192.7
June	19.3	223.3	259.1
Jully	22.5	293.1	69.6
August	21.8	289.9	60.7
September	15.7	157.6	127.7
Average / sum	18.0	1336.7	815.6

Table 2 shows the yields per vine, and the number of bunches per vine, from which we see that the quantity of grapes in creases the total surface area as well as yield per plant basis. Weather conditions did not affect the total yield per hectare.

Table 2. Number of shoots per vine, number of bunches per vine, yield per vine (kg) and total yield (kg) of Muscat in 2008, 2009 and 2010.

Year	Number of shoots / vine	Number of bunches / vine	Yield per vine (kg)	Total yield (kg)
2008	5	10	1.6	4000
2009	7	14	2.2	5500
2010	8	16	2.5	6200

Table 3 shows that weather conditions significantly affected the maturation of grapes, for 2008 we can say is an average year, for 2009 grape harvest was early, while 2010 was late harvest in rainy year. Harvest started in the span of a month, up from 12.IX to 1.X, while the late harvest of 2010 had to be at 1.XI, otherwise, the yield would be ruined by rot.

Table 3. Dynamics of maturation Muscat in 2008, 2009 and 2010.

2008	sugar (Oe°)	total acid (g/l)
01.IX	52	9.5
10.IX	65	6.9
20.IX harvest	82	5.3
10.X	91	5.2
01.XI	101	5.1
12.XI selected harvest	107	5.3
2009		
25.VIII.	61	8.3
05.IX	70	5.6
12.IX harvest	89	4.2
25.IX	95	4.0
15.X	105	3.7
01.XI selected harvest	117	3.9
2010		
07.IX	51	9.7
20.IX	68	5.5
01.X harvest	79	5.1
15.X	87	5.0
25.X	96	4.8
10.XI selected harvest	103	4.7

Table 4. The results of the physico-chemical and organoleptic analysis Muscat wines from the regular harvest for 2008, 2009 and 2010.

Year		2008	2009	2010
Relative density		0.9953	0.9905	0.9943
Total alcohol	(vol%)	12.6	13.6	12.9
The actual alcohol	vol%)	12.0	13.2	12.1
The actual alcohol	(g/l)	99.1	105.7	99.5
Total extract	(g/l)	28.8	20.4	27.9

Reducing sugar	(g/l)	10.4	3.0	5.6
Extract without reducing sugar	(g/l)	19.4	18.4	23.3
Extract without reducing sugars and Non-volatile acids	(g/l)	14.7	14.1	18.6
Ash	(g/l)	2.2	1.9	2.1
pH	-	3.41	3.40	3.47
Total acidity as tartaric	(g/l)	5.1	4.8	5.2
Volatile acidity as acetic	(g/l)	0.3	0.4	0.5
Non-volatile acidity as tartaric	(g/l)	4.7	4.3	4.7
Free SO_2	(mg/l)	21	15	11
The total SO_2	(mg/l)	102	109	103
Organoleptic	point	80	77	79

CONCLUSION

Cultivar Muscat is extremely sensitive on climate change especially on the amount of aromatic components in the wine. Pruning and limit the yield per plant may be partially affect on the final quality of wine.

Determination the date of harvest is of crucial importance for the final quality of wine. If it happens that acid in the grape to much fall, parallel to that it will affect on wine aroma, and despite the high alcohol and a large amount of residual sugar does not leave a good impression on evaluator.

Monitoring of cultivar Muscat in Kutjevo vineyards can conclude that it is not always possible to achieve good results, it is very doubtful what will be the outcome of the rainy and cold years, and in particular it is important to take care about trends of sugar and acid in grapes, which partly can be regulated by restrictive pruning or green pruning.

Fermentation of this demanding varietes for wine making must be conducted under controlled conditions and with selected yeast.

It is desirable for all other technological processes in cellar, clarification, filtration and filling to do in winter when lower temperatures are suitable for wine handling. Only one inadequate procedure, like an open flow, significantly reduces the level of volatile aromatic components, and evaluators can easily identify that like wine flaw.

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Food Quality, Safety and Regulation

BEER FLAVOR STABILITY IN DIFFERENT TYPES OF PACKAGING

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ABSTRACT

The beer flavour stability is one of the biggest problems in brewing industry. The flavour of beer alters with time due to the breakdown of existing components or the synthesis of new flavour compounds. The effect of three types of packaging on development of flavour profile in beer during storage has been investigated. The beer was packed in glass brown bottle, aluminium can and glass transparent bottle. The brown bottles and cans were stored for one year while the transparent bottle for two months. The concentration of flavour active compounds such as vicinal diketones, dimethyl sulfide, ethyl acetate, isoamyl acetate and n-propanol was determined by headspace gas chromatography (HS-GC). Concurrently to instrumental measurements, a sensory evaluation of all three types of beer was performed by trained panellists. The sensory properties of beer were correlated with appearance of off-flavour compounds. Of all kind of packaging, the cans provided the highest stability to the flavour of beer. The beer in glass transparent bottles was evaluated with the lower scores by the panellists.

KEYWORDS: *beer; flavour chemistry; off-flavours; head space gas chromatography; sensory analysis*

INTRODUCTION

Along with the progress in brewing technology, the appearance of hazes and beer spoilage microorganisms are no longer considered as the main trouble-causing phenomena in beer production. Instead, the beer flavour became the most important quality parameter of the product (Vanderhaegen et al., 2006). Beer has a complex flavour and aroma, but shows limited flavour stability compared to other alcoholic beverages. It depends on both formation and degradation reactions as well as on the interactions among different compounds. The flavour profile of beer changes during storage. Maintaining the flavour stability by preventing the off-flavour formation in beer is one of the biggest problems in brewing industry.

The flavour chemistry, which examines the mechanisms of reactions in all stages of the production and storage process, combined with sensory analysis provides good information on how to improve the process of beer production and how to take corrective measures since the early stages of the process thus avoiding the appearance of off-flavours.

The goal of this study was to examine the effect of packaging on development of flavour profile in beer during storage. The beer was packed in glass brown bottle, glass transparent bottle and aluminium can. In addition, a sensory evaluation of beer was performed and the efforts were made to correlate the sensory perception with the appearance of off-flavour compounds.

MATERIALS AND METHODS

Lager beer, produced in standardized brewing process, was packed in three types of packaging: glass brown bottles, glass transparent bottles and aluminium cans. Samples of beer were kept in a dark chamber without effect of UV light, at 20°C.

Beer analyses were performed using a PerkinElmer® TurboMatrix™ automated headspace sampler and a Clarus® 500 gas chromatograph equipped with two columns. Elite-5 column was used for analysis of nonpolar compounds (vicinal diketones, diacetyl and 2,3-pentandione, and dimethyl sulphide (DMS), while Elite-WAX column was used exclusively for polar compounds (ethyl acetate, isoamyl acetate and n-propanol). The gas chromatograph was configured with both a flame ionization detector (FID) and an electron capture detector (ECD), and was connected to a PC with special software, Total Chrome. Beer in glass brown bottles and aluminium cans was analyzed every month during one year, while beer in glass transparent bottles was assayed every 10 days over a two-month period. Four samples of each type of beer packaging were analyzed. Results are presented as arithmetic mean of the measured values. The relative standard deviation does not exceed the value of ± 2.8%.

Beer was sensory evaluated concurrently with HS-GC analysis. The trained panelists used sensory description and evaluation technique commonly used in the company. Assessment of the beer characteristics and verification of its quality was made with a description of the sensory wheel of the beer flavours, as well as sensory assessment scale between 5 and 7 (Table 1).

Table 1. Categories for beer sensory evaluation

Category for beer sensory quality	Sensory assessment
very poor	5.0 - 5.5
poor	5.5 - 6.0
neither good nor poor	6.0 - 6.5
good	6.5 - 7.0
very good	7.0 - 7.5

RESULTS AND DISCUSSION

The results of the HS-GC analysis for off-flavour compounds vicinal diketones (diacethyl and 2,3 pentandione) and dimethyl sulfide in beer packed in brown bottles and cans are shown in Figures 1 and 2. The concentration of the compounds increased slightly as a result of oxidation processes due to the presence of oxygen in the packaging. Diacetyl contributes to thev “buttery” while 2,3-pentanedione to a “honey” flavour of beer. Beer in transparent bottles was monitored for only two months, given its instability compared with the other two types of packaging (Fig. 3). In this type of product the processes with negative effect on flavour stability were facilitated and the off-flavours were formed much faster. The taste threshold for DMS (50-60 μ g/L) was maintained in all types of packaging for the corresponding storage periods. If the DMS concentration is too high, beer will have a relatively objectionable taste and aroma of cooked vegetable.

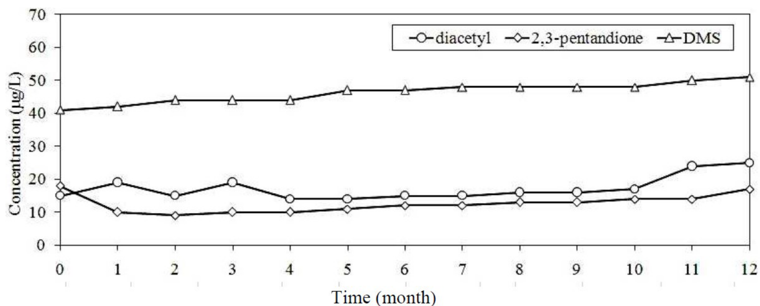

Fig. 1. Concentration profile of vicinal diketones and dimethyl sulphide in beer packed in glass brown bottle

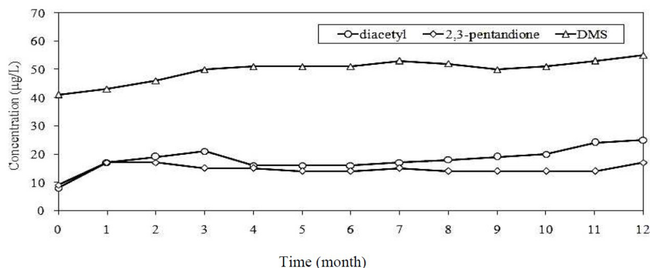

Fig. 2. Concentration profile of vicinal diketones and dimethyl sulphide in beer packed in can

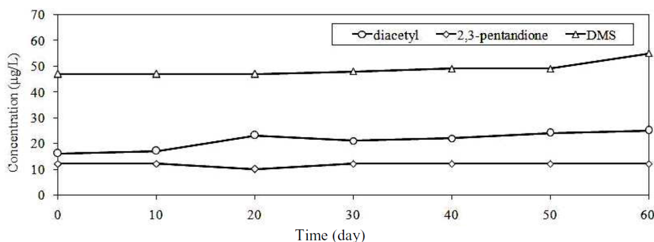

Fig. 3. Concentration profile of vicinal diketones and dimethyl sulphide in beer packed in glass transparent bottle

The concentrations changes of ethyl acetate, isoamyl acetate and n-propanol in beer as a function of storage time are given in Figure 4, 5 and 6. Esters are volatile constituents of beer that create the major part of its flavor (Verstrepen et al., 2003). A slight decline of the ester concentration can be noted contributing to the loss of fruity and estery flavour of beer. In beer packed in transparent bottles these changes happened in the very first days, which is the reason why this beer losses its estery flavour much faster than the other two. n-propanol, which causes "rough" flavour and harshness to beer did not exhibit significant changes during the storage period.

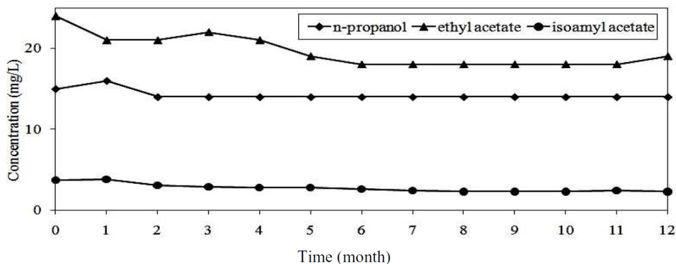

Fig. 4. Concentration profile of esters and n-propanol in beer packed in glass brown bottle

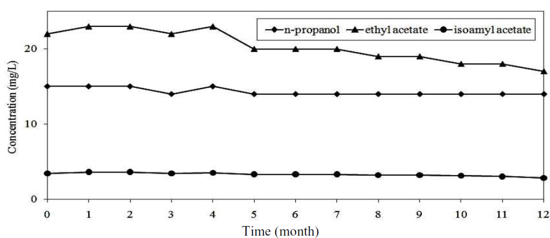

Fig. 5. Concentration profile of esters and n-propanol in beer packed in can

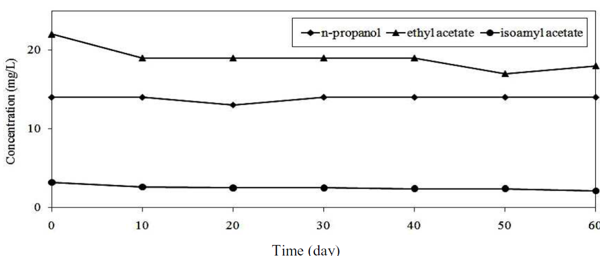

Fig. 6. Concentration profile of esters and n-propanol in beer packed in glass transparent bottle

Although HS-GC analysis is very important for the evaluation of beer quality, without sensory assessment, beer flavour profile can not be completed. Only trained tasters are able to determine off- flavours which can not be detected by gas chromatography. Figures 7 and 8 show intensities of some sensory properties of beer in glass brown bottles and cans as a function of storage period. Beer in glass brown bottles did not show significant changes for the first six months of storage. Staling changes in lager flavor congeners originate in oxidations and are accelerated by in-package residual oxygen (Vanderhaegen, 2003). In the 12th month, beer had developed sweet and aged taste, due to the formation of trans-2-nonenal and oxidation processes. Increasing sweet taste is observed during aging and this is partly due to sensory masking by decrease in bitterness. By intensifying off-flavours in beer, estery and fruity flavour (originated from ethyl acetate and isoamyl acetate) was masked. The can of beer was much more protected from external influences, and therefore there were less pronounced changes in its sensory quality. However, in this type of packaging metal taste was notable indicating aluminium migration from can to beer (Ivušić et. al., 2006).

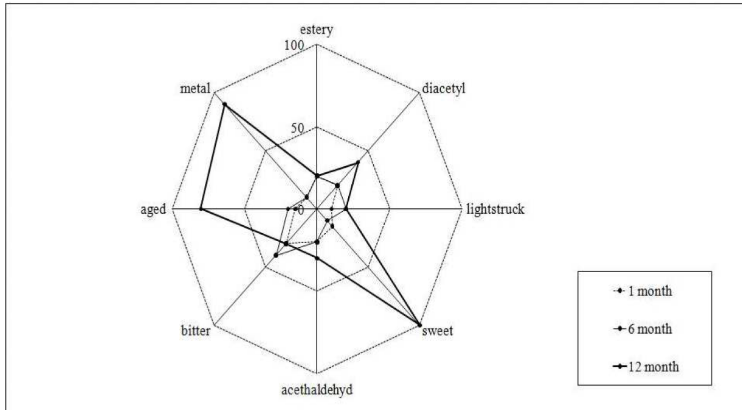

Fig. 7. Intensity of the sensory characteristics of beer in glass brown bottle

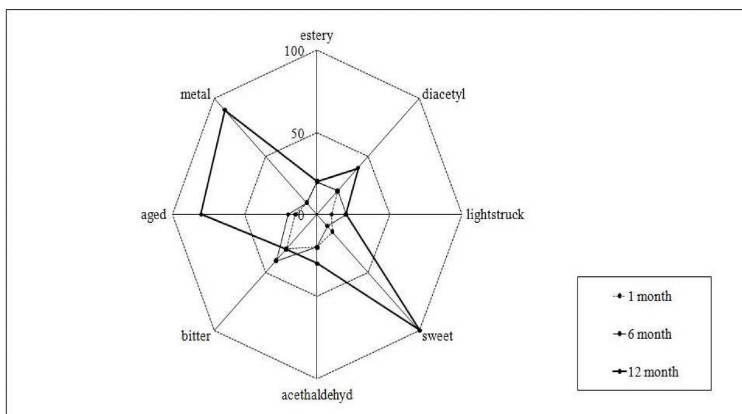

Fig. 8. Intensity of the sensory characteristics of beer in can

In beer in glass transparent bottles (Fig. 9), even after a month, a presence of a distinct lightstruck off- flavour can be seen which results from of photochemical reactions of isohumulones (Huvaere et al., 2004). These reactions in beer in glass transparent bottles are going for just a few nanoseconds (De Keukeleire, 2001). In this type of packaging due to the dominant taste of the 3-methylbut-2-en-1-thiol, the major flavour source in lightstruck beer, the other off-flavours could not be felt.

The sensory assessment of beer by trained panelists is given in Tables 2 and 3. As can be seen, beer in glass brown bottle was graded as poor (<6) after the 10th month, beer in can after the 11th month while beer in glass transparent bottle was graded as poor after only 10 days.

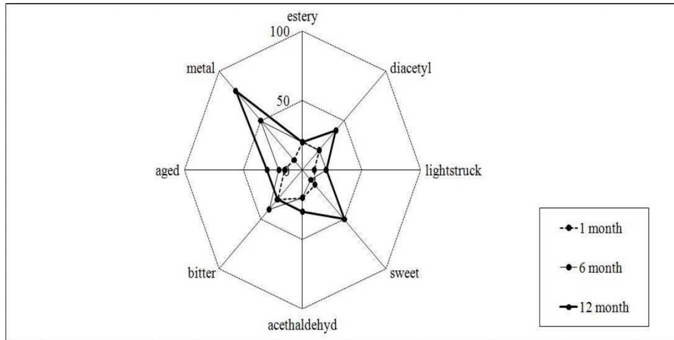

Fig. 9. Intensity of the sensory characteristics of beer in glass transparent bottle

Table. 2. Sensory grades of beer in glass brown bottle and can

Time (month)	0	1	2	3	4	5	6	7	8	9	10	11	12
Brown bottle	6.1	6.4	6.1	6.1	6.1	6.1	6.1	6.0	6.0	6.0	5.9	5.8	5.8
Can	6.5	6.4	6.1	6.1	6.3	6.5	6.3	6.2	6.3	6.0	6.0	5.8	5.9

Table. 3. Sensory grades of beer in the glass transparent bottle

Time (day)	0	10	20	30	40	50	60
Transparent bottle	6.0	5.8	5.5	5.5	5.3	5.2	5.0

CONCLUSION

From the conducted HS-GC and sensory analysis of beer in different kind of packaging it is obvious that beer in cans is the most stable and with the best sensory quality over time. On the other hand, foreign tastes in beer are not always regarded as off-flavours and often are accepted by consumers as flavours characteristic for a certain brand of beer.

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BUFFER CAPACITY AS A PARAMETER FOR HONEY CHARACTERIZATION

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ABSTRACT

Honey contains mainly carbohydrates and water. Minor compounds found in honey are various organic acids, amino acids, proteins, minerals, aromatics and many others. To characterize the composition of honey, various physical chemical and chemical parameters can be used. One of these parameters is buffer capacity.

The aim was to study possible use of buffer capacity to characterize honey composition and quality, and to determine possible correlation of buffer capacity with electrical conductivity or mineral content and free acids.

Buffer capacity and free acids content were determined with volumetric analysis methods, electrical conductivity - conductometrically, pH – potentiometrically, water content– refractometrically, but mineral content - gravimetrically. To determine electrical conductivity and buffer capacity, honey solutions of 20g/100 mL were used.

Obtained results provide evidence that buffer capacity of honey at least partially depends on electrical conductivity of honey. Buffer capacity of honey, when the amount of acid used is calculated varies from 10 to 20 mmol of HCl per 1 kg; when the amount of base used is calculated, buffer capacity varies from 3 to 9 mmol of NaOH per 1 kg. Free acids content varies from 11 to 27 mmol per 1 kg, but mineral content from 0,1 to 0,6%.

Obtained results suggest that buffer capacity can be used only to characterize overall honey content (minerals and acids content). Buffer capacity of honey is characterized not only by acid and mineral content.

KEYWORDS: *honey; buffer capacity; electrical conductivity*

INTRODUCTION

Special features of honey composition can be characterized by many chemical, physical and physical

chemical indicators. One of these indicators is buffer capacity. The buffer capacity can be characterized with liquid's ability to resist the effects of acid or base, that is, the ability to resist changes of pH level. The buffer capacity is determined by the proportion of acid and salts or base and salts in it, as well as the level of water in the honey.

The acids form in honey during its ripening. Bees, processing the nectar and "sweating", bring enzyme glucose oxidase in the honey, from which one of honeys main acids- gluconic acid- is formed. Honey also contains many other organic acids, such as malic, acetic, oxalic, butyric, citric, lactic, formic acid. The level of organic acids in honey is about 0,3 %. Honey also contains inorganic acids (HCl and H_3PO_4), which make up to 0,03 % of honey (Belitz et al., 2004).

The level of acids in honey is also one of its quality parameters. According to the EU regulations, the free acids content in honey must not surpass the mark of 50 mmol kg^{-1} (Council Directive 2001/110/EC).

The level of minerals in honey is insignificant- on average about 0,2 %, though the level of minerals can vary in a wide range starting from 0,1 to even 1,6 %. From the metallic elements in honey, potassium, calcium and sodium can be mentioned as macroelements, whereas iron, zinc, copper, cobalt and manganese as microelements, but lead, cobalt, nickel and chromium as ultra-microelements (Chepurnoy, 2002). The average content of metallic elements in honey is described in Table 1.

Table 1. Composition of metallic elements in honey[1]

Metallic element	Content, $\mu g\ g^{-1}$	Metallic element	Content, $\mu g\ g^{-1}$	Metallic element	Content, $\mu g\ g^{-1}$
Aluminium	1.4 – 40	Lithium	0.54 – 0.81	Strontium	0.27 – 0.81
Barium	0.27 – 2.7	Magnesium	3.1 – 300	Antimony	0.8 – 1.8
Vanadium	0.03 – 0.08	Manganese	0.15 – 40	Titanium	2,7 – 8.1
Bismuth	0.005 – 0.01	Molybdenum	0.003 – 0.8	Chromium	0.003 – 1.6
Gallium	0.01 – 0.03	Copper	0.02 – 4.8	Zinc	0.003 – 69
Germanium	0.003 – 0.01	Sodium	6 – 400	Zirconium	0.008 – 0.03
Iron	0.27 – 34	Nickel	0.003 – 0.81	Cobalt	0.01 – 0.27
Potassium	100 – 4700	Tin	0.003 – 27	Silver	0.003 – 0.54
Calcium	5 – 1780	Lead	0.02 – 6.3		

To describe the mineral content in honey, electrical conductivity is used. According to the EU regulations, electrical conductivity must not surpass 0,8 mS cm^{-1} (Council Directive 2001/110/EC).

The buffer capacity of honey is also influenced by water. Ripen honey with less water will have a greater buffer capacity compared to honey with a larger amount of water.

It is characteristic for unripe honey to have an increased water content, which, whilst storing honey, makes it mildew and ferment. Thus the proportion of the acids and salts in the honey changes, which has a great effect on the buffer capacity.

For some samples the buffer capacity changes in a wide range. The buffer capacity according to the used amount of acid is average from 3,5 to 47 mmol kg^{-1}, but according to the used base- from 1,3 to 30 mmol kg^{-1} (Malyutenkova, 2004).

Hypothesis of research: the buffer capacity of honey depends from the salts content (minerals) and acids in honey. The aim was to study possible use of buffer capacity to characterize honey composition, and to determine possible correlation between buffer capacity and mineral content or free acids.

MATERIALS AND METHODS

Buffer capacity and free acids content were determined with volumetric analysis methods. The electrical conductivity was measured conductometrically, but water content - refractometrically.

For determination of free acids, the honey sample is dissolved in water, the pH measured and the solution titrated with 0,1 M sodium hydroxide solution to pH 8,30.

For determination of buffer capacity the honey solutions was titrated with 0,01 M sodium hydroxide and 0,01 M hydrochloric acid. Results were calculated on 1 kg of honey.

The electrical conductivity of a solution of 20 g honey in 100 mL distilled water is measured using an electrical conductivity cell. For volumetric analysis was used automatic titrator. pH of honey solutions was determined potenciometrically.

RESULTS AND DISCUSSION

Various samples of honey (lime blossom, rape flowers, sweet clover, heather blossom, meadow – sweet,

1 Chudakov W.G. Apiculture product technology (in russian) - http://paseka.su/books/item/f00/s00/z0000024/index.shtml - resource used on 18.02.2011.

phacelia, hogweed, spring flowers, wild flowers, various flowers) have been analyzed and the following parameters have been determined - the free acid content of the honey, its electrical conductivity, water and mineral content, buffer capacity according to the amount of the used acid or base.

In researches was analyzed possible correlation between buffer capacity of honey or calculated buffer capacity of dry honey (by base and acid addition) and mineral content in honey or honey electrical conductivity (Figures 1, 2, 3, 4).

