3. FOOD CHEMISTRY & BIOTECHNOLOGY

3.1. Lectures

L01 NONSACCHAROMYCES YEAST IN GRAPE MUST – ADVANTAGE OR SPOILAGE?

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Introduction

The fresh grape must consists of spontaneous microflora formed from 90 to 99 % by yeasts. The most important genus is without doubt *Saccharomyces cerevisiae* which is responsible for successive fermentation and good wine quality. Recently the contribution of non-*Saccharomyces* yeasts have been widely discussed as there is not definitive opinion on their contribution to the wine quality, especially aroma. *Hanseniaspora osmophila and Kloeckera apiculata* should be considered detrimental yeast species, by higher acetic acid, acetaldehyde, ethyl acetate and acetoin production[1]. To avoid spoilage it is recommended to inoculate the grape must by *Saccharomyces cerevisiae* immediately after pressing. On the other hand, the apiculate non-*Saccharomyces* yeast is a natural indigenous microflora which contributes to the wine origin. Thus the question wheather to allow the apiculate microflora to start fermentation or not has not yet been solved.

The aim of this study work was to determine the aroma profile of isolated non-*Sacaharomyces* yeast from the chemical as well as the sensorial viewpoints. The yeasts of the genera *Rhodotorula*, *Sporobolomyces*, *Pichia*, *Hansenula, Issat-chenkia,* and *Torulospora*, were tested from the viewpoint of their contribution to the wine aroma. The results were than exploited and tested in the real wine-making process.

Experimental

Following yeast strains were isolated from the grape must and degraded products: *Rhodotorula mucilaginosa* (2 strains), *Sporobolomyces pararoseus*, *Pichia membranefaciens*, *Pichia anomala* (2 strains), *Candida intermediata*, *Torulospora delbruecki,* and *Issatchenkia orientalis*. For comparison 3 *Saccharomyces cerevisiae* strains were also used. Two of them were isolated from the grape must, the third was a commercial one (Lallemand).

Consequently the isolated yeast strains were inoculated to the first cultivation medium: the sterile grape must had been femented for 10 days at 10 °C under semiaerobic conditions or 4–6 weeks at 10 °C under anaerobic conditions.

Second cultivation medium: the sterile Vinea drinks were inoculated by studied yeast strains and cultuivated at 20 °C for 10 days under semiaerobic conditions.

The samples were than sensorially evaluated by a group of degustators. The same samples were than analysed by gas chromatography for the aroma compounds production. Each sample was analysed on the GC MS (Shimadzu QP 2010) equipment and also on the GC FID equipment (GC 8000 CE Instruments).

Two methods of sample preparation were done:

The samples (20 ml) were extracted by ether (2 ml), and centrifuged prior to analysis. The etheric extract was used for analysis (liquid – liquid extraction). This method was used for higher alcohols (propanol, isoamylacohol, ethyl ester and higher alcoholos esters) determination

The samples were extracted by Tenaq (solid phase microextraction) and than 10 min sampling according to[6]. This method was used for monoterpenic compounds determination.The same column and the same conditions were used by both analysis: Column: DB WAX 30 m, 0.25 × 0.25, temperature programme: 30 °C, hold 2 min, increase by 4 $°C\,min^{-1}$ up to 230 °C, hold 10 min, 1 ml of sample was injected to injection port at 200 °C, detector temperature 220 °C, carrier gas: helium, injektion mode: split 1 : 100, flow control mode: pressure 70 kPa

Results

After inoculation and fermentation of the grape must and Vinea drink the number of aroma compounds increased significantly, in both cases and more than 60 compounds were found. Most of them were recognized as typical fermentation products, etc. ethanol, izoamylacohol, propanol, etylester of caproic, caprylic and caprinic acids, ethylacetate, isovaleric acid, pentylacetate, 2,3-butandiol, furfural, 3-hydroxybutyrate, methionon, 1,4-butandiol. 2-metyl a 3-metylbutanoic acid, 2-phenyletylacetate, izoamylacetate, cis 3-hexenylacetate, etylbenzoate, α-terpineol, etyl isobutyrate, etyl butyrate, etyl 2-metylbutyrate, etyl isovalerate, isoamyl acetate, ethyl hexanoate, cis-3-hexenol, ethyl octanoate, furfural, linalool, ethyl furoate, ethyl decanoate, ethyl benzoate, α-terpineol, fenylethyl acetate, and geraniol. The increased production of typicall glycolysis products were also confirmed by several authors[2,3,4]. Ethyl propionate and propyl acetate, characterized the sample of the grape must fermented by *Kloeckera apiculata*, and 2-propanol and 2-hexanone characterized the sample of the grape must fermented by *Pichia membranefaciens*[2]. Rojas[3] studied analysis of non-*Saccharomyces* yeast fermentation products and found higher acetate content, especially 2-phenylacetate and isoamylacetate for *Pichia* yeasts. Albertazzi[4] also described higher levels of phenylacetate by other yeast - *Pichia pastoris* and *Kloeckera saturnus* (700–1700 $mg\,dm^{-3}$). From our results we can confirm incereased ester production by all studied microorganisms, especially increased content of ethyl acetate.

However, we have found that some microorganisms produced special compounds, which were not recognised by other yeasts. After the fermentation of the grape must by *R. mucilaginosa* and *Sp. pararoseus,* aroma compounds significantly increased. Acetate, hexanal, heptanal, octanal, cyclopentanone, thiazole, decalactone, propyl-3-dimethyl aminopropyl, nonanone, heptanon, and butanediol were formed. *P. anomala* produced especially isoamyl benzyl ether. The medium fermented by *Sporobolomyces* was rich in sabinylacetate, 3,4-hexandion and eicosane. *R. mucilaginosa* generated cyklopentanol and, α-cyklogeraniol. *S. cerevisiae* produced vericaldehyd and γ-nonalakton.

We have found out that yeasts of the genera *Pichia*, *Rhodotorula*, and *Sporobolomyces* did not produce the linalool acetate, contrary to *S. cerevisiae*.

The differences in the compound production within the same yeast species were also observed. *S. cerevisiae* strain 8 produced caproic aldehdyd, trans-pinocamphon, dodecanal, 5-methyl-3-heptanon, izo-menthylacetate, contrary to *S. cerevisiae* strain 5 which did not produce any of these compounds. *R. mucilaginosa* strain 11 produced higher amounts of 2,3-butandiol, but did not produce any izopentylformiate and 3,4-hexandion. These compounds produced strain *Rhodotorula mucilaginosa* strain 3.

Table I
Aroma compounds typically produced by various yeast species and flavoural characterisation of founded compounds

Yeast strain	compound	Flavour fragrance
R. mucilaginosa	cyklopentanol, alfacyklogeraniol	mint aroma spice flavour carnation odour
Pichia anomala	izoamyacetat	banana flavour
Sp. pararoseus	sabinylacetate	fruity aroma
S. cerevisiae	valericaldehyd, γ-nonalakton	coffee aroma coconut odour

Table II
Sensorial evaluation of fermented grape must by various yeast strains, + positive impression, − negative impression

	Anaerobic conditions		Semiaerobic conditions	
Yeast strain	aroma	perc.	aroma	perc.
C.intermediata	yeast	+	acidic	−
R.mucilaginosa 3	socks smelly	−	acethone	−
T.delbruecki	pleasant	+	vanilla	+
I.orientalis	autolyses	−	acethone	−
Pichia anomala	acethone	−	lime	+
P.membranefaciens	autolyses	−	yeasty	+
R. mucilaginosa 11	autolyses	−	honey	+
Pichia anomala	acethone	−	honey	+
Sp. pararoseus	not recognised	−	yeasty	+
S.cerevisiae 5	acidic	−	ferment	+
S.cerevisiae 8	fruity	+	ferment	+
S.cerevisiae 16	honey	+	ferment	+

Table III
Evaluation of Vinea fermented under semiaerobic conditions

	Semiaerobic conditions Vinea 20 °C	
Yeast strain	Aroma	Percept
C. intermediata	acidic	+
R. mucilaginosa 3	acethone	+
T. delbruecki	yeasty	+
S. cerevisiae 5	yeastly	+
I. orientalis	grape must	+
S. cerevisiae 8	honey	+
P. membranofaciens	vinea	−
R. mucilaginosa 11	acethone	+
P. anomala	honey	+
Sp. pararoseus	acethone	+

One of the most important factors in wine proofing is the sensorial evaluation of wine aroma. It is very difficult to estimate which from all above mentioned compounds will prevail over the other ones and wheather the wine could give positive or negative impression. It is due to the complexity of wine aromas, the heterogenity of perception and recognition thresholds for each one compound as well as many interactions occuring within and after fermentation[5].

As shown in Table II, under anaerobic conditions only *T. delbruecki* from apiculate microflora developed a pleasant aroma. All the other yeast strains evolved unpleasant, smelly aroma. However, the situation was radically changed when the fermentation occured under semi-aerobic conditions. *Torulospora delbruecki* produced the pleasant aroma, exactly defined as vanilla. Also both strains of *P. anomala*, *Pichia membranefaciens, Sp. pararoseus* and one strain of *R. mucilaginosa* evoked very pleasant aroma, some of them with honey notes, some of them fruity or increased fermetative impression.

The results of better sensorial evaluation under semiaerobic conditions were subsequently tested by real winemaking fermentation. The problem which usually occurs in real process is that the present microfloras consists of several yeast genera which can possitively or negatively contribute to the wine quality.

The group of degustators confirmed that the wines were better evaluated when they were fermented under semiaerobic conditions, with the inocoluation by *S. cerevisae* not directly after fermentation, but several hours after grape pressing. The main advantage of this system is its ability to improve the originality of the wine.

Conclusions

We have found compounds which were typically produced by some non-*Saccharomyces* yeast strains – cyklopentanol, alfacyklogeraniol for *R. mucilaginosa,* sabinylacetate, for *Sp. pararoseus,* and izoamylbenzylether for *P. anomala*.

We can confirm that under anaerobic conditions, most of the apiculate microflora, except *T. delbruecki*, negatively affected wine aroma because they produced higher amounts of aldehydes – pentanal, hexanal, heptanal, 3,4-hexanedione, eicosene which caused the buttery and waxy odour.

However under semiaerobic conditions apiculate yeast species promoted positive aroma perception in products. *Torulospora* and *Pichia* yeast strains increased the fruit and/or coconut aroma by higher production of sabinyl res. isoamylacetate.

Semiaerobic conditions applied several hours prior inoculating by *S. cerevisiae* improve sensorial evaluation of wine and increase the support of originality and variety typicity.

However, the fermentation under such conditions is still very hazardous because oxidative defects or microbiological defaults of wine could occured.

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L02 APPROACHES TO MINIMIZATION OF ACRYLAMIDE LEVEL IN FOODS

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Introduction

Thermal treatment of foods is a common way for improvement of digestibility, safety, quality and sensory properties of many foods which is used for ages. Besides unambiguous desirable aspects of this treatment some detrimental effects are still emerging e.g. a loss of nutrition-worthy compounds and an undesirable generation of contaminants.

In 2002, Swedish researchers have first reported the formation of acrylamide in foods processed at elevated temperatures[1]. Recent assessment by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2005[2] confirmed that a risk cannot be excluded for dietary intake of acrylamide because it is classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC)[3]. In that assessment JECFA concluded that the margin of exposure for average and high consumers were low for compound that is genotoxic and carcinogenic and that this may indicate a human health concern. Therefore the Commission Recommendation since 2007 announced that "appropriate efforts to reduce acrylamide concentrations in foodstuffs should continue"[4]. Moreover, with respect to the last observations confirming the association between acrylamide intake and endometrial, ovarian[5], and breast[6] cancer risk, the concern on the acrylamide mitigation activity is very urgent.

Occurrence of Acrylamide in Thermally Treated Foods

After the discovery of acrylamide, a lot of studies confirmed the presence of acrylamide in nearly all fried, baked and roasted foods. Acrylamide exposure varies depending upon the population´s eating habits and the way the foods are processed and prepared. Generally, fried potato products, ready-to-eat breakfast cereals, baked goods and roasted coffee are the most important food categories that contribute most to acrylamide exposure. An average long-term exposure of acrylamide was estimated of 0.3 to 0.8 μg (kg body weight) $^{-1}$ day^{-1} on the base of the few data which were available at the FAO/WHO Consultation in 2002[7]. Based on the reported data, the Committee JECFA in 2005[2] noted that children may have intakes of acrylamide around two or three times higher those of adult consumers when expressed on a body weight basis. It is expected that children and adolescents have consumption patterns different from adults. Most of the types of foods in which acrylamide was detected are popular among children and adolescents, such as French fries, snacks, biscuits and breads. Moreover, they have a lower average body weight and, consequently, a higher average food intake per kilogram body weight than adults. For that, acrylamide intake by these individuals is considered a concern.