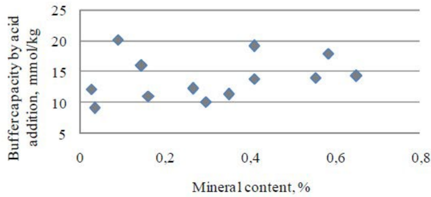

Figure 1. Relationship between honey buffer capacity by acid addition and mineral content in honey

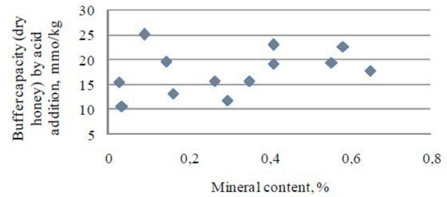

Figure 2. Relationship between calculated buffer capacity of dry honey by acid addition and mineral content in honey

From the data in the Figures 1 and 2 we can see that there isn't direct correlation between mineral content and the buffer capacity of the honey and dry honey (according to the used acid). However it is possible to make conclusions, that there is a tendency towards correlation between buffer capacity of honey and the mineral content in honey. Basically, excepting separate cases, it is possible to conclude: it means that greater content of salts in the honey also increases the buffer capacity of the honey according to the acid, because the amount of salts within the honey determines its ability to resist the effects of the added acid.

Buffer capacity of honey (according to used acid) depends not only on the content of normal salts in honey, but also from the content of hydrogen salts in honey. It is possible to explain some cases, when the mineral content in honey is low, but buffer capacity of honey is high. It is presumed that the buffer capacity of honey depends also from a proportion of normal and hydrogen salts. The symmetry of Figures 1 and 2 indicate the low influence of water on buffer capacity of honey (according to used acid).

From the data in the Figures 3 and 4 we can see that there isn't direct correlation between mineral content and the buffer capacity of the honey and dry honey (according to the used base). However it is possible to make conclusions, that there is a tendency towards correlation between mineral substances in honey and the buffer capacity of the honey according to the used base. Similar in the previous case, weak correlation between mineral substances in honey and the buffer capacity of the honey according to the used base, are explained with content of hydrogen salts in honey. Buffer capacity of the honey according to the used base also depends from content of organic and inorganic acids in honey.

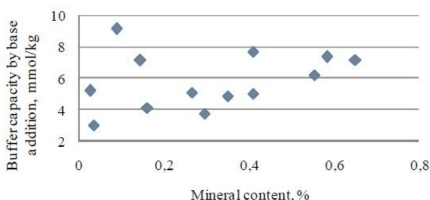

Figure 3. Relationship between honey buffer capacity by base addition and mineral content in honey

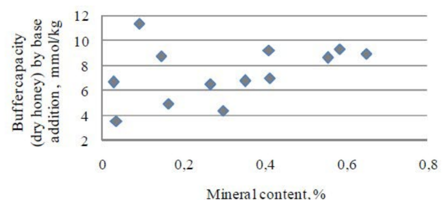

Figure 4. Relationship between calculated buffer capacity of dry honey by base addition and mineral content in honey

The analysis of the data between possible correlation for buffer capacity of honey and dry honey (by base and acid addition) and free acids content in honey are shown in Figures 5, 6, 7 and 8.

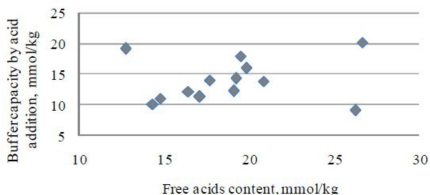

Figure 5. Relationship between honey buffer capacity by acid addition and free acids content in honey

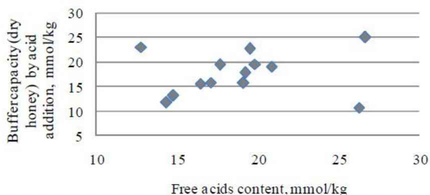

Figure 6. Relationship between calculated buffer capacity of dry honey by acid addition and free acids content in honey

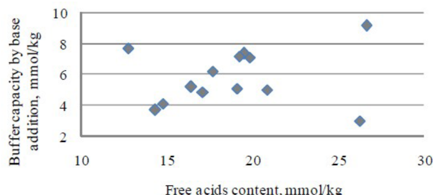

Figure 7. Relationship between honey buffer capacity by base addition and free acids content in honey

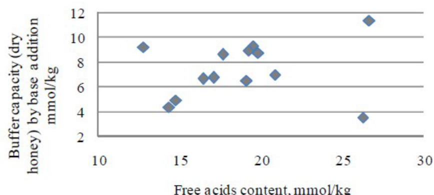

Figure 8. Relationship between calculated buffer capacity of dry honey by base addition and free acids content in honey

From the data in Figures 5, 6, 7 and 8 we can see that there is a tendency towards correlation between buffer capacity of honey and dry honey and free acids content. Similar in previous cases, weak correlation between free acids content in honey and buffer capacity of honey, are explained with proportion of the normal and hydrogen salts, organic and inorganic acids and others factors.

As known, that the electrical conductivity of honey is good indicator for salts content in it. The relationship between electrical conductivity of honey and its buffercapacity was characterised in Figures 9, 10, 11 and 12.

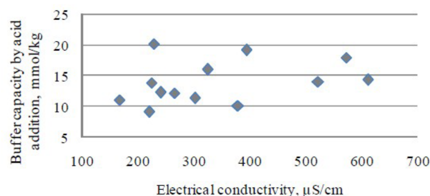

Figure 9. Relationship between honey buffer capacity by acid addition and electrical conductivity of honey

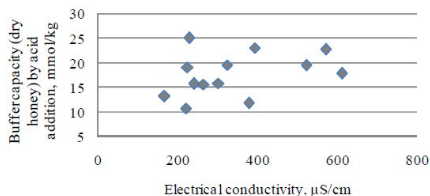

Figure 10. Relationship between calculated buffer capacity of dry honey by acid addition and electrical conductivity of honey

From Figures 9, 10, 11 and 12 we can see that there isn't direct correlation between content of honey buffer capacity (according to used acid and base) and its electrical conductivity, but is tendency.

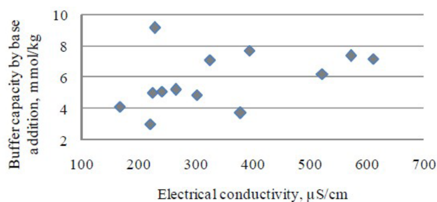

Figure 11. Relationship between honey buffer capacity by base addition and electrical conductivity of honey

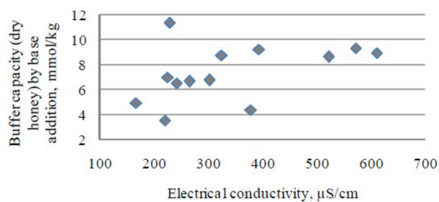

Figure 12. Relationship between calculated buffer capacity of dry honey by base addition and electrical conductivity of honey

From all relationsships between honey buffer capacity (according to acid and base) and it electrical conductivity, mineral content and acids, we can see, that there isn't direct correlation.

CONCLUSION

The buffer capacity of honey according to the effect of acid is greater than the effect of base.

The buffer capacity of honey according to the used base or acid was not in direct correlation with mineral content or free acids content in honey.

The buffer capacity of honey was not in direct correlation with electrical conductivity.

The buffer capacity of honey depends not only from free acids content of honey or mineral content, but also from others factors.

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CHARACTERIZATION OF BLACK LOCUST (ROBINIA PSEUDOACACIA) HONEY FROM THREE GEOGRAPHICAL REGIONS OF NORTH-WEST BOSNIA AND HERZEGOVINA

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ABSTRACT

The most important quality characteristics of honey are necessary to identify and track over time, in order to conduct its characterization and to qualify for protection of geographical origin. The aim of this study was to analyse the physicochemical characteristics of Black Locust (*Robinia Pseudoacacia l.)* honey collected from three climate ecological-vegetation geographical regions of North-West Bosnia and Herzegovina, which differ according to regional, geographic and climate factors and composition of soil. According to the place of honey production, collected samples were designated as GROUP (1) from North-West Bosnian region; GROUP (2) from Northern Bosnian region; GROUP (3) from West-Bosnian limestone-dolomite region and the physicochemical characteristics of honey were analysed. In the study we investigated and compared the nineteen physicochemical parameters (water content, ash, content of Zn, Cd, P, Fe, Cu, Mn, Mg, K, Na, refraction index, density, electrical conductivity, pH, free-acidity, total sugars, fructose, glucose and sucrose) in 39 samples of Black Locust honey from three geographical regions of North-West Bosnia and Herzegovina. A description of the Black Locust honey and its physical, chemical and sensory characteristics for the three defined geographical regions could be used in further work on protection of the honey geographical indications.

KEYWORDS: *honey; physicochemical characteristics; geographical origin*

INTRODUCTION

Honey is nectar collected from many plants and processed by honey bees (*Apis mellifera*). The composition of honey is variable that depend of the differences in plant types, climate, environmental conditions, and contribution of the beekeeper (Anklam, 1998; Grujić, Popov-Raljić, 2007). It is important to ensure control and protection of quality of honey that are produced in particular geographic regions and offered on the market. This can be achieved by defining the values and allowed deviations of the individual parameters of quality of honey produced from plant species in a particular geographic area (Mateo, Bosch-Reig, 1998; Grujić, Popov-Raljić, 2007). The most important quality characteristics of honey are necessary to identify and track over time, with aim to conduct its characterization and to qualify for geographical origin protection. It is possible to create a quality database for selected quality parameters of black locust honey, produced in different regions where acacia trees grow (Golob and Plestenjak, 1999; Popek, 2002; Devillers et al., 2004; Persano et al., 2004; Kenjerić et al., 2007; Šarić et al., 2008). It is important to expand and complement it with new knowledge.

The aim of this study was to determine the physicochemical and sensory characteristics of black locust (*Robinia Pseudoacacia l.)* honey produced and collected from three climate ecological-vegetation geographical regions of North-West Bosnia and Herzegovina, to characterize the honey samples and to define quality of the honey with regard to EU regulations. In these regions of honey production, which differ according to regional, geographic, and climate factors and composition of soil, there are good conditions for the production of honey from a diverse wild honey plants. Regarding that it is very important to highlight possible relationships between the composition of honey and its botanical and/or geographical origin.

MATERIALS AND METHODS

The analytical work was carried out on totally 42 samples of black locust, also known as robinia or acacia (*Robinia Pseudoacacia l., Fam. Fabaceae)* honeys produced and collected from beekeepers in two years, 2000 and 2001. Collected samples were from three geographical regions, six locations of North-West Bosnia and Herzegovina: **Kostajnica, Banja Luka, Gradiška, Srbac, Brod and Novi Grad.** According to the information from the beekeepers regarding geographic origin, location of production and belonging to the ecological-vegetation regions (Stefanović et al., 1983), all 42 analyzed black locust honey samples were classified in three groups, of which 9 honey samples produced on locations municipality area of **Kostajnica and Banja Luka** belong to the **North-West Bosnian region,** were designated as **GROUP (1)**. In **GROUP (2)** are designated 18 honey samples produced at the municipality area of Gradiška, Srbac and Brod inside **Northern Bosnian region**. Inside west part of **West Bosnian limestone-dolomite region** there is Ključ-Petrovac region, and municipality area of **Novi Grad** as location of 15 honey samples production, designated as **GROUP (3)**.

Honey samples were collected from individual producers and members of the beekeepers association, according to the Association of Official Analytical Chemists (AOAC) Official Method (2000), and stored in glass containers short time on 15°C in the dark, prior to the analysis. Samples were screened by sensory analysis as soon as they arrived to the laboratory.

Honey samples were classified by beekeepers, regarding botanical origin and production regions (Stefanović et al., 1983). All analyses were performed in triplicate at the same time and the results are given as mean values. Physicochemical analyses were done according to the AOAC Official Methods (2000): water content; content of total sugars, glucose, fructose and sucrose; total acidity; active acidity (pH) on pH Meter (Model 3310, Jenway, Engl.); specific electrical conductivity (on Palinest Conductivity Meter, PT 115, Palinest Instruments); ash content; refractive index (on Leica Abbe Mark II Refractometer Model 10480, Leica, USA); density of honey (expressed in g/cm^3) and free-acidity (expressed in meq/kg) were measured in accordance with the standard methods (*Official Gazette BA, 37*/2009a,b). Trace elements in honey (Zn, Cd, Fe, Cu, Mn, Mg, K, Na) were analysed on atomic absorption spectrometer with flame detector (AAS Unicam 969), and measurements of phosphorus (P) content were done on UV- VIS Spectrophotometer (Milton Roy Spectronic 1201) in accordance with AOAC (2000) methods.

Basic statistics (median, standard error, standard deviation, minimum, maximum and range of values) and multivariate statistical analysis were carried out using StatistiXL Toolpak SPSS 1.8 for Microsoft Excel Analysis. To verify if there is variability between honey samples of same botanical origin as result of impact of geographical origin, an analysis of variance (ANOVA) was realized and appropriate F-tests applied. To determine if the difference in the mean values of analysed parameters of the GROUPS was significant, a t-test was also performed.

RESULTS AND DISCUSSION

Honey as a natural product of limited production and relatively high prices, has always been expressed to the risk of adulteration. The chemical composition of nectar, as well as concentration and ratio of certain constituents present in it, are just some of the reasons that is almost impossible to find honeys of different geographical origin which have identical botanical origin and completely identical content of ingredients (Anklam, 1998; Persano et al., 2004). External factors, such as different geographical, climatic and other factors specific to the locality in which the honey plants growth, season of collection and honey storage conditions, affects to the chemical composition and physical properties of honey. Identification of the botanical origin of honey is usually based on determining the pollen contents, colour, smell and taste of honey and honey collection season and hives location.

Characterisation of black locust honeys samples produced in three regions in North-West part of Bosnia and Herzegovina grouped regarding their geographic origin performed the data set of measured and compared physicochemical parameters for 39 samples of honey designated as GROUP (1), GROUP (2) and GROUP (3).

In the study the nineteen physicochemical parameters (water content, ash, content of Zn, Cd, P, Fe, Cu, Mn,

Mg, K, Na, refraction index, density, electrical conductivity, pH, total acidity, total sugars, glucose, fructose and sucrose), grouped regarding origin in three geographical regions of North-West Bosnia and Herzegovina were analysed and compared. An overview of the mean values for analysed parameters grouped in the three geographical regions, and median, standard error, standard deviation, minimum, maximum and range of values for parameters analysed Black Locust honey are shown in Tables 1-6, and results complied with the national regulations (*Official Gazette BA, 37*/2009a, b) and European regulations relating to honey (Council Directive 2001/110/EC). Similar analysis of acacia honey was carried out by Golob and Plestenjak (1999), Popek (2002), Persano et al. (2004), Piana et al. (2004), Šarić et al. (2008).

Black locust honey is one of the highly prized types of honey for taste and flavour. Slowly crystallizes under usual conditions of storage at room temperature, and after crystallization becomes white, with fine grain crystals. The liquid honey is crystal- transparent, almost colourless to light yellow hue and the colour can have a noticeable greenish tint barely. The black locust honey samples were analysed applying descriptive sensory method (Grujić, 2008). Black locust honey has very light colour with more or less yellow impact on tone, weak, pleasant warm odour and, the aroma is fruity, luscious, light and refreshing. The ratio of sweet and sour taste modalities is well balanced, so it gives the impression of slightly sweetish sour honey.

A statistically significant impact of regional, geographic and climatic factors on certain quality characteristics of honey confirmed a numerous publications (Mateo and Bosch-Reig, 1998; Gonzalez Pamaras et al., 2000; Persano et al., 2004). If look at the quality parameters of acacia honey samples grouped by geographic origin in GROUP (1); GROUP (2) and GROUP (3), it can be observed the differences between them in average values for each parameter. To obtain more complete and accurate database of the chemical composition, physicochemical and sensory quality characteristics of honeys produced in the three observed geographic regions, analytical data are used for statistical analysis and comparison. A statistically significant effect of geographic origin on the parameters measured for black locust honey produced in three geographic regions in north-western part of the Bosnia and Herzegovina in one-way ANOVA were confirmed for acidity ($F=4.435$, $p<0.05$), Cd ($F=9.954$, $p<0.05$), and for ash ($F=2.942$, $p<0.1$) and for pH ($F=2.431$, $p<0.1$). ANOVA was followed up by Multiple Comparisons Tukey's HSD test for comparing every group mean with every other group mean, and find statistically significant difference ($p<0.05$) between the GROUPS (three geographic regions) for the content of cadmium in honey, between GROUP (1) and GROUP (3), the same as between GROUP (2) and GROUP (3). There was statistically significant difference in acidity ($p<0.05$) and in ash content ($p<0.1$) between GROUP (1) and GROUP (2), as well as between GROUP (1) and GROUP (3).

Table 1. Content of Zn, Cd, P, Fe, Cu, Mn, Mg, K, Na, ash and water in black locust honey samples from GROUP (1)

Parameters	Zn mg/100g	Cd mg/100g	P mg/100g	Fe mg/100g	Cu mg/100g	Mn mg/100g	Mg mg/100g	K mg/100g	Na mg/100g	Ash g/100g	Water g/100g
Mean	0.945	0.186	4.995	0.435	0.179	0.206	1.405	9.640	6.095	0.25	17.10
Std Error	0.127	0.019	0.985	0.032	0.077	0.095	0.514	0.776	0.132	0.059	0.411
Std Dev.	0.358	0.053	2.786	0.090	0.217	0.269	1.453	2.194	0.374	0.167	1.161
Minimum	0.596	0.116	2.556	0.254	0.037	0.032	0.050	6.292	5.496	0.08	15.80
Maximum	1.785	0.265	10.128	0.551	0.661	0.845	3.394	12.967	6.639	0.54	18.40
No. of samples	8	8	8	8	8	8	8	8	8	8	8

Table 2. Content of Zn, Cd, P, Fe, Cu, Mn, Mg, K, Na, ash and water in black locust honey samples from GROUP (2)

Parameters	Zn mg/100g	Cd mg/100g	P mg/100g	Fe mg/100g	Cu mg/100g	Mn mg/100g	Mg mg/100g	K mg/100g	Na mg/100g	Ash g/100g	Water g/100g
Mean	0.928	0.105	4.934	0.410	0.271	0.098	1.770	9.589	6.070	0.16	17.38
Std Error	0.138	0.015	0.594	0.028	0.193	0.007	0.255	0.279	0.061	0.013	0.229
Std Dev.	0.585	0.063	2.519	0.118	0.819	0.031	1.084	1.184	0.261	0.054	0.971
Minimum	0.595	0.018	2.536	0.247	0.036	0.057	0.201	7.612	5.676	0.08	15.90
Maximum	3.171	0.253	11.778	0.775	3.535	0.164	3.689	11.573	6.481	0.26	18.80
No. of samples	18	18	18	18	18	18	18	18	18	18	18

Table 3. Content of Zn, Cd, P, Fe, Cu, Mn, Mg, K, Na, ash and water in black locust honey samples from GROUP (3)

Parameters	Zn mg/100g	Cd mg/100g	P mg/100g	Fe mg/100g	Cu mg/100g	Mn mg/100g	Mg mg/100g	K mg/100g	Na mg/100g	Ash g/100g	Water g/100g
Mean	1.178	0.180	4.451	0.424	0.141	0.145	1.174	9.579	6.137	0.16	17.01
Std Error	0.116	0.011	0.579	0.017	0.037	0.051	0.312	0.349	0.062	0.022	0.225
Std Dev.	0.447	0.044	2.244	0.066	0.145	0.199	1.210	1.350	0.239	0.086	0.871
Minimum	0.709	0.077	1.007	0.320	0.036	0.016	0.048	7.708	5.798	0.06	15.60
Maximum	2.399	0.252	8.981	0.550	0.610	0.653	3.408	11.797	6.604	0.34	18.40
No. of samples	15	15	15	15	15	15	15	15	15	15	15

Table 4. Physicochemical parameters of black locust honey samples from GROUP (1)

Parameters	Refraction index	Density (g/cm^3)	Electrical conductivity (mS/cm)	pH	Acidity (mmol/kg)	Fructose + Glucose (g/100 g)	Sucrose (g/100 g)	Total sugars (g/100 g)
Mean	1.4937	1.423	0.25	3.82	16.37	70.46	4.72	75.18
Std Error	0.0009	0.002	0.059	0.066	2.481	0.491	0.383	0.544
Std Dev.	0.0027	0.006	0.177	0.197	7.442	1.473	1.150	1.633
Minimum	1.4907	1.413	0.09	3.49	6.18	68.25	2.35	72.45
Maximum	1.4972	1.432	0.56	4.17	27.15	73.55	6.00	77.71
No. of samples	9	9	9	9	9	9	9	9

Table 5. Physicochemical parameters of black locust honey samples from GROUP (2)

Parameters	Refraction index	Density (g/cm^3)	Electrical conductivity (mS/cm)	pH	Acidity (mmol/kg)	Fructose + Glucose (g/100 g)	Sucrose (g/100 g)	Total sugars (g/100 g)
Mean	1.4932	1.421	0.20	3.95	10.73	69.85	4.92	74.77
Std Error	0.001	0.002	0.019	0.042	0.679	0.241	0.189	0.227
Std Dev.	0.002	0.009	0.081	0.180	2.882	1.023	0.800	0.965
Minimum	1.4893	1.411	0.10	3.62	5.09	68.25	2.58	73.27
Maximum	1.4968	1.442	0.37	4.35	16.17	72.22	6.00	76.45
No. of samples	18	18	18	18	18	18	18	18

Table 6. Physicochemical parameters of black locust honey samples from GROUP (3)

Parameters	Refraction index	Density (g/cm^3)	Electrical conductivity (mS/cm)	pH	Acidity (mmol/kg)	Fructose + Glucose (g/100 g)	Sucrose (g/100 g)	Total sugars (g/100 g)
Mean	1.4930	1.422	0.27	3.84	12.05	69.17	5.02	74.19
Std Error	0.001	0.003	0.033	0.043	1.128	0.463	0.143	0.504
Std Dev.	0.002	0.013	0.126	0.168	4.367	1.794	0.554	1.951
Minimum	1.4903	1.388	0.08	3.52	5.10	63.45	4.16	68.00
Maximum	1.4978	1.438	0.54	4.16	22.16	71.00	6.00	76.10
No. of samples	15	15	15	15	15	15	15	15

CONCLUSION

In this study were determined the physicochemical characteristics of black locust (*Robinia Pseudoacacia l.)* honeys produced and collected from three geographical regions of North-West Bosnia and Herzegovina. The black locust honeys had quality that complying with the European regulations relating to the honey. In studied regions of honey production, which differ according to regional, geographic and climate factors and composition of the soil, there are good conditions for the production of honey from a diverse wild honey plants. Regarding that, relationships between the composition of honey and its botanical and/or geographi-

cal origin were highlighted. The results obtained in research represent important data that can be used to define the quality characteristics of acacia honey and in further work on protection of the honey geographical indications, as well as for control of quality, purity and authenticity of honey produced within the regions in the North-West part of the Bosnia and Herzegovina.

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HYDROXYMETHYLFURFURAL FORMATION IN MUESLI WITH HONEY ADDITIVE

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ABSTRACT

Hydroxymethylfurfural (HMF) is a thermal degradation product of sugars, and it is present in many everyday foods. One of these products is honey and other sugars containing products. High HMF content can indicate a high temperature heating or long storage of honey. The detection of HMF has been started 70 years ago.

The aim of this work was to study effect of production conditions on HMF content in muesli made with honey or sugar syrup additive.

HMF was determined by liquid chromatography. Honey and sugar syrup was additionally tested for pH – potentiometry and sugar content by chromatography. HMF content was analyzed in muesli with honey or sugar syrup additive prepared at three different heat treatment temperatures (150, 180 and 200 °C) and three different heating times (5, 10, and 15 min).

HMF content in muesli heat treated at 150 °C for 5, 10 or 15 min was similar, but it differs between products made with honey or sugar syrup heated at 180 °C or 200 °C for 15 min. HMF content in muesli with honey or sugar syrup additive heated for 15 min at 180 °C w as 37.37 and 3.01 mg kg^{-1}, respectively. HMF content in product with honey was 112 mg kg^{-1} and 17.31 mg kg^{-1} in product with sugar syrup if heated for 15 min at 200 °C.

Conclusion can be drawn that HMF content is many times higher in a product with honey if heated at temperature above 150°C comparing to product with sugar syrup additive.

KEYWORDS: *Muesli; honey; hydroxymethylfurfural; sugars; heating.*

INTRODUCTION

Very often people use foods without paying serious attention to their nutritive value and wholesomeness. In heat-treated products can be formed various hazardous substances which can have adverse effect on human health if consumed in large quantities. One of these harmful compounds is hydroxymethylfurfural (HMF).

HMF is an organic compound which is derived in dehydration of monosaccharides. Particularly it is formed from ketose and fructose at the presence of acids.

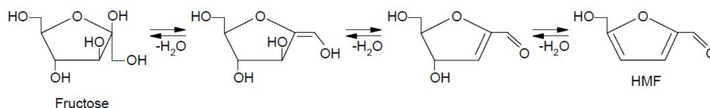

Figure 1. Formation of hydroxymethylfurfural 1

In gradual dehydration of fructofuranose through several intermediate stages of dehydration HMF is formed. HMF is not found in fresh and unprocessed foods. Formation of HMF starts during long-term storage of foods rich in sugars and especially rapid increase in HMF content is observed after heat treatment of these products. HMF is found in dried fruits and caramel candy – about 1 g kg^{-1}. HMF sources can be the foods whose ingredients are caramel, flavours and honey. HMF can be used as a marker for control of heat

treatment temperature applied to foods such as honey, coffee, cereals, pasta, biscuits, bread, and breakfast cereals. HMF is also present in drinks like wine, bear and brandy. However, in each product HMF as a marker should be measured different (Stadler, Lineback, 2009). HMF in big concentrations is cytotoxic, causing eye, upper respiratory tract, skin, mucous membrane irritation (Stadler, Lineback, 2009).