Mechanism of Acrylamide Formation

Initial results on acrylamide content indicated carbohydrate-rich foods to generate relatively more acrylamide[1]. Several researchers have established that the main pathway of acrylamide formation in foods is linked to the Maillard reaction and, in particular, the amino acid asparagine[8,9]. The link of acrylamide to asparagine, which directly provides the backbone of the acrylamide molecule, has been established by labelling experiments[9,10]. Study to date clearly shows that the amino acid asparagine is mainly responsible for acrylamide formation in heated foods after condensation with reducing sugars or a carbonyl source. Moreover, the sugar-asparagine adduct, N-glycosylasparagine, generates high amounts of acrylamide, suggesting the early Maillard reaction as a major source of acrylamide[9]. In addition, decarboxylated asparagine (3-aminopropionamid), when heated can generate acrylamide in the absence of reducing sugars[10]. A good evidence supporting the early Maillard rection as a main reaction pathway involving early decarboxylation of the Schiff base, rearrangement to the resulting Amadori product, and subsequent beta-elimination to release acrylamide has been presented[11].

Factors Affecting Acrylamide Formation in Foods

The resulting acrylamide concentration in foods ultimately depends on both products and process variables. Acrylamide formation requires the amino acid asparagine and a carbonyl compound as the Maillard reaction precursors. The concentration of acrylamide precursors and temperature mainly affect the rate of acrylamide formation. It is a fact that formation and degradation of acrylamide occurs in the same time during heating at elevated temperatures, it means that measured acrylamide content of a food is net result of two consecutive reactions occurred during thermal processing[12].

Based on the current knowledge of the mechanism of acrylamide formation, many parameters affecting the level of acrylamide in foods were investigated, e.g. heat intake, the level and type of saccharides and amino acids, moisture and water activity, additives, processing steps etc.[13], and consequently various ways of acrylamide minimization in foods have been proposed. Many of them are summarized in a "living" document "The Acrylamide Toolbox" published by experts associated in the Confederation of the Food and Drink Industries of the European Union (CIAA)[14].

The mitigation approach is divided in two strategies:

- Prevention of acrylamide formation through a modification of food composition (a decline of asparagine and reducing saccharides contents), processing conditions (thermal input, pH, moisture), an addition of compounds suppressing the formation of acrylamide (acids, enzymes, proteins, antioxidants etc.) and an enhancement of processing steps (pre-treatment, blanching, fermentation etc.)[15].
- Facilitation the acrylamide elimination through storage conditions or the initialization of acrylamide polymerization[16,17].

Temperature plays an important role in the formation and elimination of acrylamide. It is well known that acrylamide forms in foods that are cooked at high temperatures (>120 °C)[8,13,18]. For shorter heating times as in the frying operation of potato chips or strips, lowering the frying temperature may significantly reduce the amount of acrylamide formed (Fig. 1.). The same may not be true for longer heating periods as in the roasting of coffee beans where extending the operation may result in a decrease in the amount of acrylamide persisted in the final product[19]. They may be a critical temperature/time zone where acrylamide is formed at a greater rate than it is destroyed, at temperatures outside of this zone little acrylamide is present.

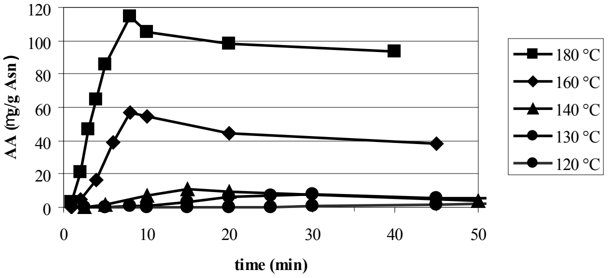

Fig. 1. **Amount of acrylamide after heating of equimolar mixture of glucose and asparagine at different temperatures**

The fact that acrylamide is not formed during boiling indicates that higher temperatures and/or low moisture conditions are needed for its formation. During heating under atmospheric conditions, higher temperatures can be reached only if simultaneous drying takes place, which is the case in frying, baking and roasting. The loss of water as the food dries during heating extracts a large amount of the incoming energy, and hence a bulk of the product is at a temperature very much lower than that of the heating medium. In this respect, temperature, time and moisture are key drivers of acrylamide formation in foods during heating (Fig. 2.). The moisture content determines the physical state and mobility of chemical constituents in food matrix. In addition, water alone affects the chemical route and the mechanistic pathway for acrylamide formation[13].

Concerning reducing sugars as carbonyl source, fructose has been found more effective than glucose in forming acrylamide (Fig. 2.). Both the chemical reactivity of sugars and their physical state play an important role in acrylamide formation. The melting point of fructose and glucose are 126 °C and 157 °C, respectively[13]. This explains why fructose is more reactive than glucose on acrylamide formation during heating. Frying, baking and roasting are simply characterized as open processes in which heat and mass transfer occur simultaneously. As the moisture reduces due to evaporation, sugars initially dissolved in water begin to form a saturated solution and then crystallize. After crystallization, melting is required to change their state to liquid, so to make them chemically reactive. In this respect, reducing sugars having a lower melting point is expected to form acrylamide earlier during heating.

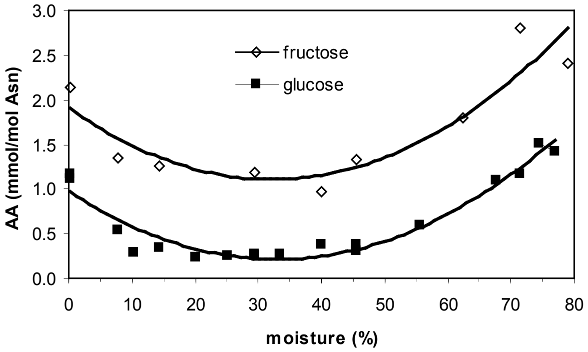

Fig. 2. **Amount of acrylamide after heating of equimolar mixture of asparagine and glucose/fructose at 180 °C for 20 min with different addition of water**

Enzyme Treatment Leading to Acrylamide Reduction

One of the most effective ways to avoid acrylamide formation is removing the precursors, particularly amino acid L-asparagine. L-asparaginase as an enzyme of the hydrolases group (EC 3.5.1.1.) selectively hydrolyses the amide bond of L-asparagine which results in the formation of aspartic acid and ammonia. Because the acrylamide formation correlates strongly with a free asparagine concentration, the reduction of L-asparagine in raw materials leads to the reduced level of acrylamide in final products[20]. The safety of asparaginase application is guaranteed by approving of GRAS status of *Aspergillus oryzae* asparaginase enzyme from Novozymes A/S[21], and *Aspergillus niger* asparaginase enzyme from DSM[22], and a positive evaluation from the JECFA in 2007[23] as well. Moreover, this enzyme is inactivated by high temperature in the process of proteolysis.