HMF content in honey is an indicator of honey purity (freshness, naturalness). High concentration of HMF indicates overheating, poor storage conditions and long storage time of honey (Khalil, Sulaiman, at.al., 2010; Maria, Nozal, at.al., 2001). In natural honey shortly after harvest HMF is not detected. HMF content in honey increases, depending on the pH value and storage temperature at about 2–

3 mg kg^{-1} per year. According to the European quality standards and codes, HMF content in honey should not exceed 4 mg 100 g^{-1} (Council Directive 2001/110/EC, 2002).

One of the most widely used products containing sugar is muesli. Usually muesli is a blend of oat flakes and dried fruit, seeds, and nuts. Ingredients of muesli can be honey, chocolate pieces, nuts, spices. Muesli is exposed for heat treatment. To protect cereals from burning down there must be added saturated oils and sugar to the muesli and it is flavoured with caramel, chocolate, and honey [2]. Honey is a sugar-rich product, where increased content of HMF can be formed in heat treatment or long-term storage process. The main sugars in honey include the D-fructose and D-glucose. Fructose

content in honey is about 35 to 38%, while the glucose content is about 35%. The sucrose content in honey does not exceed 7% (Belitz, Grosch, at.al., 2009).

As a honey substitute in muesli can also be used many of syrups - "glucose syrup" (produced by the hydrolysis of starch), the "sugar syrup" (produced by products, refining of beet or cane). Sugar syrup

is a viscous sugar solution, where part of the sucrose is converted to fructose and glucose. Syrups are suitable for making various products. They are used in place of regular sugar in the desserts and meat dishes and sauces, and drinks. Syrups are especially useful for the confectionery because they improve the swelling of dough, make the dough softer and are excellent moisture holders[3].

The work has a hypothesis: dynamics of HMF formation in muesli with honey and muesli with added sugar syrup additives is different. Therefore, the aim of this work was to study effect of production conditions on HMF content in muesli made with honey or sugar syrup additive.

MATERIALS AND METHODS

For the research were prepared nine samples of muesli, which consisted of oat flakes, hazelnuts, almonds, olive oil, salt, sugar, and as a binder honey of various flower 'Vinnis' (15%) was added (produced in Cesis, Latvia), and nine muesli samples with sugar syrup 'Dansukker' (produced in Denmark) additive.

The samples were heat-treated at 150, 180 and 200 °C temperature for 5, 10 and 15 minutes. Muesli samples were ground, extracted in distilled water. The obtained extract was centrifuged (10 min,

6000 rpm) and filtered through a 0.45 μ m nylon filter. Content of HMF and sugars was analysed using

high pressure liquid chromatography (SHIMADZU, Japan). Chromatography conditions for HMF determination were as follows.

The content of HMF in samples was determined by the calibration curve method. The filtrate of the sample (10 μ l) was injected into the HPLC, and analysed by using (10/90) acetonitrile (CH_3CN/H_2O) by flow rate of 1.3 ml min^{-1} as a mobile phase, and Alltech C18 column (4.6×250 mm) connected to a UV/VIS detector (SPD-20A) set at 280 nm. Data were acquired and processed using Shimadzu LaBSolutions software (LaBsolution Version 1.21 SP1).

The content of sugars in samples was determined by the calibration curve method. The filtrate of sample (10 μ l) was injected into the HPLC, and analysed by using (80/20) acetonitrile (CH_3CN/H_2O) by flow rate of 1.3 ml min^{-1} as a mobile phase, and Altima Amino column (4.6×250 mm) connected to a refractive index detector (RID-10A). !F!

The pH level of samples was determined by potentiometry.

RESULTS AND DISCUSSION

For establishing differences between raw materials used in the research, the composition of mono-and disaccharides (Figure 2) in honey and sugar syrup as well as pH was determined.

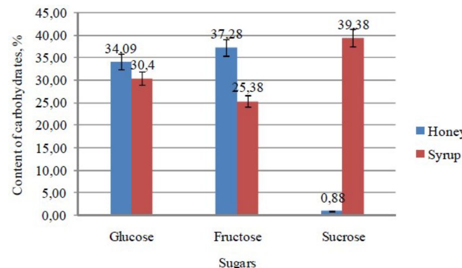

Figure 2. Content of sugars in honey and syrup.

As can be seen from Figure 2, glucose and fructose content in honey is higher compared to the sugar syrup, while the sucrose content in honey is considerably lower than in sugar syrup. Formation of HMF is more strongly affected by presence of fructose. Since honey has more fructose and glucose, then one would expect that in the muesli with honey added will be more HMF than a product with syrup additive.

The pH was determined in honey and glucose syrup samples. In honey it was about 3.9, while in the glucose syrup 4.3. A lower pH can also contribute to the formation of HMF.

HMF content was determined in unheated honey and sugar syrup. In honey it was – 2,44 mg 100 g-1,

but in sugar syrup – 2,01 mg 100 g-1. As can be seen, there are no significant differences between the

HMF content in honey or sugar syrup. In honey it is almost twice lower, compared to the European quality standards, which limits HMF content in honey to 4 mg 100 g-1.

In the research HMF content increase was analyzed in honey and sugar syrup, depending on the heat-

treatment conditions. Honey and sugar syrup samples were heated at a temperature of 180 °C for 5, 10 and 15 minutes. HMF content changes in honey and sugar syrup samples can be seen in Figure 3.

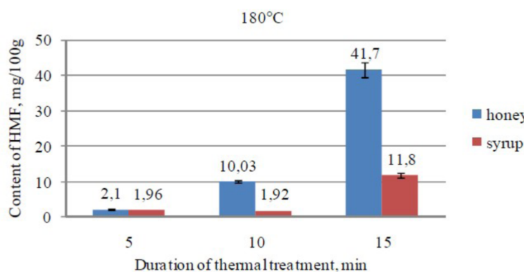

Figure 3. HMF content changes in honey and sugar syrup upon heating at 180°C.

As we see in Figure 3, HMF content increase in honey is a fivefold already after 10 minutes of heating, and it exceed the limit set by European quality standards2.5 times. After 15 minutes of heat- treatment, the limit has been already exceeded 10 times. In ten minutes HMF content in sugar syrup did not change, but after 15 minutes it increased almost six-fold.

Studies on the HMF content in muesli, depending on heat treatment duration, were carried out at 150 °C, 180 °C and 200 °C.

The dynamics of HMF content changes upon heat treatment at 150 °C is shown in Figure 4.

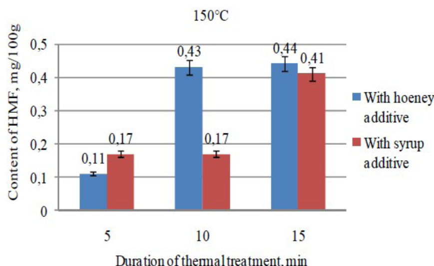

Figure 4. Changes of HMF content in muesli at 150 oC.

HMF content in muesli with honey added increase (4 times), if samples were heat-treated for more than 10 minutes. HMF content in muesli with glucose syrup additive increased significantly (2.4 times), when heat treatment is 15 minutes, compared with the heat-treatment duration of 5 minutes. Although the HMF content changes significantly, but over the entire study area it is much smaller than 4 mg 100 g^{-1} and therefore it can be considered that at the temperature 150 °C there is no significant difference between the muesli made with honey or muesli with sugar syrup additive. Dynamics of HMF content changes during heat treatment at 180° C is shown in Figure 5.

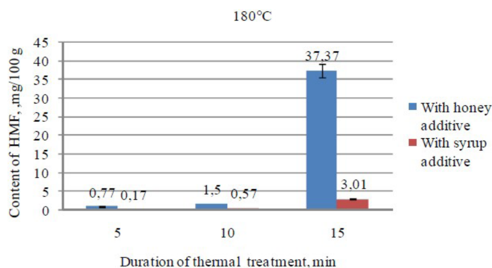

Figure 5. Changes of HMF content in muesli heat-treated at 180 C

HMF content in muesli with honey additive increases significantly throughout studied range, compared to the heat treatment at 150 °C (Figure 4). After 5minutes of heating the HMF content increased 7 times. Very big increase in HMF content is observed after 15 min of heat treatment 37.37 mg 100 g^{-1}, it is nine times higher than it is permitted in honey (4 mg 100 g^{-1}). While in the muesli with sugar syrup additive, HMF content increases significantly less. After 15 minutes of heat- treatment the HMF content increased 18 times compared to the HMF content in product after 5 minutes of treatment.

Changes in HMF content at a temperature of 200 °C are presented in Figure 6.

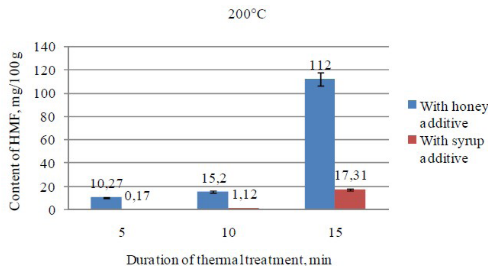

Figure 6. Changes of HMF content in muesli by 200oC

In all tested muesli samples with honey additive heat-treated at 200 °C HMF content is from 2.5 to 28 times greater than 4 mg 100 g^{-1}. After 15 minutes of heat-treatment, in muesli with honey additive HMF content is increased a thousand times over muesli, made 5 minutes at 150 °C (Fig. 4). In muesli with glucose syrup additive only after 15 minutes of heat-treatment the HMF content is more than 4 times higher than 4 mg 100 g^{-1}, but it is still much lower than in muesli with honey additive.

CONCLUSIONS

- If muesli is prepared at temperature 150 °C, there is no significant difference in HMF content whether honey or sugar syrup additive is used.
- Muesli with sugar syrup additive has lower HMF content than muesli with honey additive, if the heat treatment temperature is set above 180 °C.
- To protect muesli from high concentration of HMF, the heat treatment process shouldn't exced 10 minutes at temperature 200 °C if sugar syrup is used as an ingredient.
- Muesli with honey additive is not recommended for young children due to possible HMF presence.

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INTERNET RESOURCES

1. Hydroxymethylfurfural - http://www.thefullwiki.org/Hydroxymethylfurfural - resource used on 10.05.2011. Musli - it really that healthy (In Latvian) - http://www.ekozeme.lv/partika/musli-%E2%80%93-tiesam-tik-veseligs – resource used on 10.05.2011.
2. What are the health concerns about high – fructose corn syrup? - http://www.mayoclinic.com/health/high- fructose-corn-syrup/AN01588 - resource used on 10.05.2011.

INFLUENCE OF ALBANIAN OLIVE VARIETIESON THE QUALITY OF ORGANIC EXTRA VIRGIN OLIVE OILS

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ABSTRACT

„Kalinjot" and „Tirana White Olive" are two olive varieties very important for the production of organic extra virgin olive oil in Albania. We were particularly interested to create/evaluate the profile of main qualitative components of organic extra virgin olive oils produced from these two varieties as well as the comparison among them, in regard with quality attributes and sensorial properties.

12 olive oil mean samples, produced from 2005-2009, were analyzed. Free acidity, peroxide value, ultra violet absorbency (K 232 and K 270 nm), ΔK, sensory evaluation and fatty acid composition in compliance with IOOC standards for oils, as well as insecticides (organ phosphorous), were determined.

Fatty acid composition was measured by gas chromatography. The report of fatty acid composition of olive oil produced from „Kalinjot" variety was different from „Tirana White Olive"; regarding sensorial attributes of fruity flavor, bitterness, pungency and astringency, these attributes were more intense at Kalinjot olive variety.

*K*EYWORDS: *organic extra virgin olive oil, fatty acids, phenolic compounds, sensory evaluation*

INTRODUCTION

Olive trees in Albania, are considered an important point of biologic diversity. Albania is included in the seventh Mediterranean climate centre, based on the origin and formation of the cultivated plants (Dodona, Ismaili et al, 2010). Main centers of olive trees are: Antic olive trees of Tirana region located in Preze, Ndroq, Petrele, Linze, Brar, Kruje and olive trees of the Vlora region in Kanina, Bestrove, Narte, Panaja, Trevllazer, Dhermi, Himare, Palase, Qeparo, Borsh. In Albania there are 5 olive varieties, which are considered economically viable; the planted area of these varieties is about 5 % of the total olive area. The most popular variety is Kalinjot", which covers 45 % of the total area planted with olive trees (Dodona, Ismaili et al, 2010). White Olive of Tirana is a local autochthonous olive variety in this region. It has a good productivity and is known for high oil yield and a very good quality.

Virgin olive oil produced from these varieties has a delicate and unique flavour. Quantity and quality of substances existing in the extra virgin olive oil, such as fatty acids, phenolic compounds, chlorophyll are affected by various factors including the type of the olive cultivar (D. Boskou 1996), climatic conditions, ripening stage, irrigation management (Minguez-Mosquera, Perez-Galvez, 1998) and the extraction methods Among these factors, cultivar is the most important (Thomai, 2001).

Our scope of work was to create the profile of main qualitative attributes of organic extra virgin olive oils, produced from these two varieties as well as the comparison between them, in regard with the quality and sensorial properties.

MATERIALS AND METHODS

Fruit samples

Healthy olive fruit mean samples of 'Kalinjot' and 'White Olive of Tirana' cultivars were picked at industrial optimum ripening stage. The experiment was conducted during the crop season of 2007- 2009 in olive orchard in "Vlora Olive Research Institute", located in south of Albania, for Kalinjot variety and olive orchard

of "Shkalla" Factory, in the central part of Albania, in Tirana for White Olive variety.

Extra virgin olive oils were products of two oil factories: "Mullai" in Vlora and "Shkalla" in Tirana, using continuous extraction system with two centrifugations (first horizontal and then vertical).

Free acidity, peroxide value and UV Spectrophotometric indices (K232, K270)

The quality indices of olive oil, degree of acidity, peroxide value, specific extinction coefficient of K232 and K270 and ΔK were calculated according to the European Commission Regulation EEC/2568/91 (Commission Regulation (EC), 1991), AOAC method (Edition 16, 2005) and Codex Alimentarius.

Sensory Assessment

The sensory assessment of virgin olive oil was done by a Panel of expert tasters, who appraised the aroma and flavour attributes of the virgin olive oil, following European standards. Favourable attributes were bitterness, spicy and fruity flavour. An olive oil was considered extra virgin when the defect points (average) given by the Tasting Panel was 0 (zero).

Determination of chlorophyll

Pigment of chlorophyll was determined with a spectrophotometric method (Techcomp - 7500 UV Visible spectrophotometer), (Giovani Lerker, et al, 2009). 1 g of olive oil was dissolved in 10 ml of isooctane. The absorbance of the diluted solution was measured at λ 630, 670 and λ 710 nm and the content of the chlorophyll was calculated using the formula: A 670 – (A630-A710/2) / 0.0964x1(cm).

Fatty acids analysis

The fatty acid composition of olive oils was determined as acid methyl esters (FAMEs). FAMEs were prepared by vigorous shaking of a solution of each olive oil sample in hexane (0.2 g in 3 ml) with 0.4 ml of 2 N methanol potassium hydroxide solution, then 2 μl were injected into the GC (HP5890N, Hewlett Packard, USA) with a FID detector. The carrier gas was helium at a flow through the column (50 m length×0.25 μm i.d.×0.25 mm film thickens, HP 5) of 1 ml/min, according to the method of European Regulation Commission 2568/91.

The temperature of the injector and detector were set at 250°C and the oven temperature at 210°C. The results were expressed as relative area percent of the total FAMEs.

Phenolic compounds and Insecticides content

Total polyphenols were measured according the method of Gutfinger, using spectrophotometer at 760 nm and the results were expressed in mg/l caffeic acid.

Insecticides I and insecticides VI (organ phosphorous in mg/kg), was determined according to UNI EN 1528-1, 2, 3, 4:1997.

Statistical analysis

The complete data were evaluated by randomized block design, with three blocks (replications) each, containing three oil samples every season, for each olive variety, using SAS software (version 9.1). The results were displayed in mean values and standard deviation (n=3). Significance of the

differences among the numbers was determined by the analysis of variance, using Duncan's multiple range tests. The level of significance was determined as $P<0.05$.

RESULTS AND DISCUSSION

Free acidity, peroxide value and UV spectrophotometric indices

All analyzed oils showed very low values for physic-chemical parameters (acidity ≤0.8%; peroxide value ≤20 mev O2/kg oil; K270 ≤0.22; K232 ≤2.5); as well as sensorial evaluation (Aguilera M.P., et al. 2005), which categorized oils as "extra virgin olive oil", (IOOC), (**Table 1**).

Table 1: Quality indices of virgin oils from cultivars Kalinjot and White Olive of Tirana

Quality indices of organic extra virgin olive oils	Variety "Kalinjot"	Variety "White Tirana"
Free acidity (% oleic acid)	0.35±0.003a	0.2775±0.002a
Peroxide value (mev. O2/kg oil)	4.9±0.8 a	10.4 ±0.6 a
K 232	1.451±0.03 a	1.9±0.025 b
K 270	0.11±0.03 b	0.12±0.01 a
ΔK	0.001	-0.001
Sensory evaluation	8.5	6.8
Total phenols (mg/l caffeic acid)	298±0.2 a	175±0.24 a
Chlorophyll content (mg/kg)	8.90±0.12a	4.75±0.10 a

EVOO EEC, 2003: Free acidity ≤0.8, Peroxide value ≤20,K 232 ≤2.5, K 270 ≤0.22, ΔK ≤0.1
Means in each column with the same letters are not significantly different at 5% of probability by Duncan's multiple range test.

Chlorophyll content, total phenols and insecticides

Chlorophyll level of organic extra virgin oils from the two cultivars showed differences ($P<0.05$). Oils of 'Kalinjot' cultivar had two times more chlorophyll (8.90 mg/kg) than oils produced from other variety 4.75 mg/kg), the difference of total phenols content was distinguished also for Kalinjot variety, (Lerker, G., Cerretani, L 2009) (Singleton V. L,1965) Table 1. These changes are due to different

characteristics of olive varieties and climatic as well as pedological conditions of the areas where they are cultivated (Ryan D, 2003). Insecticides were not valuable in all oil samples produced from two olive varieties, (Tsatsakis A. M. et. al. 2003), that is a confirmation for the organic content of two olive oils, during the three seasons of the study **Table 2.**

Table 2. Insecticides I and VI content (organ phosphorous in mg/kg),

Insecticides I (organ phosphorous) (mg/kg)	Kalinjot	White Olive of Tirana	Insecticides VI (organ phosphorous) (mg/kg)
Azinfos-etil	NV	NV	Acefat
Azinfos-metil (1)	NV	NV	Dimetoato
Bromofos-etil	NV	NV	Etoprofos
Bromfos-metil	NV	NV	Etrimphos
Carbofenothion	NV	NV	Fenthion 1
Clorfenvinfos	NV	NV	Metamidofos
Clorpirifos-etil	NV	NV	Metidathion 1
Clorpirifos-metil	NV	NV	Mevinfos
Diazinon (1)	NV	NV	Monocrotofos
Diclorvos	NV	NV	Ometoato
Disulfoton	NV	NV	Pirimifos-etil
Eptenofos	NV	NV	Pirimifos-metil
Ethion	NV	NV	Triclorofon
Fenamifos	NV	NV	
Fenitrothion	NV	NV	
Fonofos	NV	NV	
Forate	NV	NV	
Formothion	NV	NV	
Fosfamidon	NV	NV	
Parathion-etil	NV	NV	
Paration-metil	NV	NV	
Phosmet	NV	NV	
Insecticides VI	NV	NV	

NV- non valuable

Fatty acid compounds

Fatty acid composition of olive oils produced from two olive varieties was different (**Table 3**). Oleic acid (C18:1) was the main monounsaturated fatty acid, represented high concentrations for both cultivars (70.4%-74.8%). Palmitoleic acid (C16:1) was another monounsaturated fatty acid, with mean values (0.9%) in the oil of 'White olive of Tirana' cultivar and less (0.5 %) in the oil of 'Kalinjot' cultivar. Palmitic acid (C16:0), the major saturated fatty acid in olive oil, had mean values (13.0%) in the oil of 'White Olive of Tirana' cultivar, in comparison with oils of 'Kalinjot' cultivar, in which the mean value was (11.3%) respectively (**Table 3**). Stearic acid (C18:0) which is an important saturated fatty acid in olive oil, varied from 2.1% in Kalinjot variety to 3.2 in White Olive variety. The levels of linoleic acid (C18:2) and linolenic acid (C18:3), which are considered as polyunsaturated fatty acids, have also differences between two cultivars.

Table 3. Fatty acids composition of organic extra virgin olive oils from Kalinjot and White Olive of Tirana

Fatty acid composition (% mm of methyl esters)	Kalinjot variety	White Olive of Tirana variety	IOOC
Myristic acid (C 14:0)	0.01±0.0	0.01±0.0	Max 0.05
Palmitic acid (C 16:0)	11.3±0.2a	13±0.3a	7.5-20.0
Palmitoleic acid (C 16:1)	0.5±0.1b	0.9±0.2a	0.3-3.5
Heptadecanoic acid (C 17:0)	0.1±0.02a	0.1±0.01b	Max 0.3
Heptadecenoic acid (C 17:1)	0.2±0.1a	0.1±0.1a	Max 0.3
Stearic acid (C 18:0)	2.1±0.14a	3.2±0.4b	0.5-5.0
Oleic acid (C 18:1)	74.8±0.6b	70.4±0.3a	55.0-83.0
Linoleic acid (C 18:2)	9.1±0.5b	8.4±0.4a	3.5-21.0
Linolenic acid (C 18:3)	0.5±0.2a	0.3±0.1a	Max 0.9
Arachidic acid (C 20:0)	0.60±0.23a	0.4±0.15a	Max 0.6
Gadoleic acid (C 20:1)	0.3±0.1a	0.2±0.1a	Max 0.4
Behenic acid (C 22:0)	0.1±0.0a	0.1±0.0a	Max 0.2
Lignoceric (C 24:0)	0.1±0.0a	0.1±0.0a	Max 0.2
Saturated fatty acids	14.0±1.2a	15.0±1.3a	
Monounsaturated fatty acids	74.2±2.6a	70.4±1.8a	
Polyunsaturated fatty acids	8.9±2.1a	9.4±1.8b	

IOOC -International Olive Oil Council

Differences also show the content of Linolenic acid (C18:3) and Arachidic acid (C20:0); Kalinjot content is higher than White Olive of Tirana Oil.

The results confirmed that there were significant differences in the characteristics of organic extra virgin olive oils of 'Kalinjot' and 'White olive of Tirana' cultivars from South and Central part of Albania. This study showed that the high content of oleic acid (unsaturated fatty acid) of both cultivars (little bit higher at olive oil of 'Kalinjot' variety) is positively correlated with the stability of olive oil (Hashempour A, et.al 2010).

Levels of fatty acids in the oils of these two cultivars for all samples were in accordance with figures of International Olive Oil Council. Variations observed in fatty acid composition in the olive oil samples were probably related to genetic-varietal factors, climatic and environmental conditions. The color of olive oils (the content of chlorophyll) is an important quality attribute for the consumer acceptability in the market (Crido, Maria et al, 2008) (Roca M., 2001). Olive oil produced from" Kalinjot" has high concentration compare to other variety "White Olive of Tirana"; this because of biosynthetic characteristics or catabolic pathways (Brenes, Rejano et al, 1995) (Cardoso, Guyot, et.al, 2005).

Total phenols are very important for the quality of extra virgin olive oil, because of their involvement in its resistance to oxidation, bitterness and pungency of olive oil taste. The difference of total phenols was high in the oil samples produced from Kalinjot variety (Esti M., et al 1998)

The determination of insecticides is important for the definition of organic olive oils. From the analyses; there were not valuable figures in all oil samples produced from two olive varieties that confirm the organic content of them.

From all results put in evidence, this study evaluated that the quality of organic extra virgin oils produced from "Kalinjot" and "White Olive of Tirana", is very good and consistent during the three years of the production studied.

CONCLUSION

The results of this study showed some differences of qualitative attributes of organic extra virgin olive oils, produced from two regional varieties cultivated in South and Central part of Albania: "Kalinjot" and "White Olive of Tirana". Variations in fatty acid composition, chlorophyll content and phenolic compounds were observed at the olive oil samples, these probably due to genetic factors, climatic conditions, cultural agriculture practices and other environmental conditions. From all analytical parameters determined in this study was showed that the quality of organic extra virgin oils produced from "Kalinjot" and "White Olive of Tirana" is very good for both varieties, but with a small difference regarding sensorial evaluation: nice fruity flavor with a pleasant bitterness, pungency, and astringency; characteristics more, for Kalinjot variety.

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INFLUENCE OF THERMAL TREATMENT ON NUTRITIONAL VALUE OF SEVERAL FROZEN BAKERY PRODUCTS AND SIDE DISHES

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ABSTRACT

Frozen products are convenient and time sparing alternative of classic bakery products and side dishes, requiring only thermal treatment prior to be served. Due to cooking the proportions of nutrients and water content in the product may alter. From the declared nutritional information it is often not clear whether the data are related to raw or thermally treated product. Thirteen samples of frozen food

products were sampled in triplicates. Products were basing on leavened or potato dough, and puff, danish or filo pastry, respectively. All samples were analysed using standard physicochemical methods prior thermal treatment and after the thermal treatment, for which the instructions, labelled by the producer were strictly followed. The contents of water, protein, fat and minerals were determined by physicochemical methods. The content of carbohydrates and the energy values were calculated from analytical data. Due to great diversity of products and cooking methods used, the contents of nutrients in thermally treated samples greatly varied. The water content in products decreased during baking and frying and increased during boiling. Consequently the content of nutrients increased in baked or fried products, with exception of three samples made with puff pastry, where the content of fat was lower after thermal treatment. The energy value of baked and fried samples increased by 4-21%, and the energy value of boiled samples was lower for 17-40% in comparison to raw. Although consumer may not always follow the producer's instructions for preparation of frozen bakery products, it was found relevant that nutrition facts label contains data for thermally treated product as it is ingested.