The application of L-asparaginase solution in a simulated potato matrix resulted in 50 to 90 % reduction of acrylamide content depending on the conditions (enzyme dose, time and temperature of incubation). No significant differences in impacts on L-asparagine conversion into L-aspartic acid in model samples between bacterial and fungal originated enzymes were observed. The positive effect of enzyme on the decrease of acrylamide content was confirmed also after L-asparaginase application on raw potato mash as well as dehydrated potato-wheat semi-products (Fig. 3. and Fig. 4.)[20].

It is known that each intervention in the technology can be accompanied with consequences on the quality and sensory properties of final products which are strongly connected with the acceptability by consumers. For that reason the preliminary sensory evaluation of thermally and enzymatically treated products was done by a panel of trained judges. They described the main properties important for these kinds of products such as darkness, yellowness, appearance, stickiness, crispness, oiliness, flavour, off-flavour, saltiness, sweetness and overall acceptability. Changes in colour were observed in pancakes prepared under different heating programmes, where darkness and crispness were more intensive in pancakes prepared at higher temperature of frying. No differences in evaluated sensory properties mentioned above were found out in any case of L-asparaginase application (P = 99 %) that was consider as a great advantage of the presented effective way of acrylamide reduction in food products[24].

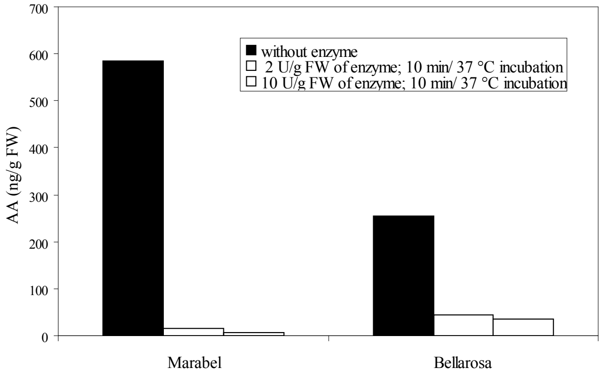

Fig. 3. **Amount of acrylamide (AA) in raw potatoes (varieties Marabel and Bellarosa) after enzymatic treatment (L-asparaginase produced by A. oryzae applied at concentration of 2 and 10 U g^{-1} FW and incubated at 37 °C for 10 min) and following heat treatment at 180 °C for 20 min**

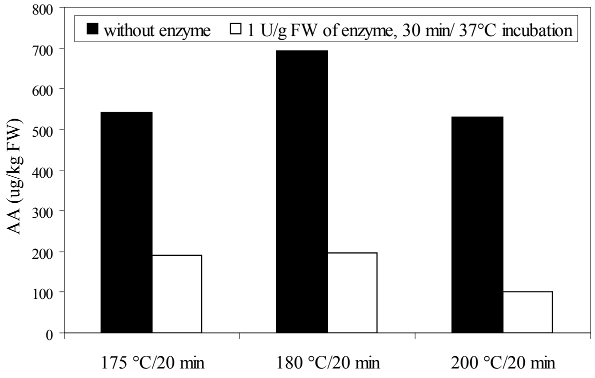

Fig. 4. **Amount of acrylamide (AA) in pancakes prepared from potato-wheat powder at different heating temperatures (175 °C, 180 °C and 200 °C) for 20 min with previous enzymatic treatment (L-asparaginase produced by A. oryzae applied at concentration of 1 U g^{-1} FW and incubated at 37 °C for 30 min)**

Conclusions

Since the acrylamide occurrence in foods and its potentiality to cause detrimental affects on human health attracts attention in all over the word, the effort to minimize its level in foods and consequently the human exposure to acrylamide is extremely advisable. Among many ways of acrylamide reduction the application of enzyme in order to prevent acrylamide formation is feasible and effective without any undesirable effect on sensory quality of final products. For that reason, this procedure is protected by the patent application filed with the Industrial Property Office of the Slovak Republic under the number 5027-2006.

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L03 SOLID STATE FERMENTATION AS A TOOL FOR PREPARATION OF BIOPRODUCTS ENRICHED WITH POLYUNSATURATED FATTY ACIDS

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Introduction

Increasing demand for high-value lipids has focused commercial attention on the provision of suitable biosynthetic framework for their production. One of the main target for microbial oil transformation is construction of healthy and dietary important polyunsaturated fatty acids, such as γ-linolenic acid (18 : 3 ω-6; GLA), dihomo-γ-linolenic acid (20 : 3 ω-6; DGLA), arachidonic acid (20 : 4 ω-6; AA), eicosapentaenoic acid (20 : 5 ω-3; EPA) and docosahexaenoic acid (22 : 6 ω-3; DHA). Their applications in biomedical, nutritional and pharmaceutical fields coupled with their inadequacy from conventional agricultural and animal sources has looked for developing suitable biotechnologies to produce these compounds[1].

Particularly active in the synthesis of PUFAs are species of fungi belonging to *Zygomycetes*[2]. Oleaginous fungi producing PUFA could be economically valuable because the most of their PUFAs occur in the triacylglycerol fraction of their lipids. Two basic processes have been developed for microbial production of PUFAs: submerged and solid state fermentations[3,4]. However, the principal difficulty that has been experienced with submerged PUFA-riched oil production has been in its marketing rather than in developing the large-scale fermentation and oil extraction process. Therefore, the association of oleaginous fungi with solid state fermentations (SSF) has been developed in order to improve commercial potential of microbial oils and thus to create new perspectives for the economic competitiveness and market of microbial polyunsaturated fatty acids (PUFAs). Solid state fermentation is a process in which microorganisms grow on a moist solid substrate in the absence of free water[5]. SSF simulates fermentation reactions occurring in the nature and allows microbial utilization of raw agro-materials or byproducts of the agro-food industries. Because some oleaginous fungi simultaneously decrease anti-nutrient compounds in the substrates (e.g. phytic acid) and partially hydrolyze substrate biopolymers, prefermented mass with a high content of PUFAs may be used as inexpensive food and feed supplement[6]. Thus, SSF might provide the other opportunity to fill marketing claims in food, feed, pharmaceutical, veterinary and environmental fields.

This paper deals with effectivity of several lower filamentous fungi to synthesize various PUFAs during their utilization of cereals by solid state fermentations.

Experimental

Microorganisms

Thamnidium elegans CCF 1456, *Cunninghamella echinulata* CCF-103, *Cunninghamella elegans* CCF-1318, *Mortierella isabelina* CCF-14, *Mortierella isabelina* CCF-1098, *Mortierella alpina* CCF 185 were obtained from the Culture Collection of Fungi (Charles University, Prague, Czech Republic). The culture was maintained on modified Czapek-Dox agar slants with yeast extract (2.5 g dm^{-3}) at 4 °C.

Substrates and Cultivation Conditions

Depending on the microorganism, various types of substrates were employed during SSF experiments. Spent malt grains (SMG) were added to some substrates. Autoclavable microporous polypropylene bags (160 × 270 mm^2) were filled with 10 g of dry substrate, moistened by the addition of 10 ml distilled water, soaked for 2 h at laboratory temperature and sterilized in autoclave (120 kPa, 120 °C, 20 min). In order to increased yield of PUFAs, sunflower or linseed oils were added to some substrates. In addition, various amounts of 10% acetone or ethanol solutions of selected plant extracts were tested with the aim to activate enzymes involved into PUFA biosynthesis. The substrates were inoculated and mixed with 2 ml of spore suspension (1–2 × 10^6 spores per ml). Then each bag was closed with sterile cotton plugs, inoculated substrate was spread in the bags to obtain substrate layer of about 1 cm and incubated statically at 25 °C for 4–6 days (*T. elegans*, *C. echinulata*, *C. elegans*, *M. isabellina*) and 10–14 days (*M. alpina*). Triplicate SSF experiments for each substrate were prepared to assess reproducibility and average results are presented.

Lipid Extraction and Fatty Acid Analysis

Prefermented cereal materials (bioproducts) were gently dried at 65 °C for 10 h and weighed. Lipids from homogenized bioproducts were isolated with chloroform/methanol (2 : 1, v/v) and purified according to Čertík et al.[7] and total lipids were determined gravimetrically. Fatty acids of total lipids were analyzed as their methyl esters[8] by gas chromatography according to Čertík et al[9]. Gas chromatograph (GC-6890 N, Agilent Technologies) was equipped with a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies) and a FID detector (constant flow, hydrogen 35 ml min^{-1}, air 350 ml min^{-1}, 250 °C). Analyses were carried out under a temperature gradient (130 °C for 1 min; 130–170 °C at program rate 6.5 °C min^{-1}; 170–215°C at program rate 2.7°C min^{-1}; 215 °C for 7 min; 220–240 °C at program rate 2 °C min^{-1}; 240 °C for 2 min) with hydrogen as a carrier gas (flow 2.1 ml min^{-1}, velocity 49 cm s^{-1}, pressure 174 kPa) and a split ratio of 1/50 (inlets: heater 230 °C, total hydrogen flow 114 ml min^{-1}, pressure 174 kPa). The fatty acid methylester peaks were identified by authentic standards of C_4–C_{24} fatty acid methylesters mixture (Supelco, USA) and quantified by an internal standard of heptadecanoic acid

(C17:0, Supelco, USA). Fatty acid concentration was evaluated with ChemStation software B0103 (Agilent technologies, USA).

Results and Discussion

The extensive research and development of PUFA production by SSF is basically aimed at improving the economic competitiveness of that microbial process compared to plant- and animal-derived oils. Emphasis is put on increasing the product value, using inexpensive substrates, screening for more efficient strains and reducing the processing steps. Therefore, it is necessary to optimize the potential of microorganisms for transformation of agroindustrial materials and oil residues into desired metabolites.

Screening of microorganisms has led to selection of *T. elegans*, *C. echinulata*, *C. elegans* and *Mortierella isabellina* as producers of GLA[6,9] and *Mortierella alpina* as a producer of DGLA, AA and EPA[10]. Generally, the surface of substrates was not only covered by the fungal mycelium during cultivation, but the fungal hyphae also penetrated into the substrates. Thus, fungal PUFAs were accumulated in the newly formed bioproduct and their amount depended on the substrates, microorganisms and cultivation conditions used.

Depending on the microorganism, various types of cereal substrates were employed during SSF experiments (Table I). Spent malt grains (SMG) served as an internal support. Substrates without SMG in most cases led to agglomeration of substrate particles and created more compact mass which in turn interfered with microbial respiration and affected substrate utilization negatively. Presence of SMG improved bioconversion of linoleic acid from substrates to GLA[9]. Substrates with internal support not only provided better respiration and aeration efficiency due to an increased inter-particle space but also helped to remove the heat generated during fermentation. It should be noticed that although PUFAs were synthesized more effectively by SMG addition to substrates, total PUFAs yield was also dependent on substrate/SMG ratio. Unbalanced substrate/SMG ratio might provide limited surface for microbial attack and thus poorer availability of assimilable compounds (including oils) from substrates.

Growth of fungi on a carbohydrates-containing substrates resulted after optimization of cultural conditions in constant lipid yield with the demanded fatty acid profile. Further improvement of PUFAs formation was achieved by physiological regulation of the SSF process employing following steps[10]: a) gradual elevation of carbon/nitrogen ratio with addition of appropriate carbon source; b) optimization of water activity, temperature and oxygen availability; c) transformation of exogenously added oils consisting of precursor of PUFAs. There is a stock of relatively cheap vegetable oils containing individual fatty acid precursors and SSF was applied for microbial utilization of renewable agricultural oils with the aim to modify their properties for production of value-added bioproducts with enhanced biological characteristics. Thus the ability of the strains to utilize exogenous fatty acids opens new possibilities to prepare PUFAs in high yield. Moreover, because fungi possess active oil-biotransforming system, these strains were also tested for their ability to convert directly oil-rich substrates (corn, sunflower seeds, linseeds, rapeseeds) to PUFAs.