KEYWORDS: *convenient frozen food products; nutritional value; energy value; thermal treatment; physicochemical analyses*

INTRODUCTION

The life of today's society is strongly denoted by time scarcity, the feeling of not having enough time to do all duties needed or wanted to be done in a day. The time pressure has been implicated in changes in dietary habits, e.g. decrease of time spent for food preparation and consumption at home, increase in consumption of convenience or ready-to eat food products (Jabs and Devine, 2006). The food industry's respond to the modern pace is enlarging assortment of foods that require no or only basic thermal treatment and are easily accessible in food stores. Since these products are in majority pre-packed, their producers often provide nutritional information according to Directive on nutritional labelling of food stuffs (90/496/EEC, 1990), although it is not mandatory at present. The label may include the main four (energy, protein, carbohydrate and fat content) or eight (main 4 plus the content of sugar, saturated fat, fibre and salt/sodium) parameters referred to 100 grams or millilitres of the food. As reported by Hoefkens et al. (2011) the importance of nutrition information in the form of food label is raising, because it helps consumers to choose food beneficial to their health. Namely, the awareness about the health, food choice and intake has increased significantly, although the interest in nutritional information has not spread in the same extent. The most frequent users of the nutritional label tend to be women, parents of children living at home, older consumers and consumers in North and Central Europe (DG SANCO, 2005).

Frozen products are convenient and time sparing alternative of classic bakery products, important components of breakfast or snacks (croissant, pockets with different fillings, burek) and side dishes (gnocchi,

tortellini), requiring only thermal treatment prior to be served. Although they are often associated with the presence of saturated fatty acids or trans fatty acid, their consumption is quite popular, promoted greatly by their palatability (Quílez et al., 2006). It has been observed that consumers prefer frozen products at most due to their longer shelf-life and storage time, higher safety and flexible distribution (Kindt et al., 2006). The cooking of frozen convenient food products may involve wet methods, like boiling in water, or dry methods such as baking in oven, broiling etc. The cooking method applied alters the proportions of macronutrients and water in the food product (Gokoglu et al., 2004).

As discussed already in the first paragraph, nutritional labels are welcome among consumers since they assist choosing products that are more health beneficial. The impacts of the appearance, complexity and other characteristics of the labels on consumers' understanding, education and dietary intake are being thoroughly studied (Flabel, 2011). Nevertheless, the nutritional label bears important information; it is generally not clear whether the values refer to raw or thermally treated food product as intended for consumption. The purpose of the present study was to analytically determine and compare nutritional value of different convenient frozen bakery products before and after thermal treatment proposed by the producer. Labelled values, where available, were supposed to be compared with analytical as well.

MATERIALS AND METHODS

Different types of frozen ready products, available on the market in Slovenia, were included into the study. Products were made with puff, danish or filo pastry, or leavened or potato dough, respectively. In total 13 different samples of frozen food products were sampled in triplicates in different supermarkets. Samples, classified according to the type of dough/pastry are listed in Table 1.

Table 1. Samples of frozen bakery products and thermal treatment applied in their preparation

Sample	Sample description	Type of dough/pastry	Sample code	Thermal treatment applied
1	bends with chocolate-hazelnut filling	danish	1R	-
			1T	oven baking
2	buttery snails with apples	danish	2R	-
			2T	oven baking
3	mini pockets with coconut filling	danish	3R	-
			3T	oven baking
4	pockets with walnut filling	danish	4R	-
			4T	oven baking
5	whole-wheat croissant with diabetic marmalade	danish	5R	-
			5T	oven baking
6	burek with cottage cheese filling	filo	6R	-
			6T	oven baking
7	strudel with sour cherry	filo	7R	-
			7T	oven baking
8	bends with walnut filling	puff	8R	-
			8T	oven baking
9	buckwheat strukljі with walnut filling	leavened	9R	-
			9T	boiling
10	tortellini with spinach	pasta	10R	-
			10T	boiling
11	dumplings with plums	potato dough	11R	-
			11T	boiling
12	potato gnocchi	potato dough	12R	-
			12T	boiling
13	grits gnocchi	potato dough	13R	-
			13T	frying

R: raw subsample; T: thermally treated subsample

Sample preparation

Each sample was divided into two subsamples, one was analysed without thermal treatment (indexed with R), while the other was prepared according to the instructions on the package and then submitted to the analyses (indexed with T). Prior the analyses subsamples were homogenized in a blender, dried at 40°C, milled and stored in glass jars in the dark.

Physicochemical analyses

The contents of fat, protein, water and ash were determined according to the AOAC methods (2002). Kjeldahl method and nitrogen-protein conversion factor 6.25 were used for protein determination, while extraction with petroleum ether after acid digestion of the sample was applied for the analysis of total fat. The water amount was determined gravimetrically using drying at 105°C, and the content of inorganic residue with ashing in a muffle furnace at 550°C. The carbohydrate content was calculated by difference (*100 – water – ash – protein – fat*) and expressed as total carbohydrate (FAO, 2003). The energy value of the samples was calculated as well, using the Atwater general factor system (90/496/EEC, 1990; FAO, 2003).

RESULTS AND DISCUSSION

The samples were very diverse regarding the type of dough/pastry they were prepared with, as well as were

the cooking methods applied in preparation of the samples for the consumption. Therefore the variability of the nutrients content and energy value was expected, as already reported also by other authors (Quílez et al., 2006). Still, it was of great interest to evaluate the extent of the effect of thermal treatment on nutritional value of the selected food products.

The comparison of mean values of analysed (water, ash, protein and total fat content) and calculated (amount of total carbohydrate and energy value) parameters in the raw and cooked subsamples of thirteen bakery products is presented in Table 2.

Table 2. Chemical composition (mean ± SD) of raw and thermally treated samples

Sample code	Nutrient content in g/100 g					Energy value (kJ/100 g)
	Water	Ash	Total fat	Protein	Total carbohy-drate	
1R	26.3±0.8	1.01±0.06	23.7±0.7	6.01±0.19	43.0±1.3	1710±35
1T	20.5±0.6	1.09±0.05	26.8±0.8	6.46±0.21	45.2±1.3	1869±36
2R	46.4±1.3	0.91±0.03	14.4±0.4	4.75±0.14	33.5±1.0	1184±24
2T	40.2±1.2	1.00±0.04	15.2±0.5	5.01±0.20	38.6±1.1	1304±26
3R	32.3±0.9	1.06±0.05	25.0±0.8	5.83±0.27	35.8±1.1	1633±32
3T	23.3±0.6	1.14±0.04	27.1±0.8	6.40±0.29	42.1±1.2	1827±38
4R	34.7±1.1	1.08±0.05	24.8±0.8	7.41±0.35	32.0±0.9	1588±32
4T	28.8±0.5	1.18±0.05	22.9±0.7	8.13±0.39	39.0±1.1	1648±34
5R	35.6±1.0	1.17±0.06	16.6±0.5	6.34±0.29	40.3±1.2	1407±29
5T	27.5±0.9	1.28±0.06	16.2±0.6	7.18±0.35	47.8±1.4	1535±31
6R	57.5±1.6	1.70±0.07	10.6±0.4	11.54±0.28	18.7±0.6	906±21
6T	47.8±1.5	1.53±0.05	12.5±0.4	11.84±0.30	26.2±0.7	1109±26
7R	50.3±1.6	0.97±0.03	12.8±0.4	4.08±0.16	31.9±0.9	1084±23
7T	48.1±1.4	1.04±0.04	13.2±0.4	4.36±0.17	33.3±1.1	1129±26
8R	31.9±1.1	1.11±0.05	40.6±1.2	8.19±0.38	18.2±0.5	1951±39
8T	16.7±0.3	1.29±0.05	39.9±1.2	8.98±0.38	33.1±1.0	2192±44
9R	35.8±0.9	1.15±0.06	20.1±0.6	9.57±0.46	33.4±1.1	1474±29
9T	46.8±1.2	0.89±0.05	15.2±0.5	8.25±0.37	28.9±0.9	1193±25
10R	38.5±1.0	1.45±0.07	5.1±0.2	12.73±0.59	42.2±1.3	1123±23
10T	63.5±1.7	1.03±0.06	4.0±0.2	7.59±0.33	23.9±0.7	683±14
11R	49.5±1.5	1.54±0.06	3.9±0.1	5.69±0.26	40.5±1.2	888±20
11T	57.1±1.7	1.28±0.06	2.8±0.1	5.43±0.25	32.3±0.9	786±16
12R	48.5±1.4	1.60±0.08	6.4±0.2	5.49±0.18	38.0±1.1	976±19
12T	62.5±1.8	1.08±0.06	4.4±0.2	4.47±0.14	27.6±0.8	707±16
13R	54.2±1.4	2.30±0.11	6.6±0.3	9.43±0.25	27.5±0.8	872±18
13T	47.2±1.3	2.64±0.11	9.9±0.3	10.84±0.31	29.4±0.9	1051±23

Raw products contained on average from 4.08 to 12.73 g/100 g protein, from 2.8 to 40.6 g/100 g total fat and from 18.2 to 43.0 g/100 g total carbohydrate. Their energy value ranged from 872 to 1710 kJ per 100 gram of the product.

The variability in nutritional value of thermally treated samples was in similar ranges; however the increase or decrease of nutrient concentrations was influenced by the cooking method applied. The protein content ranged from 4.36 to 11.84 g/100 g, the amount of total fat from 2.8 to 39.9 g/100 g, and total carbohydrate from 23.9 to 47.8 g/100 g. The amounts of macro nutrients: protein, fat and carbohydrate, and water in raw and thermally treated samples were found to be correlated as seen in Figure 1. Moreover, the highest correlation coefficients (r): 0.995 (protein), 0.986 (fat), 0.897 (carbohydrate) and 0.953 (water) among values on raw and cooked samples were found in the products that were baked in the oven.

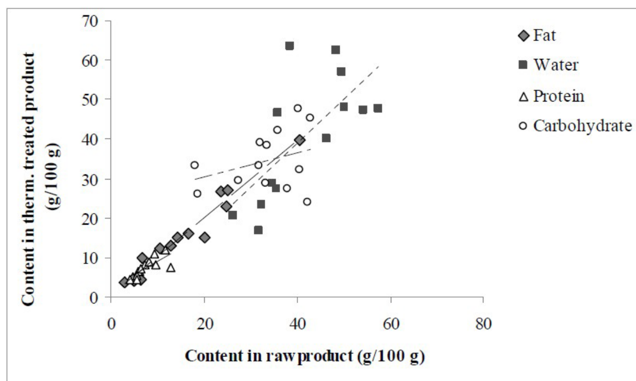

Figure 1. Correlation between the content of nutrients in raw and thermally treated samples.

During dry cooking such as oven baking, the water evaporates affecting the content of dry matter in the food product, which increased for 4.4 to 22.8% in oven baked products and 15.3% in fried product, respectively. Higher content of dry matter, i.e. protein, fat, carbohydrate, influenced also the energy value per 100 gram of baked or fried products, which was from 3.9 to 21.1% higher than in the raw subsamples.

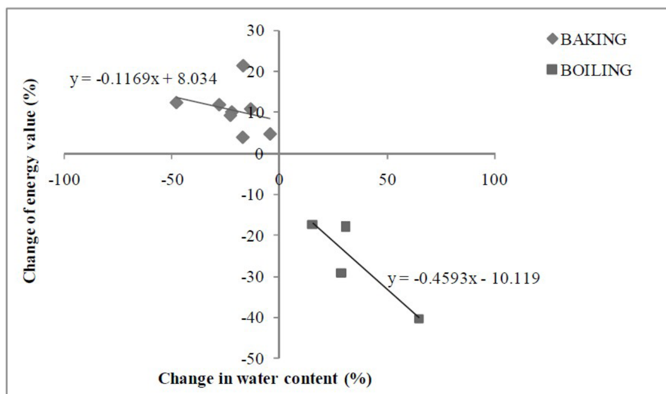

Figure 2. The influence of change in water content on change of energy value in thermally treated sample.

On the contrary, during boiling in water the proportion of the dry matter diminishes on account of water absorption. The content of water increased in boiled samples by 15 to 65%, and the energy value of 100 gram of the product was lower for 13 to 40%. The changes in the water content and in energy value, presented in Figure 2, were found strongly related (correlation coefficient, r=0.891) for the four boiled products, while for baked samples such relation was weak (r=0.275). It was suspected that the increase or decrease in energy value of thermally treated samples is influenced also by the change of the fat content, however the relation, shown in Figure 3, was proved to be strong (r=0.729) in baked and moderate (r=0.382) in boiled samples. This suggests that the energy value of baked or boiled bakery products was not influenced solely by the change of fat content.

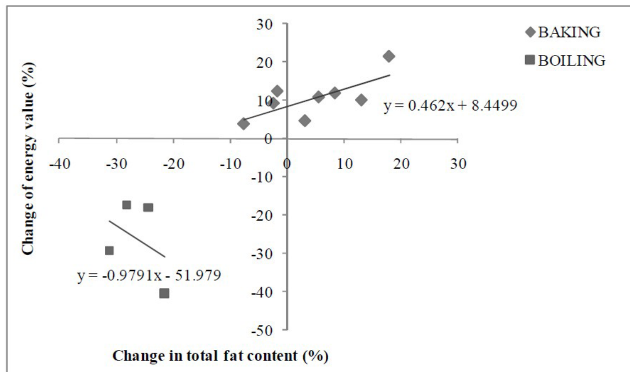

Figure 3. The influence of change in fat content on change of energy value in thermally treated sample.

As already reported (Gokoglu et al., 2004; Murniece et al., 2010), the method of thermal treatment of the samples affected the content of the main nutrients, which increased in baked and fried products, with exception of the three samples: 4T, 5T and 8T, made with puff or danish pastry, respectively, where the content of fat diminished with baking. The change of the fat content influenced by dry cooking method was not found statistically correlated neither to the fat or water content in the raw sample nor to the extent of water loss during baking. The leakage of fat observed in the three samples was contributed to characteristics (defects) of the product rather than cooking method applied, since producers' instructions for the preparation were strictly followed.

Table 3. Differences in analysed and labelled values for water, macronutrients and energy value

Sample	A Water (%)		A Fat (%)		A Protein (%)		A Carbohydrate (%)		A Energy value (%)	
	R-L	T-L	R-L	T-L	R-L	T-L	R-L	T-L	R-L	T-L
6	18.1	-1.8	4.1	22.8	22.0	25.2	-81.3	-74.4	-3.4	17.3
5	-6.8	-28.0	-17.2	-19.2	38.5	56.9	6.1	26.3	0.1	9.3
11	10.2	27.1	32.7	-4.7	26.4	20.6	-27.6	-39.8	-11.7	-27.0
12	-4.4	23.2	51.6	4.2	-9.3	-26.2	-5.9	-34.1	7.5	-23.9
13	-10.7	-22.2	5.5	58.3	15.2	32.4	6.2	6.2	7.2	24.4

L: labelled value; R: value in raw sample; T: value in thermally treated sample

The nutritional value was labelled on five of the analysed samples. Comparison with analytical values showed the greatest discrepancies in the content of fat, followed by the protein and dry matter content. The differences were calculated as the fraction of difference between value of raw (cooked) sample and labelled value with the labelled value (*(R-L)/L*100* and *(T-L)/L*100*). As observed from the Table 3 clear conclusions are difficult to be made, but the tendency of the discrepancies is indicating that the labelled values were most likely referring to the raw product. The source of labelled values was not stated; however they might have been obtained in the analyses or from food composition tables, which would partly explain the variability.

CONCLUSION

For the present mandatory nutrition labelling in the European Union is still under reconciliation; however it was already agreed that eligible nutritional information ought to refer, for better transparency, to 100 grams or millilitres of food and optionally to the portion in the packaging.

Although thermal treatment of frozen convenient products may greatly change the content of particular nutrient, e.g. the fat content in grits gnocchi fried in oil increased by 50%, the state of the food that nutritional label refers to, is deemed irrelevant according to applicable law. It has also been overlooked in the latest Impact assessment report of Commission of the EC, although mandatory nutrition labelling, which is said to become European standard, is meant to assist consumers to make informed dietary choices.

Analysing thirteen convenient frozen food products, which require thermal treatment prior ingestion, and comparing their nutritional value prior and after the cooking, showed that the energy value of Danish pastry products increased by 10% and by 21% in cottage cheese burek with filo pastry. On the other hand the energy of 100 grams of boiled products of potato dough was lower on average by 21%, while that of spinach tortellini made of pasta decreased by 40%. In similar range diminished also the contents of nutrients in the boiled products.

The nutrition labelling on frozen food products that need to be thermally treated bears relevant information only if values refer to the state of the product as it is consumed, i.e. baked, boiled, fried. In opposite case the label ought to include a remark that nutrition value refers to the raw product and might change during cooking.

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PHENOLS IN OLIVE PASTE AS MODULATORS OF VOLATILES FROM LOX PATHWAY IN VIRGIN OLIVE OILS FROM BUŽA CULTIVAR

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ABSTRACT

Biochemical synthesis of virgin olive oil volatile substances during olive processing depends on activity of endogenous enzymes involved in the LOX pathway. Phenols present in olive paste could be one of the inhibitory factors, since it is known that phenolic compounds are able to bind and inhibit various enzymes. Olive paste of Buža cultivar, known to have low phenols content and a relatively high C6 volatiles mass ratio in oil, was fortified with phenolic extract from olive fruits during processing, in order to study the effect on volatile compounds in oil. Oil samples were obtained by a laboratory plant from olive pastes containing two levels of phenols content (real and increased by 20%). Phenols in oils were determined by RP HPLC with UV-DAD detection using tyrosol, apigenin and luteolin (calibration standards) and syringic acid (internal standard). Volatiles were analysed by SPME-GC-MS with 4-methyl-2-pentanol as an internal standard. The increase of total phenols mass ratio in olive paste by 20% has led to a much higher rise of phenols in oil samples (hydroxytyrosol 185%; oleuropein and ligstroside derivatives by 175% and 750%, respectively). Oils from fortified pastes had up to 10 times reduced values of Z-3-hexenal and 2 times of E-2-hexenal, E-2-hexen-1-ol and hexyl acetate. Other substances (hexanal, E-3-hexen-1-ol, Z-3-hexenyl acetate and C5 compounds) showed minor changes in relation to the increased phenols mass ratio in olive paste. A relatively low odour threshold value of Z-3-hexenal, E-2-hexenal and E-2-hexen-1-ol suggests that a radical decrease of their mass fraction in oil should have an evident negative impact on the perception of green odour notes. Results confirm the hypothesis of inhibitory effect of phenols compounds in olive paste on the biosynthesis of the most important volatile compounds of pleasant virgin olive oil aroma.

KEYWORDS: *olive oil; olive paste; phenols; volatiles; Buža*

INTRODUCTION

High quality virgin olive oils (VOO) are characterised by intense and pleasant green and fruity odour notes. Biochemical synthesis of volatile substances responsible for these characteristics mainly occurs during milling of olive fruits (Sánchez-Ortiz et al., 2008; Inarejos-García et al., 2011) and depends on activity of several endogenous enzymes involved in the lipoxygenase (LOX) pathway (Kalua et al., 2007). The first substrates are phospholipids and glycerolipids in membrane structures from which acyl hydrolase detaches polyunsaturated fatty acids. Linoleic and linolenic acid are than oxidised by lipoxygenase and cleaved by hydroperoxide lyase into C6 aldehydes, which can be later reduced to C6 alcohols by alcohol dehydrogenase and transformed to C6 esters by alcohol acyl transferase (Olias et al., 1993).

Although this enzymatic process is in general considerably slowed down during kneading of olive paste, operating conditions of this producing step (time, temperature, oxygen availability) could influence on the concentration and composition of VOO volatile compounds (Sánchez-Ortiz et al.,

2008; Angerosa et al., 2001; Servili et al., 2003). Some authors (Sánchez-Ortiz et al., 2008; Padilla et al., 2009; Padilla et al., 2010) have hypothesised that one of the inhibitory factors in this step could be the phenols and their oxidized products present in olive paste. Numerous reports describe the

inhibition of various enzymes by various phenolic compounds, attributed both to the redox properties and the ability of phenols to directly bind to target proteins (Quideau et al., 2011). From this aspect, the most studied among mentioned enzymes were lipoxygenases from different natural sources, such as soybean, rabbit reticulocytes (Sadik et al., 2003) or fish muscle (Banerjee, 2006). Since lipoxygenases contain an iron moiety at the active site, phenolic inhibitors can act by complexing ferric iron or by reducing an active ferric (Fe^{3+}) form to an inactive ferrous form (Fe^{2+}). Furthermore, phenols in oxidized forms (quinones, semiquinones or phenoxy radicals) may inhibit enzymes by binding to their amino or sulfhydryl groups. The main phenol oxidizing agents in olive paste are free radicals (possible intermediates of lipoxygenase activity), as well as endogenous polyphenol oxidase and peroxidase (Garcia-Rodrigez et al., 2011). As regards other olive enzymes involved in the LOX pathway, there is no study related to their inhibition by phenolic compounds, although Olias et al. (1993) have observed that in crude extracts prepared from fresh olive fruits, from which only a part of phenolics was removed, alcohol dehydrogenase and alcohol acetyl transferase activity was inhibited.

Based on these assumptions, olive cultivar Buža, known to have the low phenols content and a relatively high C6 volatiles mass ratio in oil (Brkić Bubola, 2011; Koprivnjak et al., 2003; Koprivnjak, 1996) was chosen in order to verify the relation between the increased phenols content in oil paste and biosynthesis of volatile compounds from the LOX pathway.

MATERIALS AND METHODS

Olive fruits and preparation of virgin olive oil samples

Olive fruits of cultivar Buža, grown in the Istria region (Croatia) were handpicked in the middle of October 2008. The laboratory plant for oil extraction consisted of the hammer crusher, vertical olive paste mixers placed in the thermostated water bath and centrifuge (MC2 Ingenieria y Sistemas, Spain). Immediately after milling, an appropriate volume of aqueous solution of phenolic compounds was added to olive paste, while the same volume of water was added to the control sample. Olive pastes were malaxed for 45 min at 26±0.5 °C. After centrifugation at 3600 rpm for 70 sec, extracted oils were stored at room temperature in fully filled and taped dark bottles until analyses. Two independent preparation procedures of each of the two cases (with and without addition of phenolic compounds in olive paste) were carried out.

Preparation of aqueous solution of phenolic compounds

Aqueous solution of phenolic compounds was obtained from fresh de-stoned olive pulp of cultivar Istarska bjelica. The pulp was homogenised with two portions of methanol (mass/volume ratio = 1:5 at each portion) using an Ultraturrax homogenizer (5 min at 1100 rpm). After 30 min of settling in the darkness, supernatant layers were filtered through filter paper and methanol was eliminated under reduced pressure at 40 °C until dryness (viscous consistency). The residue was immediately dissolved in water, and the concentration of total phenols in the obtained aqueous solution was determined colorimetrically.

Colorimetric determination of total phenols concentration in olive paste and aqueous solution

Olive paste was homogenised with methanol (mass/volume ratio = 1:10) using an Ultraturrax homogenizer (5 min at 1100 rpm). After 30 min of solids deposition in the darkness, the suspension was filtered through filter paper. An aliquot of the obtained methanolic extract, or of aqueous solution obtained from olive pulp, was used for colorimetric reaction with Folin-Ciocalteau reagent (Merck KGaA, Darmstadt, Germany), according to the procedure described in our previous work (Koprivnjak et al., 2010). The total phenols concentration was determined from a caffeic acid (Panreac, Barcelona, Spain) calibration curve, and expressed in mg/kg, as caffeic acid equivalents.

Determination of phenols composition in olive oil samples

Phenolic compounds were extracted from olive oil and determined by RP HPLC with UV- DAD detection using tyrosol, apigenin and luteolin as calibration standards and syringic acid as an internal standard, according to a slightly modified method published by International Olive Council (2009).

Analysis of volatile compounds

For the SPME sampling procedure, the conditions tested by Vichi et al. (2003) were used. A Shimadzu 2010 gas chromatograph equipped with a quadrupole mass detector Shimadzu QP2010 and Supelco equity 5 capillary column 60 m × 0.25 mm ID, 1 µm film thickness (Sigma-Aldrich, MI, Italy), was used. Column temperature was held at 40 °C for 10 min, increased to 220 °C at 5 °C/min and to 260 °C at 15 °C/min and held for 10 min. The injector temperature was 260 °C and the time of desorption of the fiber was fixed at 1 min. The carrier gas was helium at a linear velocity of 30 cm/s. The temperatures of ion source and transfer line were 200 °C and 260 °C, respectively. Volatile compounds were quantified using the relative response factor of 4-methyl-2-pentanol and are expressed as mg/kg of oil. Each sample was analysed in two parallel repetitions.