Table I

Production of γ-linolenic acid (GLA), dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA) by solid state fermentations of selected fungi utilizing various cereal substrates. Ratio of susbtrate/SMG was 1:3 (w/w)

Strain	Substrate	PUFA	Yield [$\mathrm{g\,kg^{-1}}$ BP]
T. elegans	oat flakes/SMG	GLA	5.9
	wheat bran/SMG	GLA	5.0
	wheat bran/SMG/ sunflower oil	GLA	10.0
	crushed corn	GLA	10.0
	rye bran/SMG	GLA	4.2
	buckwheat/SMG	GLA	4.7
	millet/SMG	GLA	6.5
	amaranth/SMG	GLA	4.7
C. echinulata	barley	GLA	6.1
C. elegans	barley	GLA	7.0
M. isabellina	barley	GLA	18.0
M. alpina	rice	AA	21.4
	wheat sprout/SMG	AA	36.1
	wheat bran/SMG	AA	42.3
	rye bran/SMG	AA	21.9
	peeled barley	AA	16.2
	oat flakes	AA	31.2
M. aplina	cresed sesame seeds	DGLA	21.3
M. alpina	peeld barley/linseed oil/SMG	EPA/AA	23.4/36.3

Biosynthesis and profile of fatty acids is controlled by enzymes involved in lipogenesis, so activation or inhibition of these metabolic steps is also useful tool for improving carbon flux to individual PUFAs[11]. For example, bioconversion of DGLA to AA is catalyzed by ‚Δ^5' desaturase and inhibition of this enzyme by crushed sesame seeds was accompanied by rapid increase of DGLA/AA ratio[11]). In addition, application of various plant extracts possessing bioactive compounds seems to be promising way how to regulate fatty acid biosynthetic machinery in order to gain bioproduct with high yield of preferred PUFA. Application of ethanol extracts from ginger or sweet flag improved GLA yield by 30 % or 25 %, respectively. On the other hand, biosynthesis of GLA was reduced by 70 % when acetone extract from tansy was employed to the substrate.

Conclusions

Naturally prepared cereal based bioproducts enriched with PUFAs may be used as an inexpensive food and feed supplement. Thus, the association of selected microorganisms with solid state fermentations has created new perspectives for the economic competitiveness and market of cereal based bioproducts containing PUFAs.

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L05 PHYSIOLOGICAL REGULATION OF BIOTECHNOLOGICAL PRODUCTION OF CAROTENOID PIGMENTS

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Introduction

Carotenoids represent one of the broadest group of natural antioxidants (over 600 characterized structurally) with significant biological effects and numerous of industrial applications. Because the application of synthetically prepared carotenoids as food additives has been strictly regulated in recent years, huge commercial demand for natural carotenoids has focused attention on developing of suitable biotechnological techniques for their production.

There are many microorganisms including bacteria, algae, yeast and fungi, that are able to accumulate several types of pigments; but only a few of them have been exploited commercially[1]. From the view of yeasts, a range of species such as *Rhodotorula*, *Rhodosporidium*, *Sporidiobolus*, *Sporobolomyces*, *Cystofilobasidium*, *Kockovaella* and *Phaffia* have been screened for carotenoids formation. Yeast strains of *Rhodotorula* and *Sporobolomyces* formed β-carotene as the main pigment together with torulene and torularhodine as minor carotenoids. In contrast, *Phaffia* strains accumulated astaxanthin as a principal carotenoid. Comparative success in yeast pigment production has led to a flourishing interest in the development of fermentation processes in commercial production levels. However, in order to improve the yield of carotenoid pigments and subsequently decrease the cost of this biotechnological process, optimizing the culture conditions including both nutritional and physical factors have been performed. Factors such as carbon and nitrogen sources, minerals, vitamins, pH, aeration, temperature, light and stress showed a major influence on cell growth and yield of carotenoids.

This paper summarizes our experience with physiological regulation and scale-up of biotechnological production of carotenoid pigments by yeasts.

Experimental

Microorganisms and Cultivation Conditions

All strains investigated in this study (*Sporobolomyces roseus* CCY 19-6-4, *S. salmonicolor* CCY 19-4-10, *Rhodotorula glutinis* CCY 20-2-26, *R. glutinis* CCY 20-2-31, *R. glutinis* CCY 20-2-33, *R. rubra* CCY 20-7-28, *R. aurantiaca* CCY 20-9-7 and *Phaffia rhodozyma* CCY 77-1-1) were obtained from the Culture Collection of Yeasts (CCY; Institute of Chemistry, Slovak Academy of Sciences, Bratislava) and maintained on malt slant agar at 4 °C.

The basic cultivation medium for flasks experiments for *Rhodotorula* and *Sporobolomyces* strains consisted of (g dm^{-3}): glucose – 20; yeast extract – 4.0; $(NH_4)_2SO_4$ – 10; KH_2PO_4 – 1; $K_2HPO_4 \cdot 3H_2O$ – 0.2; NaCl – 0.1; $CaCl_2$ – 0.1; $MgSO_4 \cdot 7H_2O$ – 0.5 and 1 ml solution of microelements [(mg dm^{-3}): H_3BO_4 – 1.25; $CuSO_4 \cdot 5H_2O$ – 0.1; KI – 0.25; $MnSO_4 \cdot 5H_2O$ – 1; $FeCl_3 \cdot 6H_2O$ – 0.5; $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$ – 0.5 and $ZnSO_4 \cdot 7H_2O$ – 1]. The basic cultivation medium for flasks experiments for *Phaffia* strain consisted of (g dm^{-3}): glucose – 20, yeast autolysate – 2.0, KH_2PO_4 – 0.4, $(NH_4)_2SO_4$ – 2.0, $MgSO_4 \cdot 7H_2O$ – 0.5, $CaCl_2$ – 0.1, NaCl – 1.0. All strains grew under a non-lethal and maximally tolerated concentration of Ni^{2+}, Zn^{2+}, Cd^{2+} and Se^{2+} ions. Also, stress conditions were induced by addition of various conventrations of NaCl and H_2O_2. The cultures were cultivated in 500 ml flasks containing 250 ml cultivation medium on a rotary shaker (150 rpm) at 28 °C to early stationary grow phase. All cultivation experiments were carried out at triplicates and analyzed individually.

Flasks results were verified in bioreactors and these scale-up experiments were carried out in 2 L fermentor (B. Braun Biotech), 20 L (SLF-20) and 100 L (Bio-la-fite) fermentors with an agitation rate of 250–450 rpm and a temperature of 20–22 °C. The pH was controlled at pH 5.0 by the addition of NH_4OH and the dissolved oxygen concentration was maintained by supplying sterile air at a flow rate equivalent to 0.3–0.7 vvm.

Pigment Isolation and Analysis

Pigments from homogenized bioproducts were isolated by organic extraction and analyzed by high-performance liquid chromatography (HPLC). Analysis was carried out with an HP 1100 chromatograph (Agilent) equipped with a UV-VIS detector. Pigments extracts (10 µl) were injected onto LiChrospher® 100 RP-18 (5 µm) column (Merck). The solvent system (the flow rate was 1 ml min^{-1}) consisted of solvent A, acetonitrile/water/formic acid 86 : 10 : 4 (v/v/v), and B, ethyl acetate/formic acid 96 : 4 (v/v), with a gradient of 100 % A at 0 min, 100 % B at 20 min, and 100 % A at 30 min.

Gel Electrophophoresis

1D PAGE-SDS electrophoresis of proteins was carried out by common procedure using 10% and 12.5% polyacrylamide gels. Proteins were staining by Coomassie Blue and by silver staining. For comparison, microfluidic technique using 1D Experion system (BioRad) and P260 chips was used for yeast protein analysis too. 2D electrophoresis of proteins was optimized in cooperation with Laboratory of Functional Genomics and Proteomics, Faculty of Science, Masaryk University of Brno. 2D gels were obtained from protein preparatives isolated from lyophilized cells. After optimization of separation conditions proteomes from stressed *R.glutinis* and *R.rubra* cells were isolated, lyophilized and analyzed. Quantitative analysis was done using BioRad Laboratories 2D software. Identification of some spots was done using LC-MS/MS.

Results and Disscussion

To find suitable conditions for carotenoids production, several pigment forming yeasts have been tested for their ability to modify carotenoid biosynthesis based on media composition. Kinetic analysis revealed that yeasts strains differed from the view of growth rates and pigments formation. It must be emphasized that overall yield of carotenoids is directly related to the total biomass yield, thus, to keep both high growth rates and high flow carbon efficiency to carotenoids by optimal cultivation conditions is essential in order to achieve the maximal pigment productivity.

Carotenoid accumulation in cells was significantly activated when yeasts were treated by selected stress conditions and their combinations (heavy metals, hydrogen peroxide and salt). However, such stimulation of pigment biosynthesis was more effective if stress factors were employed to the medium in exponential growth phase than from the beginning of cultivation[2–4]. It may be explained by hypothesizing a possible activation or inhibition mechanism by metal ions on specific carotenogenic enzymes, in particular, on specific enzymes involved in carotenoid biosynthesis, in agreement with previous studies reporting activation or inhibition by metal ions in microbial enzymes. The other explanation is based on observations that presence of heavy metals results in formation of various active oxygen radicals what, in a turn, induces generation of protective carotenoid metabolites that reduce negative behaviour of free radicals[5].

During environmental stress response many red yeasts exhibite cross-protective mechanisms. Preincubation of yeast culture with low concentration of one stress factor in inoculum media (e.g. salt, hydrogen peroxide) induces adaptation pathways resulted in enhanced carotenoid production[6]. Further addition of higher concentration of either the same or other stress factor can lead to significant (5–10 times) increase of β-carotene production in *Rhodotorula glutinis* and *Sporobolomyces salmonicolor*[2]. Simple preincubation of *R. glutinis* in presence of 2% NaCl in inoculation medium followed by fermentation in inorganic production medium led to increased formation of biomass with accumulated carotenoids. This combined environmental stress using mild stress effect of salt or hydrogen peroxide could be industrially used for production of carotene enriched biomass. The results from flasks experiments are summarized in Table I.

Table I
Production of carotenoids in flask and fermentation experiments

Strain	Pigment	Pigment yields
		Flasks experiments
S. roseus	β-carotene	30 mg dm^{-3} (by Zn^{2+} + H_2O_2)
R. glutinis	torulene	6 mg dm^{-3} (by Zn^{2+} + H_2O_2)
R. glutinis	torularhodin	1 mg dm^{-3} (by Cu^{2+})
P. rhodozyma	astaxanthin	20 mg dm^{-3} (by Se^{2+})
		Scale-up fermentations
S. salmonicolor.	total carotene	41 mg dm^{-3} (by NaCl + H_2O_2)
R. glutinis	β-carotene	35 mg dm^{-3} (by NaCl + H_2O_2)
P. rhodozyma	astaxanthin	110 mg dm^{-3} (by NaCl + NaCl)

Laboratory flasks experiments were verified in semi-scale conditions (from 2 dm^3 to 100 dm^3 fermentors). It was confirmend that glucose utilization was followed by increased pigment biosynthesis. Therefore, fed-batch fermentations were applied to enhanced carotenoid production. In addition, combination of aimed environmental condition resulted in activated accumulation of selected carotenoids (Table I).

Enhancement of carotenoid production by environmental stress is also associated with changes in expression levels of various genes. Therefore, molecular changes in yeast cells on genome, proteome and metabolome level were studied using PFGE, 2D-GE, LC/MS/MS and EPR techniques. Presence of stress factors was accompanied by changes in carotenoid production as well as by alternations in protein levels. However, further analyses have to be focused on more precise characterization of proteins those displayed significant changes under increased biosynthesis of carotenoids.

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L06 ENZYMATIC SACCHARIDE ACETYLATIONS IN WATER – COMPARATION OF CATALYSTS

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Introduction

Enzymatic esterification of various hydroxy compounds is studied for about 40 years. Such reactions are usually performed in reaction systems comprising controlled amount of water to prevent product hydrolysis. Followingly, enzymatic esterification of unprotected saccharides is hampered by their decreased solubility in low water envíronment.

We had recently described acetyl esterase from *Trichoderma reesei* to catalyze acetylations of several carbohydrates and alcohols in bulk water phase[1]. Acetyl esterases may be found in several raw commercial preparations of enzymes dedicated for biomass degradation since acetylation occurs widely on natural polysaccharides and plant cell wall fragments[2]. Acetyl hydrolyzing activity occurs quite frequently in lipases (which may be result either of wider acyl specifity or of protein impurity) and some reports describe also esterase activity of serum albumins[3].

Our present work explores performance of several enzymes/crude enzyme preparations and proteins (Celluclast 1.5 L FG, Ultraflo L, lipases from *Candida antarctica*, *Candida rugosa*, *Aspergillus niger* and *Thermomyces lanuginosus*, bovine serum albumin) in acetylations of glucose (**1 a**) and methyl α-D-glucopyranoside (**1 b**), comparing product yields and position selectivity of the reaction.

Experimental

Assays of acetyl esterase activity in enzyme preparations were performed as previously described[1]. Synthetic reactions were routinely performed by dissolving 1.2 g of glucose or *alpha* methyl glucoside in 10 ml mixture of water and acetyl donor (vinyl acetate or isopropenyl acetate, 4 : 1) and the reaction was started by adding 1 ml or 300 mg of the enzyme. Reaction mixture was then stirred at 37 °C for 1–3 days and the course of the reaction was followed by t.l.c. on silica gel plates (chloroform – methanol, 3 : 1, visualisation by pouring into 5% sulphuric acid in ethanol and drying in hot air). Reaction mixture was then concentrated *in vacuo*, products separated by chromatography on silicagel column (chloroform – methanol, 3 : 1) and identified by NMR. For every reaction, a parallel experiment was executed without addition of enzyme to check occurence of a spontaneous acetylation. The spontaneous reactions were tested also separately in 0.1M acetate and phosphate buffers.