Statistical analysis

Differences among samples were tested by a one-way analysis of variance at 5% significance level. The homogeneity of variance was tested by the Brown–Forsythe test. The mean values were compared by the Tukey's honest significant difference test ($p \le 0.05$). Statistical analyses were performed using the software package Statistica 9.

RESULTS AND DISCUSSION

For the purpose of this research, the choice of Buža cultivar was motivated by its usually low content of total phenols (Koprivnjak, 1996; Škevin et al., 2004). According to habitual harvesting practice in case of this cultivar, fresh fruits were handpicked at the early stage of ripeness (most of the fruits had yellowish epidermis with light violet spots). Total phenols content in the control sample of Buža olive paste, determined colorimetrically, was 7.74 g/kg. In order to dispose of sufficient amount of phenolic compounds required for olive paste fortification, fresh de-stoned olive pulp of Istarska bjelica cultivar, known to have a high total phenols content, was used to obtain aqueous solution of phenolic compounds. In fortified Buža olive paste samples, the total phenols content was increased by 20%.

Table 1. Mass fraction (mg/kg)* of phenolic compounds in olive oil samples determined by HPLC method

Phenolic compounds	Control sample	Fortified sample**	Increase (%)***
Tyr-OH****	4.4	12.4	185
DMO-Agl-dA	51.8	77.4	49
O-Agl-dA	7.5	8.5	13
O-Agl-A	25.0	68.6	175
Tyr	9.3	12.4	33
DML-Agl-dA	50.2	53.1	6
L-Agl-dA	11.1	16.9	53
L-Agl-A	6.7	57.1	752
Luteolin	3.8	4.6	21
Apigenin	1.0	1.4	40
Methyl-luteolin	0.3	0.3	0
Vanillic+caffeic acid	3.8	4.3	13
Lignans	13.2	24.1	83
Total phenolic compounds	**350.0**	**560.5**	**60**

* results are means of two independent repetitions
**total phenols content in olive paste was increased by 20% at the beginning of malaxation;
***increase in fortified sample compared to control
****full names in ABBREVIATIONS

Mass fractions of single phenolic compounds and total phenols determined chromatographically in olive oils, obtained after malaxation and subsequent centrifugation of olive paste are shown in Table 1. The control sample of olive oil contained 350 mg/kg of total phenols that is a relatively high value for Buža cultivar when compared to available literature data, ranging from 95 to 450 mg/kg (Koprivnjak, 1996; Škevin et al., 2003). Increase of the total phenols content in olive paste by 20% with respect to the control sample has led to a much higher rise in oil samples (by 60%).

Phenolic compounds in olive fruits are mainly present in glycosidic form and are highly hydrophilic substances. However, during olive fruit processing phenolic glycosides are exposed to the action of endogenous glycosidase and esterase that gives rise to less hydrophilic i.e. more lipophilic aglycones and phenolic alcohols (Garcia-Rodrigez et al., 2011). Furthermore, similar enzymatic transformations of phenolic glycosides could take place during preparation of aqueous solution of phenolic compounds intended for olive paste enrichment. This fact could explain a much higher rise of total phenols content in oil samples obtained from fortified olive paste than it would be expected on the basis of the level of olive paste enrichment.

In the oil sample obtained from fortified olive paste, single phenolic compounds contributed differently to the increase of total phenols content. The highest increase compared to the control sample (by 752%) was evidenced for aldehydic form of ligstroside aglycone (L-Agl-A), then for phenolic alcohol hydroxytyrosol (by 185%) and O- Agl-A (by 175%). Consequently, the profile of phenolic fraction in oil changed quantitatively and this could be attributed to a different phenolic and enzymatic pattern of Istarska bjelica fruits, used as the source of phenolic compounds for olive paste fortification.

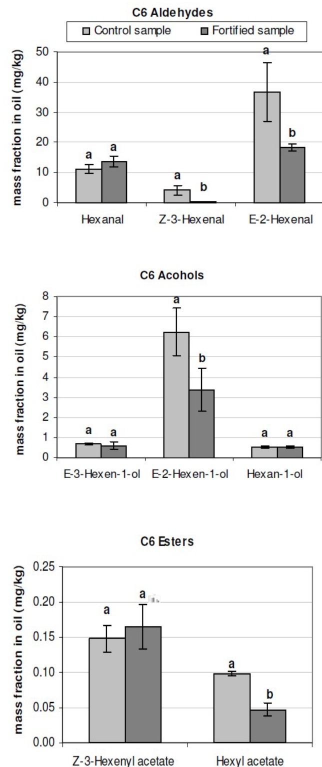

Figure 1. Mass fraction of C6 volatile compounds in olive oil samples. Results are means of four values (2 independent repetitions × duplicate analyses) ± SD; the means labelled by different letters are significantly different (Tukey's test, $p<0.05$). Fortified sample = total phenols content in olive paste was increased by 20% at the beginning of malaxation.

When C6 volatile substances are considered (Figure 1.), it could be said that the control oil sample showed a typical profile of high quality virgin olive oil, with E-2-hexenal as the predominant compound. C5 compounds, deriving from an additional branch of the LOX pathway, were present in about ten times lower mass fraction (Figure 2.). The first volatile products in the LOX pathway are Z-3-hexenal (originated from α- linolenic acid) and hexanal (originated from linoleic acid). Increase of total phenols in olive paste by 20% at the beginning of malaxation caused a decrease of Z-3-hexenal by 90%. This reduction could be the consequence of hydroperoxide lyase, lipoxygenase or acyl hydrolase inhibition by phenolic compounds

added to olive paste. On the other side, it seems that this is not confirmed for hexanal, since no statistically significant differences between control and fortified samples were found for this compound. However, it must be stressed that hexanal could also arise from chemical oxidation of linoleic acid and, in these circumstances, a decrease of hexanal caused by the inhibition of enzymes could be masked. Although there were no changes in the mass fraction of hexan-1-ol (product of alcohol dehydrogenase action on hexanal), it is noteworthy that the level of hexyl acetate (the final C6 product of linoleic acid

Similarly to hexanal, Z-3-hexenal could be transformed into Z-3-hexen-1-ol and Z-3-hexenyl acetate, but no statistically significant differences were found as the consequence of increased total phenols content in olive paste. The main branch of the LOX pathway leads to the transformation of Z-

3-hexenal into E-2-hexenal (by isomerase) and E-2-hexen-1-ol (by alcohol dehydrogenase). Mass fractions of both of these unsaturated C6 products were reduced by approximately 50% in the oil sample obtained from fortified olive paste. C5 compounds (Figure 2.) showed minor changes in relation to the increased phenols mass ratio in olive paste. A relatively low odour threshold value of Z-

3-hexenal (0.003 mg/kg), E-2-hexenal (0.420 mg/kg) and E-2-hexen-1-ol (5 mg/kg) (Luna et al., 2003) suggests that a radical decrease of their mass fraction in oil should have an evident negative impact on the perception of green odour notes.

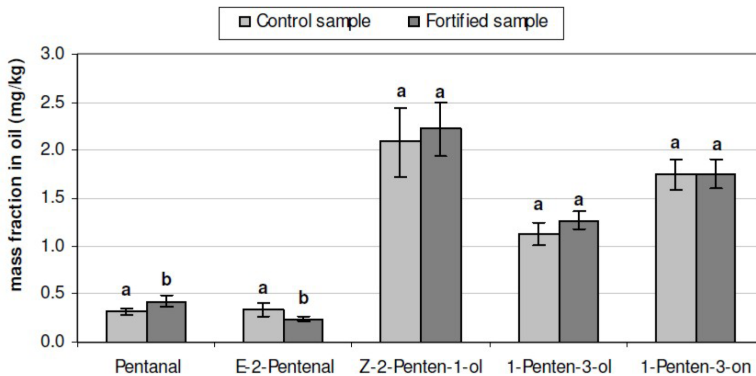

Figure 2. Mass fraction of C5 volatile compounds in olive oil samples. Results are means of four values (2 independent repetitions × duplicate analyses) ± SD; the means labelled by different letters are significantly different (Tukey's test, $p<0.05$). Fortified sample = total phenols content in olive paste was increased by 20% at the beginning of malaxation.

CONCLUSION

These results lead to the conclusion that phenolic compounds in olive paste could have an inhibitory effect on the biosynthesis of the most important volatile compounds of pleasant virgin olive oil aroma. Further research in this topic may offer interesting possibilities for targeted modulation of virgin olive oil sensory characteristics during olive processing.

ABBREVIATIONS

Tyr-OH = hydroxytirosol; DMO-Agl-dA = dialdehydic form of decarboxymethyloleuropein aglycone; O-Agl- dA = dialdehydic form of oleuropein aglycone; O-Agl-A = aldehydic and hydroxy form of oleuropein aglycone; Tyr = tyrosol; DML-Agl-dA = dialdehydic form of decarboxymethylligstroside aglycone; L-Agl-dA = dialdehydic form of ligstroside aglycone; L-Agl-A aldehydic and hydroxy form of ligstroside aglycone.

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EXPERIMENTAL RESEARCH REGARDING THE ANALYSIS OF TERPENES IN FORAGES BY HEADSPACE SOLID-PHASE MICROEXTRACTION COUPLED TO GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

The aim of this work was to analyze biochemical markers (like terpenes compounds) in forages using solid-phase microextraction (SPME) combined with gas chromatography-mass spectrometry (GC-MS) technique. There were studied 4 different types of pasture from Bucegi area, identified as plot D, C, A and T. The first three one consisted in natural fertilized meadows, while the last one was a sub-alpine pasture which underwent a rational use for 30 years. The forage samples were stored at –20°C until analysis. The samples were brought to ambient temperature and cut up roughly with scissors, the extraction being performed using tert-buthyl-methyl ether as solvent.

The headspace volatile compounds in forage samples were isolated by polydimethylsiloxane /divinylbenzene fiber, separated by GC, and identified by MS. The analysis of the 4 pastures samples by SPME/GC-MS technique showed differences among the samples. Plot T was characterized by very rich terpenes profile compared with the others. Among the terpenes identified, we mention: pinene, limonene, terpinene, terpinolene, caryophyllene. Terpenes were detected in the Selected Ion Mode by monitoring their characteristic ions at m/z 93, 136. The other samples contain other types of volatile compounds, like coumarin, butandiol, benzyl carbinol. The SPME/GC-MS method used in this work allows the identification of terpenes in forage. These compounds can be used as markers for traceability purposes.

KEYWORDS: *terpenes; gas chromatography; mass spectrometry; SPME; forage*

INTRODUCTION

Terpenes are a class of volatile compounds produced by a large number of plant species from their secondary metabolism (Pare, Tumlinson, 1999; Dillard, German, 2000; Cornu et al., 2001). They are lipophilic aliphatic compounds, of general formula $(C_5H_8)_n$. Terpenes are abundant in certain plant families, especially dicotyledons (herbs, heather, bushes and trees), while they are poor in monocotyledons (Mariaca et al., 1997). In addition, other environmental factors, such as: plant species, growth stage, soil, climate changes, geographical origin, grassland management, influence the terpenes profile and their concentration in plants. Terpenes can be transferred from herbs or forages eaten by animals into dairy products (Viallon et al., 2000), thus, these compounds have been proposed by several researchers as biochemical markers (Bugaud et al., 2001; Cornu et al., 2005), helping to characterize products made from milk obtained from cows fed forages with different composition and from different geographical areas (Viallon et al., 1999). Feeding cows with highland grass (rich in dicotyledones) during mountain pasture leads to higher concentrations of volatile compounds, as described in literature for different cheese types (Sehovic, 1999). Numerous reviews papers reported the effects of different floristic composition of pastures on milk and cheese flavour (Martin et al., 2005; Guichard et al., 2006; de Noni, Battelli, 2008; Martin et al., 2009; Chion et al., 2010). This information may be useful for recognizing a typical highland cheese (e.g. protected denomination of origin) from a lowland cheese. The detection of biochemical markers for identification of the geographical origin of food

is an attractive challenge. Several studies have been conducted on the detection of markers that could be used to identify the geographical origin of milk and cheese. Terpenes have been shown to be useful markers for the origin of dairy products (Viallon et al., 2000) particularly useful for the differentiation of highland and lowland cheeses (Bugaud et al., 2001). Analytical methods described for volatile compound identification are: headspace technique linked to a GC/MSD/FID system (gas chromatography / mass spectrometry / flame ionization detector) (Mariaca et al., 1997), steam distillation of milk and plant extraction (extractions made with diethyl ether) and analyzed with GC/MSD/FID) (Lombardi et al., 2008), Fourier transform infrared spectroscopy (FTIR) may be a good supplement to mass spectra detect volatile compounds (Carpino, 2008). The gas chromatography-mass spectrometry (GC-MS) technique is a suitable tool for analyzing volatile compounds, including terpenes. Solid-phase microextractin (SPME), which utilizes a very economical sampling procedure, has been applied in headspace analysis; its sensitivity for terpenes has been reported in literature (Schafer et al., 1995; Miller et al., 1996; Czerwinski et al., 1996). The aim of this work was to analyze biochemical markers (like terpenes compounds) in forages from Bucegi area (Romania) using SPME combined with GC-MS technique. In this research study, the influence of four different type of pasture on volatile compounds (terpenes profile) is investigated.

MATERIALS AND METHODS

Reagents

All solvents were of analytical grade. – Pinene (purity > 95% for GC), limonene (purity > 97% for GC), tert-buthyl-methyl ether, TBME, (purity > 99.8% for GC) were purchased from Merck. Standards solutions of terpenes were prepared in TBME. Helium was of 99.999% purity.

Apparatus and Operating conditions

A gas-chromatograph (GC 7890 A, Agilent Technologies, Santa Clara, CA, USA) equipped with an HP 5 capillary column (30 m long, 0.25 mm i.d., 0.25 mm film thickness) was used. The chromatograph was coupled with a mass spectrometer (MS 5975 C with Triple-Axis Detector, Agilent Technologies, Santa Clara, CA, USA) operating under electron impact mode (70eV). The samples were injected through an automatic auto-sampler CombiPAL (CTC Analytics) and the whole analytical procedure was controlled with the program ChemStation (Agilent Technologies, Santa Clara, CA, USA). The operating conditions were: injection port temperature, 230°C; carrier gas, helium with a flow rate of 0.8 mL/min; splitless mode with fiber; splitless time, 2 min; temperature program: oven temperature was initial maintained at 50°C for 4 min, then increased up to 150°C by 7°C/min, followed by 10°C/min to 240°C. The MS temperatures adopted were: source 230°C, quadrupole 150°C. The SPME fiber used to collect volatile fraction was polydimethylsiloxane / divinylbenzene (PDMS/DVB) 65 mm (StableFlex Supelco). The fiber was conditioned at 280°C for 30 min in GC injector before analysis. A single fiber was used for this study.

Sample preparation

The samples were collected from an improved subalpine pasture located at 1800 m altitude, on a low slop terrain in the Research Base Blana Bucegi and provided by the Institute of Research and Development for Grassland Brasov, Romania. The sampling was made in the second decade of August 2010 when grasses were in flower. Samples were collected using three metal cages of 2 m2; the amount of sample was around 200 g. The pasture samples have been packed in sealed plastic bags and preserved by freezing at –20°C until analysis. Before analysis, the forage samples were brought to ambient temperature and 0.5 g cut up roughly with scissors, were weighed in a 20 mL vial. An organic solvent (TBME) was added to the sample for performing the extraction. The vials were sealed with an aluminum cap provided with a pierceable septum. The performance of extraction was evaluated in order to allow the terpenes identification. The best results were obtained for direct extraction compared with extraction performed on the shaker for 12 h, followed by SPME analysis. The vial sample was allowed to equilibrate, before the SPME fiber is inserted, at 80°C for 20

min, and then the fiber was exposed to the headspace for 15 min, performing the extraction of volatiles from pasture. Blanks with fiber after GC-MS sample analysis were performed in order to indicate the absence of any compound in the fiber or column. Chromatograms were recorded in the Selected Ion Mode detection by monitoring terpenes characteristic fragments (m/z 93, 136). Identification of terpenes was done by comparing retention times with those of the pure standards. The identification was also performed by comparing the mass spectra with those of the pure standards or those stored in the National Institute of Standards and Technology library (NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library version 2.0, USA).

RESULTS AND DISCUSSION

Four experimental variants of plots considered in this study, together with the floristic composition presented in the subalpines pasture for each plot determined by Klapp-Elemberg method (Marusca et al., 2008) are reported in Table 1. The analysis of volatile fraction of the 4 forages (plot D, C, A and T) studied by SPME/GC-MS analysis showed differences among the samples. This can be attributed to the fact that plots D, C and A were consisted in natural fertilized pastures, while plot T was a subalpine pasture which underwent a rational use for 30 years. The chromatographic profile for forage samples from plot D, C, A, mainly showed the presence of other types of volatile compounds, like coumarin, butandiol, benzyl carbinol.

Plot T was characterized by very rich terpenes profile compared with the others, mainly due to the presence in higher percentage of Ligusticum mutellina species. The chromatographic profile corresponding to the analysis carried out on the forage from plot T is presented in Figure 1, the compounds identified being presented in Table 2.

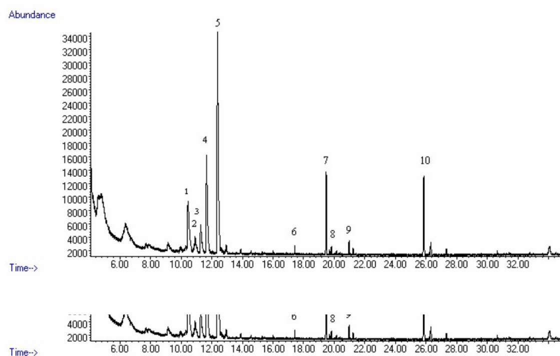

Figure 1. Chromatographic profile of forage from plot T obtained by solid-phase microextraction-gas chromatography-mass spectrometry (peaks are numbered as in Table 2).

Table 1. Floristic composition on plots from which samples were collected (%).

Species	Plot D sown pasture, limed, chemical and organic fertilized	Plot C natural pasture, limed, chemical and organic fertilized	Plot A natural pasture, chemical fertilized	Plot T natural pasture and rational utilization
Grasses:	**81**	**66**	**79**	**77**
Nardus stricta	.	+	4	14
Festuca nigrescens	5	17	27	18
Festuca ovina	.	+	.	2
Agrostis rupestris	4	4	5	9
Agrostis capillaris	39	14	30	12
Poa media	2	6	2	8
Poa pratensis	10	19	.	7
Poa annua	.	+	.	.
Deschampsia caespitosa	.	2	1	.
Deschampsia flexuosa	.	+	4	1
Anthoxanthum odoratum	.	+	.	.
Phleum alpinum	2	4	6	5
Dactilis glomerata	4	.	.	.
Fectuca pratensis	5	.	.	.
Phleum pratense	10	.	.	1
Leguminous plants	9	13	10	11
Trifolium repens	8	13	10	11
Trifolium hybridum	1	.	.	.
Lotus corniculatus	.	.	.	.
Other families	**10**	**21**	**11**	**12**
Potentilla aurea chrys.	1	2	1	4
Ligusticum mutellina	.	2	1	5
Viola declinata	.	.	+	.
Campanula napuligera	.	.	1	.
Geum montanum	1	1	1	1
Ranunculus montanus	3	3	1	1
Hieracium aurantiacum	.	.	1	.
Alchemilla vulgaris	4	2	+	+
Polygonum bistorta	+	11	5	+
Alchillea sp.	.	1	+	.
Taraxacum sp.	1	1	.	1
Cerastium. Sp.	+	.	.	.
Veronica sp.	.	.	+	.

+ / . : presence / absence of species

Table 2. Compounds corresponding to peaks shown in Figure 1.

Peak	Compound	Retention time, min
1	α – Pinene	10.4
2	Limonene	10.9
3	Benzeneacetaldehyde	11.3
4	Terpinene	11.6
5	Terpinolene	12.3
6	3’,4’,5,7 – Tetramethoxy flavone	17.4
7	Caryophyllene	19.4
8	Coumarin	19.8
9	5,7 – Diethyl-5,6-decadien-3-yne	20.9
10	Apiol	25.8

As an example, Figure 2 shows the fragmentation pattern of terpinolene in forage from plot T and that of the NIST library. These results indicate that terpenes (α – pinene, limonene, terpinene, terpinolene, caryophyllene) could be used as biochemical markers.

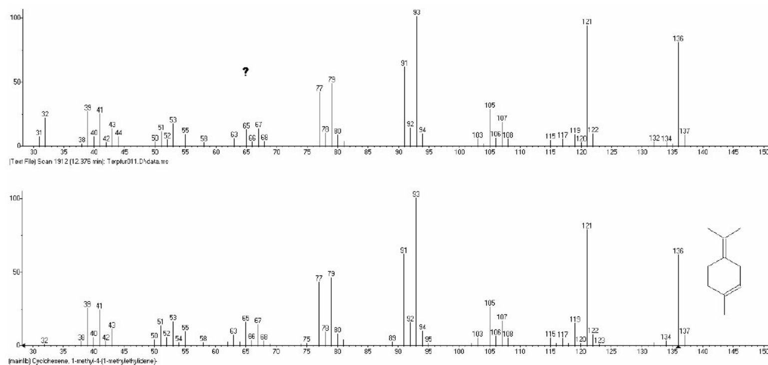

Figure 2. Mass fragmentation pattern of terpinolene in the forage sample, plot T (upper graphic) and of library reference (lower graphic).

CONCLUSION

The SPME/GC-MS method used in this work allows the identification of terpenes in subalpine forage from a sub-alpine pasture which underwent a rational use for 30 years (plot T from Bucegi area). These compounds can be used as markers for traceability purposes. This paper is part of an ongoing research project, which aim is to find a correlation between the terpenes profile contained in cow pastures and that from the corresponding dairy products.

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THE COMPARISON OF QUALITY PARAMETERS OF CONVENTIONALLY AND ORGANICALLY GROWN POTATOES

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ABSTRACT

Numerous consumers purchase organic foods because they believe that they are more nutritious than conventional foods. This claim, however, is difficult to support by the data available. Some of published data had show that organic potatoes had higher amount of starch, dry matter (DM), ascorbic and chlorogenic acid content. Therefore the aim of the research was to compare the quality parameters of organically and conventionally grown potatoes in Latvia. The experiments were carried out at the Faculty of Food Technology, Latvia University of Agriculture (LUA), and State Priekuli Plant Breeding Institute. The content of vitamin C (ascorbic acid), carotenoids, DM, starch and the colour in CIE L*a*b* system of six fresh potato genotypes – 'Imanta', Agrie dzeltenie', 'Prelma', 'S 03135-10', 'S 99108-8', and 'S 01063-5' were analyzed. All potato genotypes were grown both conventionally and organically (biologically) in one and the same location under the control of the State Priekuli Plant Breeding Institute. Results show that there were no significant differences in the carotenoid and starch contents, and the colour values L*, a* and b* between the conventional and organic genotype samples ($p>0.05$). The potato genotype 'S 01063-5' contained the highest amount of carotenoid in both growing systems. The vitamin C content was significantly higher ($p<0.05$) in the conventionally grown potatoes where considerable influence of genotype was observed. The highest content of starch and vitamin C was detected in the genotype 'Imanta'. In the research it was found that the organic potatoes did not show higher quality parameters than conventional ones.

*K*EYWORDS: *potato, genotypes, conventional, organic, vitamin C, carotenoids*

INTRODUCTION

The nutritional value of potato along with its taste and ease of cooking has made it the most popular vegetable in the world. Therefore the potato quality parameters are very important for each consumer. Potato is rich in potassium and also contains calcium, iron, and phosphorus. It is well known that potatoes are an excellent source of vitamin C. Murniece et al. (2011) have found that in the Latvian potato varieties it varies in average from 9.45 to 15.85 mg 100 g-1. While Burgos et al., 2009 has found that vitamin C concentration in freshly harvested raw, peeled tubers ranged from 6.5 to 36.9 mg 100 g- 1 on FW and from 22.2 to 121.4 mg 100 g-1 on a dry weight basis (DW). In potatoes vitamins A, B and P can be found as well (Han et al., 2004; Mishra, 1985; Mullin et al., 1991; Nordbotten et al., 2000).Potato is one of the best natural sources of starch and in Latvian varieties it ranges from 12.84 to 19.68%. The dry matter (DM) content of Latvian varieties varies from 18.92 to 25.59% (Murniece et al., 2011). Significant variation due to genotype, environment and genotype×environment (G×E) interaction was found. Numerous consumers purchase organic food because they believe that it is more nutritious than conventional food. This claim, however, is difficult to support by the data available. The dana presented by Hajslova et al., (2005) show that organic potatoes had higher amount of starch, DM, ascorbic and chlorogenic acid content. Also some other authors established that organic crops to conventional ones have higher amount of vitamin C, and different micronutrients, e.g. iron, calcium and phosphorus (Worthington, 1999). It should be noted that there are very few studies on conventionally and organically cultivated potato genotypes in Latvia. Therefore the aim of the research was to compare the quality parameters of organically and conventionally grown potatoes in Latvia.

MATERIALS AND METHODS

The experiments were carried out at the Faculty of Food Technology, Latvia University of Agriculture (LUA), and State Priekuli Plant Breeding Institute. In the research the potatoes were analysed right after harvesting keeping them for a week at temperature +5±1 °C, at the relative humidity of the air in the cellar 80±5%. The storage temperature was low, therefore, the potatoes before they were analysed, were stored at room temperature 20±3 °C for one week to promote reconditioning of carbohydrates in the potatoes, thus diminishing the reducing sugar content in the potatoes (Kazunori et al., 2003). Many researchers (Burton, van Es and Hartmans, 1992; Oltmans and Novy, 2002; Rastovski and van Es, 1987) report that up to 80% of the reducing sugars are converted back to starch during the reconditioning process. The content of vitamin C (ascorbic acid), carotenoids, DM, starch and the colour in CIE L*a*b* system of six fresh potato genotypes - 'Imanta', Agrie dzeltenie', 'Prelma', 'S 03135-10', 'S 99108-8', and 'S 01063-5' were analyzed. All potato genotypes were grown both conventionally and organically (biologically). Analyses of the peeled potatoes were done after two weeks of harvesting. The colour spectrophotometer (ColorTec PCM/PSM) was used to obtain the absorption spectra from which L*a*b* values were calculated. The measurements of the colour were repeated ten times and recorded as mean values.

The content of ascorbic acid was determined by titration with 0.05-M iodine solution (Jansons, E. 2006). Potatoes (25 g) were poured with 6-% solution of oxalic acid and homogenized. Then the sample was filtered. 2 ml of 1-% solution of starch was added to 10 ml of filtrate and the filtrate was titrated until the colour changed which does not disappeared during a 30 sec interval. For standard solution of ascorbic acid 20 mg of ascorbic acid were dissolved in 100 ml of the oxalic acid solution. Two ml of the starch solution was added to 25 ml of the standard-solution and the mixture was titrated. The content of vitamin C (ascorbic acid) mg per 100g of fresh weight of potatoes was calculated with the following equation [1]:

$$C = 5000 \cdot \frac{V_{sample}}{m \cdot V_{standard}}, \qquad [1]$$

where V_{sample} – volume of the iodine solution titrated in a sample, ml;
$V_{standard}$ – volume of the iodine solution titrated in a standard solution, ml.
m – the amount of sample, g

Each tuber's specific gravity, starch content and dry matter was measured using the weight in airweight

in water method (Smith, 1975). The starch content and dry mater content in tubers were determined indirectly via specific gravity as percentage of fresh weight. Carotenoids were analyzed by spectrophotometric method (with the UV/VIS spectrophotometer Jenway 6705) at 440 nm (Ермакова, А.И., 1987). A sample of 2g of homogenized potatoes was placed in 100 ml conic retort and 20 ml 96% ethanol was added. The sample was stirred on magnetic stirrer for 15 min then 25 ml of petrol ether was added and continued to stir for one hour. After 3–4 hours when both layers were completely divided, the top yellow layer was used for further detection of carotenoids at 440 nm. Carotene equivalent (KE) was found, using a graduation curve with K2Cr2O7. The content of carotenoids (mg 100g-1) was calculated by equation 2:

$$X = \frac{12.5 \cdot 100 \cdot KE}{36 \cdot a}, \qquad [2]$$

where 12.5 and 36 coefficients for relationship between K2Cr2O7 and carotenoids;

KE – carotene equivalent by graduation curve;

For data analysis descriptive statistics and ANOVA were performed, which was used to test significance of influence of growing system and genotype, and storage time. Software SPSS 11.0 and Excel were used for data analysis.

RESULTS AND DISCUSSION

There were no significant differences in the carotenoid, DM and starch contents, and the colour values L*, a* and b* between the conventional and biological potato samples (p>0.05). The potato genotype 'S 01063-5' contained the highest amount of carotenoid in both growing systems (in average 0.16±0.011 mg 100 g-1) (Fig. 1).

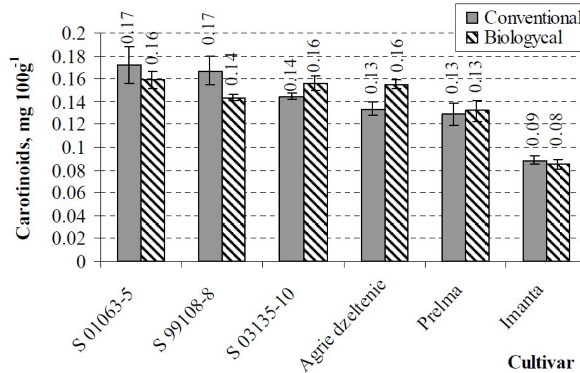

Figure 1. Total carotenoids mg 100g-1 of fresh weight (FW)

The vitamin C content was significantly higher (p<0.05) in the conventionally grown potatoes where considerable influence of genotype was observed (Table 1; Fig. 2). These results were opposite of the data given in literature (Hajslova et al., 2005; Worthington V., 1999). But as there is very little information about the vegetables grown in both growing systems in Latvia than it could be connected with some growing peculiarities in our region.

Table 1. Distribution of potato genotypes according to ascorbic acid content with the criteria of Duncan

Cultivar	N	Duncan Subset for alpha =0.05			
		1	2	3	4
S 99108-8	8	22.7762			
S 03135-10	8		25.8662		
S 01063-5	8		26.4175	26.4175	
Agrie Dzeltenie	8			29.1275	29.1275
Imanta	8				30.2075
Prelma	8				30.2087
Sig.		1.000	0.684	0.050	0.454

Means for groups in homogeneous subsets are displayed. The genotypes in one column are not significantly different.

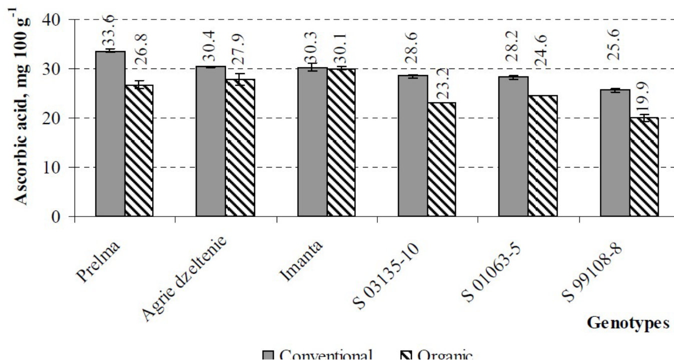

Figure 2. Vitamin C content mg 100g-1 of fresh weight (FW)

The highest content of vitamin C was detected in the conventionally grown potatoes of the genotype 'Prelma', and in the organically grown potatoes of the genotype 'Imanta' (Fig. 2). However there were not significant differences between organically and conventionally grown potatoes in DM and starch content, there was a tendency that some organically grown genotypes ('Prelma', 'S99108-8') had lower DM and starch content than in conventionally grown ones (Fig. 3 and 4).

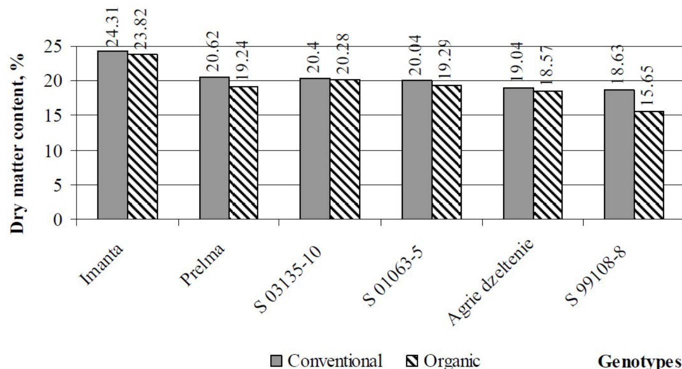

Figure 3. Dry matter content, %

The lower DM content and higher water content in organically grown potatoes also partially explain their lower vitamin C content. The genotype with the highest DM and starch content in both growing systems was 'Imanta' (with average 24.06% of DM, 17.00% of starch). Due to the very hot and rainy summer the potatoes of all evaluated genotypes contained a comparably low content of starch.

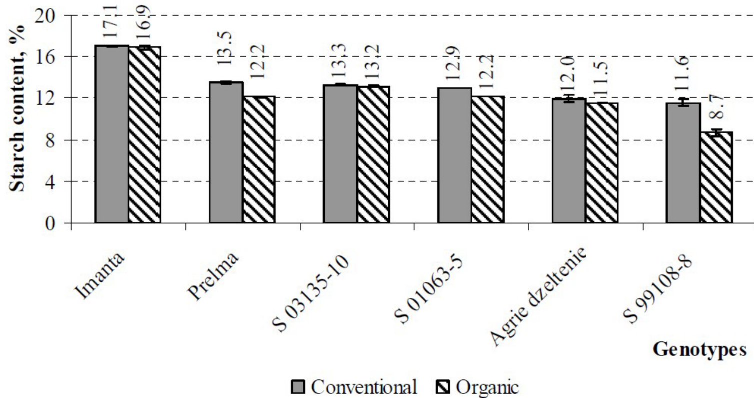

Figure 4. Starch content, % of fresh weight (FW)

There were no significant differences between different potato genotypes in measured colour parameters L* (in average 68.9) and a* (in average -4.58), but the genotype 'Prelma' and 'Imanta' had significantly lower b* value or yellow ($p<0.05$) (Fig. 5).

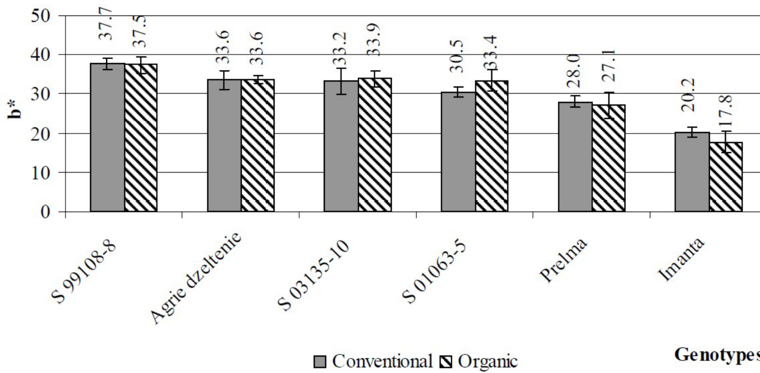

Figure 5. The b* value (in CIE Lab colour system) in potato genotypes

CONCLUSIONS

In the research it was found that the organic potatoes did not show higher quality parameters than conventional ones. There were no significant differences in the carotenoid, DM and starch contents, and the colour values L*, a* and b* in both growing systems. The vitamin C content was significantly higher ($p<0.05$) in the conventionally grown potatoes where considerable influence of genotype was observed. So the question remains are organically grown products better than conventionally grown ones.

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COMPARISON OF RACTOPAMINE AND CLENBUTEROL ACCUMULATION IN PIGS AS MEAT PRODUCTION ANIMALS

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ABSTRACT

Ractopamine and clenbuterol are β-adrenergic agonists that increase the rate of weight gain and feed conversion.

If used in meat production, they promote muscle growth of treated animals by increasing nitrogen retention, protein synthesis and lipolysis, and suppressing lipogenesis. They can be illegally used during fattening of farm animals, thus implying a risk to consumers of meat and meat products. For residue surveillance of both substances, it is essential to choose appropriate matrices and use high sensitivity analytical methods in the analysis of suspect tissues.

The aim of the study was to compare residue levels and accumulation potential of ractopamine and clenbuterol in pig tissues, liver and kidney (n=24), after subchronic repeat administration of clenbuterol and 10-fold dose of ractopamine. Ractopamine and clenbuterol residues in both matrices were determined using validated enzyme-

linked immunosorbent assay (ELISA) as a quantitative screening method.

Method validation showed good mean recoveries (*R* >70%) with acceptable inter- and intra-day relative standard deviations (RSD <10%) for both analytes and matrices. Immediately after the last dose of β-agonists, the maximal determined ractopamine concentration in the kidney (42.44 ng/g) was 2-fold that measured in the liver (20.66 ng/g) and was comparable with maximal concentration of clenbuterol in the liver (47.43 ng/g). Considering the dose difference, study results pointed to higher accumulation of clenbuterol residues in both matrices, suggesting the liver to have a significantly higher accumulation potential for clenbuterol residues and kidney for ractopamine residues. The results also suggested these tissues to be appropriate matrices to detect ractopamine and clenbuterol abuse in meat production.

KEYWORDS: *ractopamine; clenbuterol; accumulation; tissues of pigs; meat production*

INTRODUCTION

Beta-adrenergic agonists ractopamine and clenbuterol are substances with anabolic effect that cause an increased protein deposition in skeletal muscle due to enhanced protein synthesis and reduced protein degradation. Apart from their effect on protein metabolism, these substances also modify fat metabolism by inducing lipolytic processes in adipose tissue and improving food utilization (1). In European Union, clenbuterol is licensed as a bronchospasmolytic and tocolytic agent in veterinary medicine with therapeutic dose of 0.8 μg/kg body weight. If used in dosages 5-10 times higher than the therapeutic one, it provokes anabolic effects (2,3). As a repartitioning agent, clenbuterol causes reduction of body fat and promotes muscle growth, and can therefore be abused during fattening of food producing animals to yield greater muscle mass and thus higher gain in meat industry (4,5). In various animal species, the administration of clenbuterol leads to the accumulation of its residues and presence in meat products. Therefore, the use of clenbuterol in anabolic dosage is associated with a potential risk for consumer health and maximum residue limit (MRL) for clenbuterol has been set at

0.5 ng/g for liver tissue. There are a number of well documented cases where the illegal use of clenbuterol resulted in human food poisoning (6,7). Performance improvements associated with ractopamine feeding in pigs are affected by several factors, including but not limited to nutrient concentrations of the diet, dietary ractopamine concentration and duration of feeding. Literature data show that ractopamine feed concentrations of 5 to 20 mg/kg result in significant improvements in carcass characteristics (8,9). Because of food poisoning caused by clenbuterol residues, ractopamine has not been approved for use in animal feeds in Europe.

In the Republic of Croatia, ban has been placed on the use of β-agonists as anabolics, regulated by the government Decree on Prohibited Use of β-Agonists and Substances with Hormonal and Thyrostatic Effects in Farm Animals (Official Gazette 112/2008) (10), with strict control of their potential abuse through the national program of residue monitoring.

The high potency of growth promoting effects and rapid metabolism of β-agonists require highly sensitive and validated analytical methods for quantification and accumulation of data on their residues in appropriate biological material to detect their abuse in food-producing animals.

The aim of the study was to compare residue levels and accumulation potential of ractopamine and clenbuterol in pig tissues, liver and kidney, after subchronic repeat administration of clenbuterol and

10-fold dose of ractopamine. The concentrations of residual ractopamine and clenbuterol were determined using validated enzyme-linked immunosorbent assay (ELISA) as a quantitative screening method.

MATERIALS AND METHODS

The experiment was carried out in 6 male pigs, Zegers hybrid type, body mass 55-60 kg. Three pigs were treated with a daily dose of 10 mg ractopamine hydrochloride and 1 mg clenbuterol hydrochloride for 28 days each. Ractopamine and clenbuterol were administered as pure chemicals in the form of capsules admixed to feed. All animals were sacrificed immediately after receiving the last dose. Liver and kidney samples of 6 treated animals were collected, divided into two portions and stored at -20 ^{O}C until analysis for residual ractopamine and clenbuterol. The experimental protocol was designed according to the Act on Animal Welfare, as stated in the Official Gazette of the Republic of Croatia (11).

Liver and kidney samples (n=24) of experimental animals were minced and ground to a fine mass with Ultraturax, then analyzed in duplicate for ractopamine and clenbuterol concentrations. Sample preparation, clean up and ELISA test procedures for quantitative determination of ractopamine and clenbuterol were performed as described in package inserts provided by the kit manufacturer (R- Biopharm, Darmstadt, Germany). Standards of ractopamine and clenbuterol hydrochloride used for animal treatment and method validation were provided by Sigma-Aldrich-Chemie (Steinheim, Germany). All other chemicals used in the analysis were of analytical grade. ELISA was performed by use of ELx800TM microplate reader and ELx50 washer (BIO-TEK Instruments, Inc., Vermont, USA). On method validation, the method recovery was determined by spiking kidney and liver samples at the levels of 5 and 10 ng/g with clenbuterol and ractopamine (3 replicates *per* concentration level) and calculated from six-point calibration curves. On reproducibility assessment, the same steps were repeated twice with the same analysis conditions.

RESULTS AND DISCUSSION

This study compared the residue levels, i.e. accumulation potential of ractopamine and clenbuterol in pig tissues, liver and kidney, after subchronic repeat administration of clenbuterol and 10-fold dose of ractopamine. Residual tissue concentrations were determined by use of the validated ELISA as a quantitative screening method.

Results of method validation, determined by use of blank liver and kidney samples as reference materials spiked at the levels of 5 and 10 ng/g clenbuterol and ractopamine, respectively, are shown in Table 1.

Table 1. Determination of recovery and reproducibility in liver and kidney samples fortified with ractopamine and clenbuterol (n=6)

Analytical parameter	Matrices	Spiked content (ng/g)	Recovery (%)	RSD (%)	Reproducibility (%)	RSD (%)
Ractopamine	liver	5	82.3	4.6	80.1	7.2
		10	86.4	3.3	82.7	7.3
	kidney	5	76.8	4.7	75.4	8.5
		10	75.3	3.4	74.1	6.1
Clenbuterol	liver	5	82.4	3.8	80.3	7.4
		10	87.6	4.9	83.6	9.1
	kidney	5	78.3	4.1	76.7	7.2
		10	76.1	3.7	74.9	5.8

Mean recoveries ranged from 74.1% to 86.4% with inter- and intra-day relative standard deviations (RSD) ranging from 3.3% to 9.1%. Validation results demonstrated the efficiency of sample preparation and determination of ractopamine and clenbuterol in liver and kidney tissues using ELISA as a quantitative method. Therefore, both methods can be used for screening in laboratory monitoring of these substances. The mean recoveries were taken into account on calculating ractopamine and clenbuterol contents in all study samples.

Results of ractopamine and clenbuterol concentrations in kidney and liver samples of treated animals are shown in Figure 1 and Figure 2, respectively.

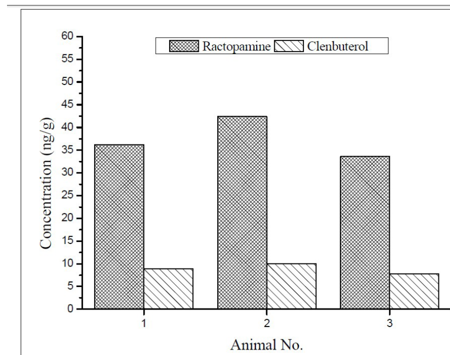

Figure 1. Kidney concentrations of ractopamine and clenbuterol

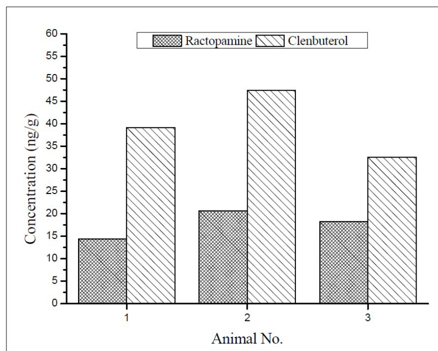

Figure 2. Liver concentrations of ractopamine and clenbuterol

Determined concentrations showed ractopamine and clenbuterol residues to accumulate in liver and kidney tissues. The mean clenbuterol concentration in the liver was 39.72 ng/g and in the kidney 8.89 ng/g. A higher mean concentration of ractopamine was determined in the kidney (37.42 ng/g) than in the liver (17.74 ng/g). The maximal clenbuterol concentration was recorded in the liver (47.43 ng/g) with about 4 times lower concentration in the kidney (10.05 ng/g). The maximal determined ractopamine concentration in the kidney (42.44 ng/g) was 2-fold that measured in the liver (20.66 ng/g) and was comparable with maximal concentration of clenbuterol in the liver. Data on the much greater kidney ractopamine residues as compared with liver residues suggest that kidney is an appropriate target tissue for routine monitoring of ractopamine illegal use. In addition, the present study revealed the liver to have a higher accumulation potential for clenbuterol in comparison to kidney. Taking into account the dose used in study animals, subchronic repeat administration of clenbuterol and 10-fold dose of ractopamine showed a significantly higher clenbuterol accumulation potential in visceral tissues than ractopamine. The results of our study indicated a higher kidney and liver accumulation potential for ractopamine and clenbuterol, respectively, pointing to these tissues as useful matrices in the control of their abuse as growth promotants in food production.

CONCLUSION

Considering dose difference, study results pointed to a higher accumulation of clenbuterol residues in both matrices, suggesting the liver to have a significantly higher accumulation potential for clenbuterol residues and kidney for ractopamine residues. The results also suggested these tissues to be appropriate matrices to detect ractopamine and clenbuterol abuse in meat production.

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FUSARIUM GRAMINEARUM AND *F. CULMORUM* CHEMOTYPES AS POTENTIAL PRODUCERS OF MYCOTOXINS IN DURUM WHEAT, GROWN IN SOME ITALIAN

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ABSTRACT

Durum wheat cultivation is economically of great importance for pasta production. In Italy Fusarium

Head Blight (FHB) is considered the most important fungal disease on wheat, especially in the center-northern regions, and *Fusarium graminearum* and *F. culmorum* are the main pathogens. They cause serious damages in yields and quality of grains in particular for mycotoxin contamination, such as deoxynivalenol (DON). DON is strictly regulated in Europe for levels in unprocessed durum wheat and pasta by CE n. 856/2005 and updated n.1126/2007 regulation. In this work, a monitoring has been performed in some Italian regions to determine the distribution of *F. graminearum* and *F. culmorum* strains, potential producers of acetylated forms of DON such as 3-acetyldeoxynivalenol (3-ADON) and

15-acetyldeoxynivalenol (15-ADON) and nivalenol (NIV). *F. graminearum* and *F. culmorum* colonies, isolated from durum wheat kernels, were morphologically identified and confirmed molecularly using specific primers. Each strain was assigned to one of the following trichothecene chemotype profiles: 3- ADON, 15-ADON and NIV by PCR. All the three chemotypes were present in the *F. graminearum* population considered in this study; in particular 15-ADON was dominated. Almost the totality of *F. culmorum* strains belonged to 3-ADON chemotype, while 15-ADON was not found. The knowledge of the distribution of *F. graminearum* and *F. culmorum* chemotypes is quite important to predict a possible durum wheat grains contamination by DON and to evaluate possible contamination risks in processed food.

KEYWORDS: *DON chemotype; Fusarium gramineraum; Fusarium culmorum; durum wheat; kernels.*

INTRODUCTION

Fusarium Head Blight (FHB) of wheat is worldwide fungal disease and in Italy strong attacks have been recorded from 1995 to date (Pancaldi *et al*., 1996). The disease is caused by several *Fusarium* species such as *F. graminearum* Schwabe, *F. culmorum* (W. G. Smith) Sacc. and *F. poae* (Peck) Wollenweb. (Parry *et al*., 1995; Pisi *et al*., 2008). In Emilia-Romagna, a region of northern Italy, from 1995 to 2007, the frequency data about the findings of *Fusarium* species from durum wheat (*Triticum turgidum* var. *durum* Desf.) confirmed that *F. graminearum* and *F. culmorum* are still the most common species causing FHB (Pancaldi *et* al., 2010). These two pathogens produce type B trichothecenes (Bottalico and Perrone,

2002) that include deoxynivalenol (DON) and its acetylated forms 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON), as well as nivalenol (NIV) and its acetylated form 4- acetylnivalenol or fusarenone-X (FUS). DON inhibits DNA, RNA and protein synthesis, it is responsible of haemorrhagic and anorexic syndromes, neurotoxic and immunotoxic effects in mammals (Bottalico and Perrone, 2002). Maximum allowed limits for mycotoxin levels in food have been established for the protection of the consumer. For instance, limits for DON are strictly regulated in the EU (CE n. 856/2005 and updated n.1126/2007). F. graminearum and F. culmorum populations can be divided into two chemotypes based on the production of the 8-ketotrichothecenes, DON and NIV. DON-producing isolates can be further distinguished on the basis of the predominant acetyl DON derivative that they produce, 3-ADON or 15-ADON (Miller et al., 1991). Although a slight difference exists between these compounds in the pattern of

hydroxylation and acetylation, the biological activities of these compounds can be different, indicating that chemotype differences may have important health consequences (Kimura et al., 1998). In the recent years, in several wheat growing Italian areas, DON has been constantly found in FHB infected kernels of durum and bread wheat and DON levels in some durum wheat kernels samples exceeded the legal limits (Pascale et al., 2010). DON is typically concentrated in the bran coat and its consistent reduction has been observed during each of the processing steps, from grains to cooked pasta (Campagna et al., 2005; Pascale et al., 2010). The knowledge on the distribution of Fusarium chemotypes is considered effective to predict a possible mycotoxin contamination risk (Jennings et al., 2004; Pasquali et al., 2010). The aim of this study was to map the chemotype presence of F. graminearum and F. culmorum in different regions of Italy during investigations conducted from 2006 to 2010 in durum wheat fields, naturally FHB infected.

MATERIALS AND METHODS

Mycological analyses were carried out on durum wheat kernels, collected from FHB infected fields, following the methodology described by Pancaldi *et al.* (2010). In total 147 samples of kernels were examined, in not homogeneous number for Italian regions (Table 1 and Figure 1). The single spore cultures of *Fusarium* genus obtained (Balmas *et al.,* 2000b) were identified as *F. graminearum* and *F. culmorum* according to the morphological criteria proposed by Leslie and Summerell (2006). The DNA of each strain was extracted using a CTAB (hexadecyl-trimethyl- ammonium bromide) method adapted from Lhodi *et al.* (1994) and subjected to PCR reactions to confirm that these strains belonged to *F. graminearum* and *F. culmorum* using specific primers (Nicholson *et al.*, 1998; Mishra *et al.,* 2003). The *F. graminearum* chemotype was assigned with a multiplex version (Starkey *et al.*, 2007) of a chemotype specific test, previously validated by Ward *et al.* (2002). Primers are designed in the region of genes *Tri*12, located in the terminal gene cluster of trichothecenes and can distinguish three subgroups, depending on the type of β- trichothecenes product. One primer is common to all chemotypes (12CON) and the others are chemotype-specific for 15-ADON (12-15F), 3-ADON (12-3F) and NIV (12NF) (Starkey *et al.,* 2007). *F. culmorum* was characterized for chemotype based on the presence of gene for monoacetylated DON derivatives (3-ADON, 15-ADON) and nivalenol (NIV) in the gene cluster *Tri3* and *Tri7* (Quarta *et al.*, 2005). PCR was carried out using the protocol reported by Prodi *et al.* (2009).

RESULTS AND DISCUSSION

Fusarium isolates (n=214), coming from durum wheat kernel samples, were selected: 157 were identified as F. graminearum and 57 as F. culmorum using traditional identification techniques and all were confirmed by species-specific PCR assays. The number of strains of each species isolated from different regions is reported in Table 1 as well as the numbers of the three chemotypes found. F. gramineraum was isolated in all the regions except Basilicata. All the three chemotypes were present in the F. graminearum population considered in this study. The most frequently isolated chemotype was 15-ADON (n =124 of 157 - 79.0%), followed by NIV (n=17 of 157 - 10.8%) and 3-ADON (n=16 of 157 – 10.2%) (Figure 2).

15-ADON producing isolates were found in all Italian regions but Sicilia, where only one strain was assayed (3-ADON). NIV-producing isolates were more frequent in Umbria (n=8 of 17) and Marche (n=5 of 17). F. culmorum was isolated from kernels coming from six Italian regions (Table 1). In F. culmorum, the 3-ADON chemotype prevailed (n=55 of 57 - 96.5%), while 15-ADON was not found and NIV chemotype was present only in two regions (Emilia-Romagna and Toscana) with value at 3.5% (Figure 2).

Figure 1. Map of Italy.

Table 1. Strains of *Fusarium graminearum* (Fg) and *F. culmorum* (Fc) isolated from wheat kernels collected in samples from different Italian regions and characterized in chemotypes.

Regions	No. samples	No. strains	No. chemotypes			No. strains	No. chemotypes		
		Fg	3-ADON	15-ADON	NIV	Fc	3-ADON	15-ADON	NIV
Basilicata	2	-	-	-	-	2	2	-	-
Campania	1	1	-	1	-	-	-	-	-
Emilia-Romagna	52	60	2	57	1	11	10	-	1
Lazio	4	9	3	4	2	1	1	-	-
Lombardia	4	5	2	2	1	-	-	-	-
Marche	25	38	2	31	5	4	4	-	-
Piemonte	2	2	-	2	-	-	-	-	-
Sardegna	4	4	-	4	-	-	-	-	-
Sicilia	1	1	1	-	-	-	-	-	-
Toscana	25	7	3	4	-	36	35	-	1
Umbria	24	27	3	16	8	3	3	-	-
Veneto	3	3	-	3	-	-	-	-	-
No. total	147	157	16	124	17	57	55	0	2

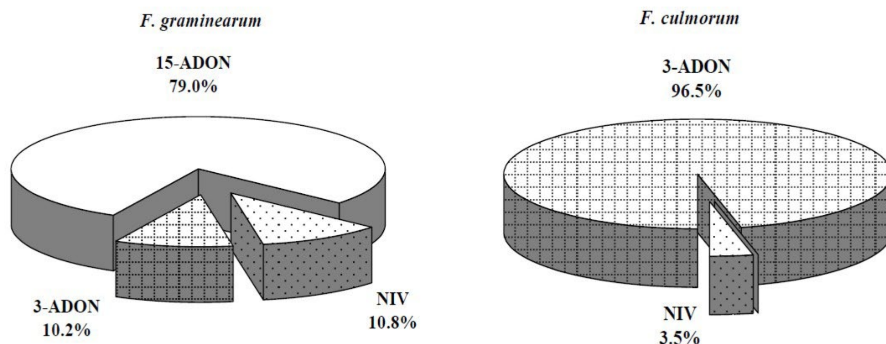

Figure 2. Frequencies of F. graminearum and F. culmorum chemotypes.

The data on 15-ADON predominance in *F. graminearum* population are according with those found in different European countries: England and Wales (Jennings *et al*., 2004), southern Russia (Yli-Mattila *et al*., 2008), a restricted area of Italy (Prodi *et al*., 2009) and Luxemburg (Pasquali *et al*., 2010) as well as in different USA areas (Gale *et al*., 2007). In contrast the isolates of *F. graminearum* recovered in Finland and northwestern Russia were exclusively 3-ADON producers (Yli-Mattila *et al*., 2009). The results obtained on *F. culmorum* are also similar to those reported in Europe: in particular in Norway, Germany, Denmark, Poland and Hungary all DON chemotypes were shown to produce 3-ADON (Langseth *et al*.,

1999; Muthomi *et al.,* 2000; Nielsen *et al*., 2001; Toth *et al*., 2004; Stepien et al., 2008), in England 3- ADON chemotype is dominating and it is present along NIV chemotype (Jennings *et al*., 2004), in Luxemburg, these two chemotypes are present in 53.2% and 46.8%, respectively (Pasquali *et al.,* 2010). In France the situation is different in fact both 3-ADON (21.6%) and 15-ADON chemotypes (8.3%) have been characterized (Bakan *et al*., 2002) as well as in USA (Mirocha *et al*., 1994) and in Japan (Sugiura *et al*., 1990).

CONCLUSION

Our data on chemotype frequency of *F. gramineraum* and *F. culmorum,* isolated from durum wheat kernels, are comparable with those reported by several authors in the world, identifying *F. graminearum* 15-ADON chemotype dominated, and in *F. culmorum*, the 3-ADON chemotype prevailed. The number of samples analyzed for each region was not homogenous however the results obtained evidenced the potentially risk of grain contamination for the presence of 15-ADON and 3-ADON chemotypes in all the regions assayed. Reminding that pasta is at the base of the Mediterranean diet, the knowledge of wheat cultivation areas at mycotoxin risk for *Fusarium* presence could be useful to protect the health of consumers.

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CAN THE GEOGRAPHICAL ORIGIN OF FLORAL HONEYS BE DETERMINED? COMPARISON OF VOLATILE PROFILES OF FLORAL HONEYS FROM BOSNIA AND HERZEGOVINA

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ABSTRACT

The volatile profile is one of the typical features of honey. The volatile's composition is specific for each type of honey and closely related to the geographical region of production. The identification of the volatile compounds is done with the aim to find marker compounds useful for the quality and authenticity control of the honeys. The studied floral honey samples were coming from two different geographical regions: Una-Sana Canton and Neretva Canton, both situated in Bosnia and Herzegovina. In order to characterize the volatile fraction of the floral honeys, Head Space-Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry and Head Space Solid Phase Micro Extraction-Gas Chromatography-Flame Ionization Detector analysis were performed. The results show a high number of volatile compounds. The compound's mean values range between 0.1-10.0 percent. Some of the compounds present in higher percentage are: benzaldehyde, n-octane, benzeneacetaldehyde, linalool, p-cymenene, n-nonanal and 1-nonanol. Unfortunately individual single volatile compounds can not be used as geographical markers, but the two groups of samples present characteristic volatile profiles., A canonical discriminant function analysis (CDA) was applied to volatile compositions of honeys from Una-Sana Canton and Neretva Canton. The results show a statistically significant discrimination between the floral honeys of the two regions. The CDA function allowed also a good classification of unknown samples (cross-validated). The difference between the locations may be due to particular geographical and botanical characteristics as well as climatic conditions. The high classification percentage indicates that the floral honeys of different geographical origin can be differentiated on the basis of their volatile profiles. Therefore, analyzing volatile profiles of honeys in combination with statistical evaluations like CDA is a good quality assurance tool enabling authenticity control.

KEYWORDS: *floral honey; volatile profiles; geographical markers; HS-SPME-GC-MS; CDA*

INTRODUCTION

The aroma profile is one of the most typical features of a food product, for both organoleptic quality and authenticity. Owing to the high number of volatile components, the aroma profile represents a "fingerprint" of the product, which could be used to determine its origin. Aroma compounds are present in honey as complex mixture of volatile components of different functionality and relatively low molecular weight (Cuevas-Glory et al, 2007). In general, the volatile composition of honey is a complex mixture of various compounds belonging to different chemical families: benzene derivatives, norisoprenoids, hydrocarbons, aldehydes, ketones, organic acids, phenolic compounds, terpenes and their derivatives (Pontes, Marques, Câmara, 2007; Guidotti, Vitali, 1998). Some of the volatile compounds are oxidation products, occurring during ripening. (Rowland, Blackman, D'Arcy and Rintoul, 1995) Several authors proposed specific volatile compounds as botanical marker, like for example: 3,9-epoxy-1-p-menthadiene for lime tree (Castro-Vázquez, Díaz-Maroto and Pérez-Coello, 2007), methyl anthranilate for citrus honey (Castro-Vázquez, et al., 2009), 1-phenylethanol for chestnut honey (Piasenzotto, Gracco and Conte, 2003; Bonaga and Giumanini, 1986), 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone for eucalyptus honey (de la Fuente, Valencia-Barrera, Martínez-Castro, Sanz, 2007). The volatile profile is one of the typical features of honey. The volatile's composition is specific for each type of honey and closely related to the geographical region of

production. The identification of the volatile compounds is done with the aim to find marker compounds useful for the quality and authenticity control of the honeys.

MATERIALS AND METHODS

The studied floral honey samples were coming from two different geographical regions: Una-Sana Canton and Neretva Canton, both situated in Bosnia and Herzegovina. In order to characterize the volatile fraction of the floral honeys, Head Space-Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) and Head Space Solid Phase Micro Extraction-Gas Chromatography-Flame Ionization Detector (HS-SPME-GC-FID) analysis were performed. The extraction step was performed as follow: 1g honey dissolved in 0.5ml water. SPME by using a fibre 50/30μm DVB/CAR/PDMS. The samples were thermostated on 50°C for 20min, absorption time 30min on 50°C, desorption time 10min. The gas chromatography analysis were carried out as follow: GC-MS, HP 6890 coupled with a HP 5972 MSD and fitted with a fused silica capillary column 30m x 0.25mm i.d., coated with DB-5MS as stationary phase, df 0.25μm. It was carried out applying the following experimental conditions: oven temperature from 40°C with 5°C/min to 230°C then with 20°C/min to 320°C, injection temperature 250°C, carrier gas (Helium) flow rate 1mL/min, split 2:1. GC-FID, Agilent 6890 fitted with a DB-5 narrow bore column (10m x 0.1mm i.d.; 0.1μm film thickness). The analytical conditions were: helium as carrier gas (average velocity 45cm/s), injector temperature 250°C, split ratio 5:1; temperature programme from 60°C with 8°C/min to 160°C then with 30°C/min to 280°C. The identification was performed with the retention Indices (RI) of the compounds that were determined on the basis of homologous n-alkane hydrocarbons under the same conditions. The compounds were identified by comparing their retention indices and the mass spectra to published data. A canonical discriminant function analysis (CDA) performed with SPSS 15.0 was applied to volatile compositions.

RESULTS AND DISCUSSION

The investigated floral honey samples are coming from Bosnia and Herzegovina. The samples from Bosnia were collected in 2008 in the Una-Sana Canton, while the ones from Herzegovina were collected in 2007 in the Neretva Canton. As far as the author is aware, no information is available regarding the volatile composition of the investigated floral honeys. Table: 1 shows the volatile compounds that were detected in the headspace of the samples. The honeys from the two Cantons present similar volatile composition with slight differences. The samples from Una-Sana Canton present some terpenes more: cis-carveol, carvotanacetone and carvacrol than the samples from Neretva Canton. On the other hand these last honeys show in their volatile fraction 3.7 % heptanol and 3.4 % benzyl nitrile that are absent in the ones from Una-Sana. A canonical discriminant function analysis (CDA) was applied to the volatile profiles to distinguish floral honeys from Una-Sana Canton and Neretva Canton. The graphical representation of the CDA (Fig. 1) shows a gap between the floral honeys from the two regions. Although from the observation of table 1 the volatile profiles of the samples are similar, the success classification (cross-validated) rate of unknown samples is 88 % for the samples from Una-Sana Canton and 64 % for the honeys from Neretva Canton. The GLM multivariate analysis indicates the volatiles that influence the differentiation between the Cantons. Table: 2 shows the compounds that significantly contribute to the classification of the samples. The differentiation between the two groups is based mainly on volatiles originating during ripening. The honeys from Una-Sana Canton present a higher percentage of volatiles that give floral notes to the honey than the ones from Neretva Canton. Moreover the last honeys present a considerable percentage of furfural that can arise from heat processing or inappropriate storage (Alissandrakis et al., 2007). In conclusion, the floral honeys samples from both Cantons present typical volatile profiles due to the different geographical and climatic characteristics.

Table: 1 Identification of volatile compounds present in the headspace of the floral honey samples

(ordered with increasing LRI)

				Area % (mean value)	
Compound	**LRI**	**ID**	**References**	**Una-Sana Canton**	**Neretva Canton**
toluene	766	a	1	0.4%	nd
1-octene	789	a	1	0.4%	0.7%
n-octane	799	a	1	2.0%	5.1%
furfural	834	a	1	1.0%	5.2%
2-methyl-butanoic acid	837	a	2	1.4%	nd
3-methyl-butanoic acid	848	a	2	1.3%	nd
n-nonane	900	a	1	0.7%	1.2%
1-(2-furanyl)-ethanone	909	a	3	1.0%	4.1%
3-methyl-pentanoic acid	943	b		nd	3.4%
benzaldehyde	965	a	1	9.0%	13.6%
1-heptanol	972	a	1	nd	3.7%
1-octen-3-ol	981	a	1	0.7%	1.4%
3-octanone	987	a	1	0.9%	nd
unknown	991			1.1%	nd
2-octanol	996	a	1	0.7%	1.2%
n-octanal	1005	a	1	1.0%	0.8%
4-terpineol	1010	a	1	4.4%	nd
1,3,5-trimethyl-benzene	1015	b		0.3%	nd
α-terpinene	1018	a	1	0.3%	0.4%
p-cymene	1027	a	1	1.8%	0.7%
limonene	1029	a	1	nd	0.5%
benzenemethanol	1038	a	4	1.7%	0.7%
benzeneacetaldehyde	1047	a	1	9.2%	13.1%
1-phenyl ethanol	1057	a	1	1.1%	nd
γ-terpinene	1061	a	1	0.7%	1.0%
acetophenone	1063	a	1	1.2%	nd
trans-linalool oxide	1073	a	1	3.1%	2.9%
cis-linalool oxide	1088	a	1	1.1%	1.7%
p-cymenene	1091	a	5	8.4%	2.9%
linalool	1099	a	1	1.1%	6.2%
HO-trienol+n-nonanal	1103	a	1	8.4%	4.5%
cis rose oxide	1110	a	1	0.6%	nd
2-phenyl ethyl alcohol	1113	a	1	1.8%	2.0%

isophorone	1123	a	1	0.4%	2.6%
N-ethyl-benzenamine	1130	a	6	1.5%	1.4%
benzyl nitrile	1141	a	7	nd	3.4%
lilac aldehyde A	1143	a	8	1.3%	2.8%
ketoisophorone	1146	a	1	0.7%	2.1%
lilac aldehyde B+C	1151	a	8	1.6%	4.7%
lilac aldehyde D	1166	a	8	0.8%	2.0%
1-nonanol	1172	a	1	3.6%	2.7%
3,5,5-trimethyl-cyclohexene	1176	b		3.9%	4.0%
unknown	1180			0.9%	5.7%
unknown	1184			1.2%	nd
p-1,8-menthadien-2-ol	1188	a	1	4.3%	0.6%
safranal	1199	a	1	1.0%	1.1%
n-decanal	1207	a	1	2.3%	1.7%
cis-carveol	1231			1.0%	nd
(+)carvotanacetone	1252	a	1	2.3%	nd
1-decanol	1273	a	1	0.9%	nd
carvacrol	1298	a	1	1.4%	nd
unknown	1302			5.1%	nd
β-damascenone	1380	a	1	0.6%	0.8%

LRI=Linear Retention Indices; ID=Identification methods; a=tentatively identified by mass spectra library research and comparison of LRI with published data; b=tentatively identifies by mass spectra library research; nd=not detected; 1=Adams, 2001; 2=Muriel, et. al., 2004; 3=Vichi, et. al., 2007; 4= www.pherobase.com; 5=Weissbecker, et. al., 2004; 6=Pérès, et. al., 2002; 7=Edris, et. al., 2008; 8= Dötterl, et. al., 2005.

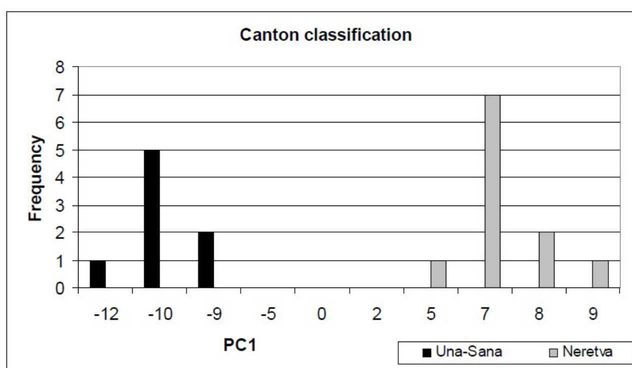

Fig. 1 Graphical representation of the CDA of floral honeys:

Table: 2 Volatiles that significantly contribute to the classification of the floral honey samples in countries: B=Bosnia

	Differentiation between country (I) and country (J)			
Compound	**Country (I)**	**Country (J)**	**Mean Difference (I-J)**	**p**
n-octane	H	B	3.1%	0.002
furfural	H	B	4.5%	0.007
2-methyl-butanoic acid	B	H	0.5%	0.028
3-methyl-butanoic acid	B	H	0.5%	0.033
n-nonane	H	B	0.6%	0.017
n-octanal	B	H	0.6%	0.022
4-terpineol	B	H	2.8%	0.032
p-cymene	B	H	0.8%	0.039
benzenemethanol	B	H	1.2%	0.006
1-phenyl ethanol	B	H	0.6%	0.019
acetophenone	B	H	0.7%	0.002
linalool	H	B	5.3%	0.003
HO-trienol+n-nonanal	B	H	3.8%	0.031
cis rose oxide	B	H	0.2%	0.043
1-nonanol	B	H	2.6%	0.015
p-1,8-menthadien-2-ol	B	H	1.6%	0.049
cis-carveol	B	H	0.4%	0.033
unknown (1302)	B	H	2.6%	0.007

CONCLUSION

The results show a high number of volatile compounds. The compound's mean values range between 0.1-10.0 percent. Some of the compounds present in higher percentage are: benzaldehyde, n-octane, benzeneacetaldehyde, linalool, p-cymenene, n-nonanal and 1-nonanol. Unfortunately individual single volatile compounds can not be used as geographical markers, but the two groups of samples present characteristic volatile profiles. A canonical discriminant function analysis

(CDA) was applied to volatile compositions of honeys from Una-Sana Canton and Neretva Canton. The results show a statistically significant discrimination between the floral honeys of the two regions. The CDA function allowed also a good classification of unknown samples (cross validated). The difference between the locations may be due to particular geographical and botanical characteristics as well as climatic conditions.

The high classification percentage indicates that the floral honeys of different geographical origin

can be differentiated on the basis of their volatile profiles. Therefore, analyzing volatile profiles of honeys in combination with statistical evaluations like CDA is a good quality assurance tool enabling authenticity control.

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THE DYNAMICS OF MICRO-ORGANISMS IN MARSHMALLOW BASED CHOCOLATE COATED CANDY MAIGUMS DURING THE STORAGE TIME

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ABSTRACT

Marshmallow based chocolate coated candy *Maigums* is sugar-candy with foam-like structure and contains components: glucose syrup, chocolate (30%), sugar, water, egg albumen powder, gelling agent (agar-agar), acidity regulator E331, vanilla 0.08%, stabilizer (carrageen). The objective of this research was to evaluate the microbial stability of hermetically in several types of polymer materials (OPP, Multibarrier 60, PP and PLA VC999 *BioPack*) packed Marshmallow candy *Maigums* during the storage time. Experiments were carried out in Latvia University of Agriculture. The microbial dynamics and water activity of the Marshmallow based chocolate coated candy *Maigums* in air medium packaging was examined. Four types of packaging materials (OPP, Multibarrier 60, PP and PLA VC999 *BioPack*) were used. The analyses were carried out before packaging and after 1; 2; 3; 4; 5 and 6 storage months. Samples were stored at the room temperature +18±3 °C. Microbial analyses were performed accordingly to: Microbiology of food and animal feeding stuffs (LVS ISO 4833:2003) and Microbiology of food and animal feeding stuffs (LVS ISO 21527-2:2008).Water activity of samples was measured directly with an AquaLab LITE model water activity meter (Decagon Devices Inc.). The obtained results indicate that several packaging materials with different barrier properties influence the water activity and growth of micro organisms in Marshmallow based chocolate coated candy *Maigums* during the storage time in air medium packaging. Environmentally friendly biodegradable packaging material PLA VC999 *BioPack* with barrier properties could be suggested. The barrier properties of packaging materials influence microbial growth during the storage time.

KEYWORDS: *Marshmallow; water activity; packaging; microbial growth.*

INTRODUCTION

Confectionery is what many people think of as „candy" or „sweets". Confectioneries are sweet, shelfstable product with low water activity (aw). Most confectioneries have aw value below 0.85, will have different microbiological issues (Downes and Ito, 2001). Confectionery belong to either of two groups, sugar confectionery or chocolate confectionery, although broader classifications sometimes are used. These products contain sugar, syrups, honey, or other sweeteners. In addition, confectionary products may contain cocoa or chocolate products; dried milk or other dairy products; nuts, cocoanut or other fruits; cereal grain product, including crisped rice; starch, gelatine or other thickeners; egg albumen; spices, colors, flavors or acidulates; or other ingredients (Downes and Ito, 2001; Kawo and Abdulmumin, 2009).The shelf life of a product can be altered by changing its composition and form, the environment to which it is exposed, or the packaging system (Raisi and Aroujalian, 2007). Microbiological contamination of foods – standards in Latvia is loaded down from the Regulation of European Commission No. 2073/2005 on microbiological criteria for foodstuffs. On the basis of this regulation, each company has developed its own guidelines to evaluate the quality of manufactured products (Karpikova, 2009). Nowadays microbiological parameters are very meaningful due to development of product manufacturing technology and variety of preservatives (Kulichenko, 2006; Sucharzewska, et al., 2003). According to Kawo and Abdulmumin, sweets most of all are contaminated from the ingredients. Additional contamination could be received from production equipment coming in contact with food, as well as from packaging materials, and from personnel. The diffusion of moisture in food

materials is of fundamental importance for processing and storage. The migration of moisture into or from food stuffs characterizes food quality, chemical reactions and microbial growth during storage (Ergun, et al., 2005). One way to slow down moisture migration is to use barrier between the domains of a food material (Ghosh, et al., 2004). Water activity aw is the intrinsic product characteristic that most of all influences the microbial ecology of confectioneries. High concentrations of sugar, especially those of low molecular weight, afford low aw. Most of bacteria cannot grow at aw below 0.85, and growth of spoilage-causing yeast and molds is unlikely at aw levels below 0.61 (Downes and Ito, 2001). A critical aw (0.6–0.7) exists below which no microorganism can grow. The moisture sorption isotherm (MSI) of composite foods is one of the most important measures affecting acceptability, shelf-life and packaging and storage requirements (Ahmed, et al., 2004). A difference in water activity, either between candy and air or between two domains within the candy, is the driving force for moisture migration in confections (Ergun, et al., 2010). Chocolate or chocolate-flavoured coatings are heterogeneous food systems that consist of a continuous fat phase with cocoa powder and sucrose as a dispersed phase. The composition and structure of this dispersed phase will certainly alter the moisture diffusivity (Ghosh, et al., 2004). The specific kind of confectioneries considered here have foam-like structures (marshmallows, soufflés, and nougats). Marshmallows are simply described as air bubbles surrounded by sugar syrup. The sugar syrup, made of sucrose, corn syrup, and water, is cooked to a temperature appropriate for the desired water content, after which air is whipped into the matrix through mechanical agitation. Proteins, such as gelatine, gum Arabic, egg albumen, agar-agar, pectin, milk or soy protein, are typically added to the sugar syrup to stabilize the foam. During whipping, the density of the product decreases as the syrup and foam mixture expands into a light, fluffy marshmallow. Marshmallows may be either ungrained or grained crystal aggregate, depending on the ratio of sucrose to corn syrup (Ergun, et al., 2010; Sucharzewska, et al., 2003). Certain glassy sugars tend to be hygroscopic, rapidly picking up moisture from the air, which causes significant changes that lead to the end of shelf life. These products need to be protected from moisture uptake during storage (Ergun, et al., 2010). The end of shelf life due to moisture loss or gain, with subsequent changes in textural and other properties, is often the main problem in confections. Thus, an understanding of water activity is important for control of shelf life and stability. A fresh-made, ungrained marshmallow has fairly high aw, above 0.7, dependent on moisture content and composition. During storage water activity (and moisture content) decrease issues (Downes and Ito, 2001; Ergun, et al., 2010). In terms of shelf life, it is well known that the difference between water activity (aw) of the candy and the RH of the surrounding environment determines whether a confection gains or loses moisture during storage. The closer aw is to the RH during storage, the less potential for moisture migration to or from the environment. Similarly, in confections with multiple domains of different aw, migration occurs between the domains until equilibrium is reached (at equal aw values). Methods to protect confections against moisture migration are continually being studied to preserve quality and extend shelf life. This includes approaches to retard migration to the environment through use of improved packaging materials and to retard migration within multi-domain candies through the use of edible films and/or reformulation to balance aw of the different domains (Ergun, et al., 2010). The aim of this research was to evaluate the microbial quality of marshmallow *Maigums* coated with chocolate during the storage time in air ambiance one by one piece in small wrapping made from various conventional polymer materials with different barrier properties and biodegradable PLA VC999 *BioPack* with barrier properties, as well as to determine the dynamics of marshmallow *Maigums* water activity during the storage time.

MATERIALS AND METHODS

Experiments were carried out in the laboratories of Latvia University of Agriculture Department of Food Technology, and in experimental laboratory of stockholder Laima, Latvia. The object of the research was Marshmallow *Maigums* by vanilla taste coated with chocolate, produced by stockholder Laima, Latvia. Ingredients of *Maigums*: glucose syrup, chocolate 30% (sugar, cocoa mass, cocoa butter, emulsifier E422, flavouring (vanillin)), sugar, water, egg albumen powder, gelling agent (agar – agar), acidity regulator E331, vanilla 0.08%, stabilizer (carrageenan). Cocoa solids in chocolate should be 52% as minimum. The study involved preliminary preparation of different conventional polymer pouches from OPP (thickness 40±2 μm), Multibarrier 60 (thickness 60±2 μm), PP (thickness 40±2 μm) and biodegradable PLA VC999 *BioPack*

(thickness 60±2 μm) with barrier properties, followed by packaging of marshmallow *Maigums* in air ambiance (one piece in each package). As a control commercially used packaging in PP polymer pouches was used with capacity 12–14 pieces of *Maigums* in one packaging, total mass 200 g.). The samples were stored at room temperature at +18±3 °C (controlled by MINILog, Gresinger electronic) and about of 40% RH for 6 months under day and night conditions. The materials for experiments were selected with different water vapour transmutation rate and various thicknesses. One piece of marshmallow *Maigums* were placed in each package. Size of each pouch was 8 x 8 mm, the total product mass in each package – 14±1g. The results were reported as an average value of all determinations. Samples were analyzed before packaging (day 0) and after 1; 2; 3; 4; 5 and 6 storage months. Water activity was determined by standard ISO 21807:2004, AquaLab LITE device. Microbial analyses were performed accordingly to: Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of microorganisms - Colony-count technique at 30 degrees °C (according LVS ISO 4833:2003) and Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of yeasts and moulds - Part 2: Colony count technique in products with water activity less than or equal to 0, 95 (according LVS ISO 21527-2:2008). The results were processed by mathematical and statistical methods. Statistics on completely randomized design were determined using the General Linear Model procedure SPSS 16.00.

RESULTS AND DISCUSSION

Currently marshmallow Maigums has been commercially realized in polymer PP pouches (12–14 pieces in one packaging) or in cardboard boxes (16 pieces in each), their shelf life is up to 6 month, what is sufficient time to meet the requirements of shopkeepers. However with target to widen the marketing activities, the stockholders of Laima care for more large-scale market and decided the marshmallow Maigums wrap up one by one piece in small packaging and sell at school coffee-bars, wending machines and in hotels and rest-houses. The aim of this research was to evaluate the quality of marshmallow Maigums during the storage time one by one piece in small wrappings made from various polymer materials. As a control commercially used packaging in PP polymer pouches was choose with capacity 12–14 pieces of Maigums in one packaging, mass in each pack 200 g. During the first period of experiments the growth of microorganisms was evaluated as well as water activity changes detected. As it was listed in the literature review, the water activity has an important influence on the quality properties of foods and their changes during storage. To evaluate the adequacy of various polymer materials for small packaging of marshmallow Maigums, the dynamics of water activity aw was detected and the results reproduced in Fig. 1. The initial value of water activity of samples was 0.713. After two month storage of samples essential difference in water activity value changes was not observed ($p>0.05$). After three month of storage some changes in values of water activity were observed: in sample packed in polymer OPP it decreases till 0.593, in PLA VC999 BioPack – till 0.629. Water activity of samples packed in other polymer materials ranged from 0.672 till 0.692, what mutually doesn't differ. Following up the storage water activity decreased even in all samples. Most of all the decrease of water activity was observed of sample packed in OPP ($p<0.05$), what could be explainable with low barrier properties of used packaging polymer material, does not preventing the migration of water vapour from marshmallow Maigums to ambient air. The least changes of water activity was observed in control sample, packed in commercially used polymer PP, providing insignificant increase in water activity of samples substantially maintaining the quality of samples in the packaging. Similar to control packaging was the influence of Multibarrier 60 polymer on the sample Maigums water activity, although in the packaging was only one piece of marshmallow. High barrier properties of used polymer provided the quality maintenance of product. According to the data presented it could be concluded that concerning the changes of sample's water activity, the influence of packaging material Multibarrier 60 is close to currently commercially used polymer PP influence on the water activity of marshmallow Maigums at long-term storage.

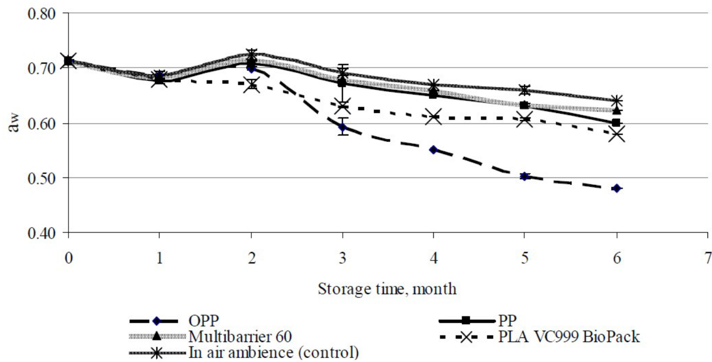

Figure 1. Water activity (aw) dynamics in air ambiance packed Marshmallow Maigums during the storage time

The dynamics of aerobic colony count (ACC), colony forming units (cfu) in the marshmallow *Maigums* samples during the storage time in small packaging made out of various polymer materials is shown in Figure 2.

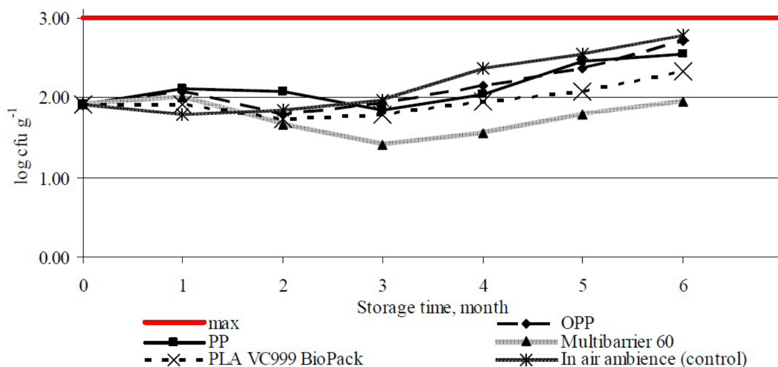

Figure 2. ACC dynamics in air ambiance packed Marshmallow Maigums during the storage time
max: Admissible level of ACC < 103 cfu g-1 according to Ltd Laima guidlines

The initial ACC in marshmallow *Maigums* samples was 1.9 log cfu g-1. During the storage up to one month at the temperature 18 ± 3 °C the ACC cfu substantially did not change (p>0.05). An insignificant increase in ACC could be observed after two-three month of storage (p>0.05). In Multibarrier 60 packed samples the ACC cfu count decreased till 1.4 log cfu g-1. In the turn, in samples packed in all other used materials the ACC count was between 1.78 log till 1.96 log cfu g-1.

The results show that up to three month the microorganisms accustom one selves to generated conditions in the packaging and their growth practically stopped, part of them even die. Substantial recession in growth of microorganisms has been observed after fourth storage month. In control sample packed in polymer PP the count of ACC increases up to 2.36 log cfu g-1, in OPP – up to 2.15 log cfu g-1, in PP – up to 2.04 log cfu g-1, in samples packed in PLA VC999 *BioPack* the ACC cfu count was close to initial – 1.95 log cfu g-1, in

the turn in samples packed in Multibarrier 60 the ACC cfu did not achieve the initial level – it was only 1.54 log cfu g-1. During the storage up to 6 month the growth of microorganisms was regular. Remarkable difference of ACC cfu count during storage time was not observed in control samples packed in PP film and as well as in samples packed in OPP (2.7 log – 2.77 log cfu g-1), somewhat less growth of microorganisms was observed in samples packed in PP (2.54 log cfu g-1) ($p>0.05$). For all that considerably less ACC cfu growth were ascertain in samples packed in PLA VC999 *BioPack* with barrier properties (2.32 log cfu g-1) and in Multibarrier 60 (1.95 log cfu g-1) ($p<0.05$). These phenomena could be explained with different barrier properties of used packaging polymers – the higher barrier properties suppress and slow down the growth of microorganisms. Allowed total colony count of yeasts is 50 cfu g-1, and of moulds – 100 cfu g-1. During the storage time the total count both of yeasts and moulds did not go beyond 10 cfu g-1.

CONCLUSION

Evaluating the obtained results the conclusion is that all investigated packaging polymer materials are applicable for marshmallow *Maigums* small packaging one by one in each pack. However as the more appropriate packaging material for marshmallow *Maigums* small packaging could be recognized laminated polymer Multibarrier 60 with high barrier properties, therefore providing slowest growth of microorganisms as well as noteless changes in water activity during 6 storage month.

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LEVELS OF CHLOROPROPANOLS IN FOODS CONTAINING HYDROLYZED VEGETABLE PROTEIN (HVP) IN BRAZIL

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ABSTRACT

Chloropropanols are a group of chemical contaminants which are formed during the treatment of protein-rich materials with concentrated hydrochloric acid at high temperatures. They have most frequently been found in hydrolyzed vegetable protein (HVP), a seasoning ingredient used in a variety of processed savory foods. The presence of some chloropropanols in foods is a concern due to their carcinogenic and genotoxic properties. Therefore, the objective of this study was to investigate the levels of these contaminants in foods containing HVP available on the Brazilian market. A total of 60 samples including seasonings, bouillon powder, soups, savory snacks, sauces, instant noodles and others were evaluated in relation to the presence of 3-monochloropropane-1,2-diol (3-MCPD) and 1,3- dichloropropan-2-ol (1,3-DCP) by using gas chromatography-mass spectrometry (GC-MS). Levels of 3-MCPD varied from not detected to 3514 µg/kg. The contaminant was found in quantifiable amounts in 36 samples and the maximum concentration was observed in a sample of frozen ready-to-eat meal. In 20 samples, the levels of 3-MCPD were higher than the regulatory limit of 20 µg/kg established for HVP by the European Commission. For 1,3-DCP, the levels ranged from not detected to 35 µg/kg and 4 samples showed results above the quantification limit. The maximum concentration was found in a sample of sauce for meats. There are no regulatory limit and no safe intake levels established for 1,3- DCP. These results demonstrated that several foods may contain chloropropanols due to the presence of HVP as seasoning ingredient.

KEYWORDS: *chloropropanols; processed foods; GC-MS; hydrolyzed vegetable protein (HVP)*

INTRODUCTION

3-Monochloropropane-1,2-diol (3-MCPD) and 1,3-dichloropropan-2-ol (1,3-DCP) belong to a group of contaminants called chloropropanols which can be formed during the thermal processing of certain foods and ingredients. They were originally discovered in acid hydrolyzed vegetable protein (acid- HVP) in the 1980s (Davídek et al., 1982) but subsequent studies demonstrated their presence also in soy sauces manufactured with acid-HVP (MAFF, 1999) as well as in several processed foods and ingredients, such as cereal-derived products, malt-derived products and smoked foods (Hamlet et al., 2002).

The presence of chloropropanols in foods is a concern due to their toxicological properties. Studies have shown that 3-MCPD can affect male fertility, kidney functioning and body weight in rats when given regularly in high doses. Carcinogenic potential has also been demonstrated in rats when 3- MCPD was administrated at high doses over a long period of time. However, the contaminant does not have significant genotoxic potential in vivo. By the other hand, 1,3-DCP presented hepatotoxic properties, induced a variety of tumours in various organs in rats and was genotoxic in vitro, being considered a genotoxic carcinogen that causes cancer by directly damage of genetic material (Baer et al., 2010).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated 3-MCPD and 1,3- DCP in its 57th meeting and assigned a provisional maximum tolerable daily intake (PMTDI) for 3- MCPD of 2 µg/kg bw/day. The Committee concluded that the establishment of a tolerable intake for 1,3-DCP was inappropriate because of the nature of toxicity (FAO/WHO, 2001). These chloropropanols were re-evaluated in the JECFA 67th meeting and the previously established PMTDI for 3-MCPD was retained (FAO/WHO, 2006).

Acid-HVPs are produced by hydrolysis of various proteinaceous vegetable and animal raw materials with hydrochloric acid. They are used as flavor enhancers and as ingredients in processed savory food products

and pre-prepared meals. Typical levels in foods range from ca. 0.1 to 0.8%. The formation of chloropropanols in acid-HVP occurs during the hydrolysis step at temperatures exceeding 100°C. The acid reacts with residual lipids and phospholipids present in the raw material, resulting in the formation of the contaminants (Velíšek, 2009).

Generally, 3-MCPD is the most widely occurring chloropropanol in foods that contain acid-HVP. Other chloropropanols can occur in smaller amounts, such as 1,3-DCP. Initial investigations made in the 1980s showed levels of 3-MCPD and 1,3-DCP in acid-HVPs of 100-800 mg/kg and 0.1-6 mg/kg, respectively. Several efforts were addressed to this issue in order to reduce the levels of these contaminants in acid-HVP and a significant decline in concentrations of 3-MCPD in acid-HVPs was achieved in subsequent years (Velíšek, 2009).

In March 2001, the European Commission adopted a rule that fixed a limit on the level of 3-MCPD in soy sauce and acid-HVP. Maximum levels were set at 20 μg/kg for a liquid product containing 40% dry matter, corresponding to a maximum level of 50 μg/kg in dry matter (EC, 2001). The level needs to be adjusted according to the dry matter content of the product.

In Brazil, there is no maximum level established for 3-MCPD in food products and ingredients. Moreover, there are no published data in the literature concerning the occurrence of chloropropanols in commercial Brazilian products. Therefore, the objective of this study was to investigate the levels of these contaminants (3-MCPD and 1,3-DCP) in foods containing HVP available on the local market.

MATERIALS AND METHODS

Standards

3-MCPD and 1,3-DCP standards were obtained from Sigma-Aldrich at a purity of 98%. 3-MCPD-d5 and 1,3-DCP-d5 internal standards were purchased from Cambridge Isotope Laboratories at a purity of 98%. Individual stock solutions of both standards and internal standards at 1 mg/ml were prepared by dissolving in ethyl acetate. Work solutions at 2 and 10 μg/ml for standards and internal standards, respectively, were prepared in ethyl acetate.

Reagents and solvents

Extrelut™ NT20 refill pack was purchased from Merck. Heptafluorobutyrylimidazole (HFBI) was obtained from Regis Technologies. Hexane, iso-octane, ethyl acetate and diethyl ether were HPLC grade. Sodium sulfate anhydrous and sodium chloride were of analytical reagent grade.

Samples

A total of 60 samples were purchased at supermarkets in the city of Campinas, SP, Brazil, including sauces (n=9), seasonings (n=8), bouillon powder (n=8), soups (n=4), snacks (n=7), instant noodles (n=5), risotto (n=1), frozen ready-to-eat meals (n=5), meat products (n=7) and canned vegetarian meals (n=6). All products were analyzed as bought.

Sample preparation

The sample preparation for determination of chloropropanols (3-MCPD and 1,3-DCP) was performed according to Brereton et al. (2001) and Lim et al. (2005). The homogenized sample (8 g) is weighed into a 250 ml beaker. Internal standards work solution of 3-MCPD-d5 and 1,3-DCP-d5 are added to the test portion, followed by NaCl solution 5M. The mixture is blended to a homogeneous consistency and, after sonication, the content of an Extrelut™ NT20 refill pack is added and mixed thoroughly. The mixture is transferred to a glass chromatographic column, and the 1,3-DCP is eluted with a mixture of hexane and diethyl ether (9:1). The 3-MCPD is then eluted with diethyl ether, and the combined extracts are concentrated to a small volume. A portion of the concentrated extract is derivatized with HFBI at 70°C during 40 minutes before injection into the gas chromatography-mass spectrometry (GC-MS) system.

GC-MS analysis

The analysis were performed into a HP 6890 gas chromatography equipped with a MSD 5973 mass spectrometer (Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1 ml/min. The Programmable Temperature Vaporizing (PTV) injector was operated in the splitless mode under the following temperature program: 100ºC, 500ºC/min to 280ºC (held until the end of the run). The separation was performed on a 60 m x 0.25 mm, df 0.25 µm HP-INNOWAX capillary column (Agilent Technologies) and the oven temperature program was: 50ºC (held for 1 min), 5ºC/min to 150ºC, 50ºC/min to 240ºC (held for 2 min). The mass spectrometer was operated in positive electron impact ionization mode (+EI) with 70 eV of electron energy. Selected ion monitoring (SIM) was used for the detection of the following ions: m/z 278*/116/79 for 1,3-DCP-d5, m/z 275*/110/277 for 1,3- DCP, m/z 294*/278/456 for 3-MCPD-d5 and m/z 289*/275/291 for 3-MCPD (*quantifier ions).

Identification and quantification

The relative retention time (RRT) and the presence of diagnostic ions were considered for identification of chloropropanols in the samples. For confirmatory purposes, an acceptable deviation of ±0.5% for RRT and ±20% for ionic relative abundance were used by comparing the sample with a standard solution. The quantification of 3-MCPD and 1,3-DCP was carried out by extrapolation from a linear analytical curve, using 3-MCPD-d5 and 1,3-DCP-d5 as internal standard.

Method validation

The method was validated in terms of linearity, selectivity, limit of detection (LOD), limit of quantification (LOQ), trueness (recovery) and precision (repeatability and within-laboratory reproducibility) according to the guidelines laid down by the Brazilian Institute of Metrology, Standardization and Industrial Quality (INMETRO, 2007). Linearity was evaluated over the range 0- 312.5 µg/kg. Selectivity was evaluated by comparison between curves set on standard solutions and on matrix. LOD and LOQ were determined by seven replicates of the matrix and calculated as 3 and 10- fold standard deviation, respectively. Recovery, repeatability and within-laboratory reproducibility were evaluated by spiking the matrix with standards at 12.5, 62.5 and 250 µg/kg (seven replicates for each concentration level). A blank sample of madeira sauce containing HVP was used in the experiments.

RESULTS AND DISCUSSION

Although chloropropanols have a relatively simple chemical structure, there are some characteristics that make them difficult to analyse sensitively. The initial methods developed for the determination of chloropropanols without derivatization showed a low sensitivity. In order to enable quantification at µg/kg levels, most of the methods currently applied are based on solid-phase extraction, derivatization and subsequent GC-MS analysis. The three most common derivatization reactions that give adequate sensitivity and selectivity are performed with HFBI, phenylboronic acid (PBA) and ketones (Baer, 2010).

In this study, the analysis of chloropropanols were carried out employing derivatization with HFBI according to the procedures described by Brereton et al. (2001) for 3-MCPD, and modified by Lim et al. (2005) to include the simultaneous analysis of 1,3-DCP as well. This method showed good linearity within the range 0-312.5 µg/kg (r2 > 0.998). A non-significant matrix effect was verified for both 3- MCPD and 1,3-DCP. LOD and LOQ were calculated as 1.1 and 3.7 µg/kg, respectively, for 3-MCPD and as 1.7 and 5.7 µg/kg, respectively, for 1,3-DCP. Recovery, repeatability and within-laboratory reproducibility were in satisfactory ranges. Figure 1 illustrate a typical ion chromatogram of a sample of bouillon powder containing 68 µg/kg of 3-MCPD and no detected level of 1,3-DCP.

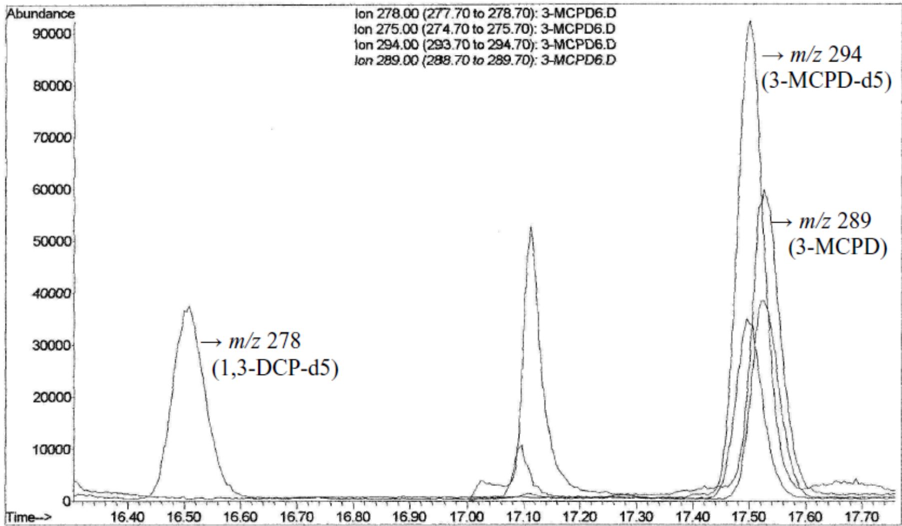

Figure 1. Ion chromatogram of a sample of bouillon powder (m/z 278, 294 and 289: quantifier ions; 3-MCPD: 3-monochloropropane-1,2-diol; 3-MCPD-d5: deuterated 3-monochloropropane-1,2-diol; 1,3-DCP-d5: deuterated 1,3-dichloropropan-2-ol; carrier gas: helium; flow rate: 1 ml/min; Programmable Temperature Vaporizing (PTV) injector: 100°C, 500°C/min to 280°C (held until the end of the run); mode: splitless; column: 60 m x 0.25 mm, df 0.25 μm HP-INNOWAX; oven: 50°C (held for 1 min), 5°C/min to 150°C, 50°C/min to 240°C (held for 2 min); mass spectrometer: positive electron impact ionization (70 eV)).

The levels of 3-MCPD found in the analyzed samples are shown in Table 1. For comparison purposes, these levels were adjusted to 40% of dry matter. The 3-MCPD content varied from not detected to 3514 μg/kg. The contaminant was found in quantifiable amounts in 36 samples (60%). In general, instant noodles, bouillon powders, seasonings, soups, vegetarian meals and ready-to-eat meals showed the highest % of positive samples. In 20 samples, the levels of 3-MCPD were higher than the regulatory limit of 20 μg/kg established for acid-HVP by the European Commission. The highest mean concentrations were observed in ready-to-eat meals, meat products, sauces and vegetarian meals.

Table 1. 3-MCPD levels in foods containing hydrolyzed vegetable protein (HVP)*

Food group	N total	N positives (%)	N > 20 μg/kg	Mean positives (μg/kg)	Maximum μg/kg
Sauces	9	4 (44)	4	378	990
Instant noodles**	5	4 (80)	3	65	97
Bouillon powder	8	6 (75)	1	9	28
Seasonings	8	8 (100)	0	5	13
Snacks	7	1 (14)	0	9	9
Risotto	1	0 (-)	0	-	-
Soups	4	3 (75)	2	24	38
Vegetarian meals	6	5 (83)	5	159	256
Ready-to-eat meals (frozen)	5	4 (80)	4	1858	3514
Meat products	7	1 (14)	1	771	775

* The levels of 3-MCPD were adjusted to 40% of dry matter.
** Only the seasoning.
N = number of samples.

CONCLUSION

The data obtained in the present study demonstrated that several foods may contain chloropropanols due to the presence of HVP as seasoning ingredient. The presence of 3-MCPD was most frequent than 1,3-DCP. Although no regulation is currently established for 3-MCPD in Brazil, it is important to control the levels of this contaminant during the production of acid-HVP.

ACKNOWLEDGEMENTS

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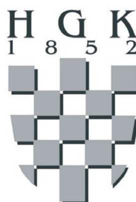

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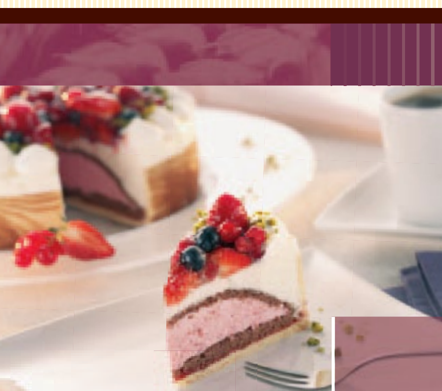

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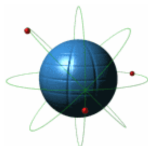

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