Results

Table I presents level of acetyl hydrolysing activities in enzyme preparations. Although water insoluble Lipolase 100 was not active in photometric assay, it catalyzed acetylations of aliphatic alcohols in stired low water reactions (data not shown).

Table I
Acetyl hydrolysing activity of enzyme preparations

enzyme (souce)	activity [U ml^{-1}]	activity [U g^{-1}]
Celluclast 1.5 L FG (*Trichoderma reesei*)	18.4	
Ultraflo L (*Humicola insolens*)	31.5	
Novozym 735 (*Candida antarctica*)	55.6	
BSA (*Bos taurus*)		4.0
Amano Lipase A (*Aspergillus niger*)		219.1
Lipase AYS (*Candida rugosa*)		621.1
Lipolyve CC (*Candida rugosa*)		319.0
Lipolase 100T (*Thermomyces lanuginosus*)		not estimated

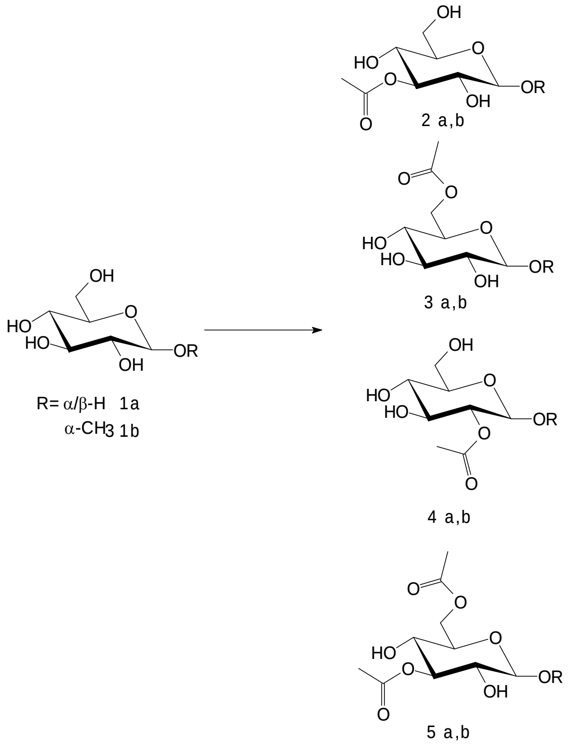
Since phosphate buffer catalyzes acetylation of saccharides by vinyl acetate[4], we tried to assess the extent of this process in our reactions (Table II).

Table II
Sponatneous acetylation of glucose by vinyl acetate (VA) and isopropenyl acetate (IPA) in buffered solutions and water

pH	Acetyl donor	Monoacetates	Diacetates
4.01	VA	+	−
4.51	VA	++	−
5.02	VA	++	+/–
5.51	VA	+++	+
5.72	VA	+++	+
6.03	VA	+++	+
Water	VA	−	−
Water	IPA	−	−
5.72	IPA	+/–	−

Increasing pH of the buffer resulted in increased creation of glucose monoacetates and eventually diacetates when vinyl acetate was used as acetyl donor. To avoid such unselective acetylations, all enzymatic reactions were executed using water instead of the buffer.

Scheme 1 summarizes in general the scale of products identified in reaction mixtures.



Scheme 1
Summarization of products created by enzymatic acetylations of glucose and methyl α-D-glucopyranoside with vinyl acetate or isoprenyl acetate

Yeast lipases gave low yields of pure **3 a,b**, thus showing high specifity for acetylation of primary hydroxyl (Tables 3,4). However, Lipolyve CC was reactive only with the glucoside **1 b** and did not acetylate free glucose within 3 days.

On the other side, Amano Lipase A (*Aspergillus niger*) as a representative of fungal lipases gave 10% yield of an equimolar mixture of **2 a**, and **3 a**. Interestingly, acetylation did continue to create only one diacetate (3,6-di-O-acetyl glucose, **5 a**), thus keeping the original specifity for positions 3-O- and 6-O- (Table III). Acetylations of methyl α-D-glucopyranoside (**1 b**) with isopropenyl acetate proceeded with the same positional specifity to produce **2 b** and **3 b**. When isopropenyl acetate was replaced by vinyl acetate as acetyl donor, selectivity increased markedly in favor of **2 b** (Table IV). No reaction was observed when another fungal lipase – Lipolase 100T – was used as biocatalyst.

Table III
Enzymatic acetylations of glucose by vinyl acetate (VA) and isopropenyl acetate (IPA) in water

enzyme	acetyl donor	mono-acetates [%]	diacetates [%]	reaction time [days]
Celluclast	VA	19.7 **2 a** : **3 a** 12 : 1	0	1
Lipolyve CC	IPA	0	0	3
Novozym 735	VA	2.0 **3 a**	9.6 not determined	3
Amano Lipase A	IPA	10.0 **2 a** : **3 a** 1:1	4.2 **5 a**	2

Acetylations of **1 b** catalyzed by bovine serum albumin and Ultraflo L (an enzyme cocktail from *Humicola insolens*) were unselective, giving mixtures of three monoacetates (Table IV).

The best results were achieved by use of Celluclast, a cellulase preparation from *Trichoderma reesei* RUT C-30 comprising the same acetyl esterase as reported earlier[1]. Acetylations gave 19.7 % and 10 % of monoacetylated glucose and methyl glucoside, respectively (Tables III, IV). The selectivity was exclusively to position 3-O- in acetylation of **1 b** while in acetylation of glucose, almost 8% of 6-O- acetate **3 a** was found in the monoacetate fraction.

Table IV
Enzymatic acetylations of methyl glucoside by vinyl acetate (VA) and isopropenyl acetate (IPA) in water

enzyme	acetyl donor	mono-acetates [%]	diacetates [%]	reaction time [days]
Celluclast	IPA	10.0 **2 b**	0	1
Lipolyve CC	IPA	2.2 **3 b**	0	0.9
Lipase AYS	IPA	1.9 **3 b**	0	0.16
Amano Lipase A	IPA	10.5 **2 b** : **3 b** 2,5 : 1	1.7 **5 b**	1
Amano Lipase A	VA	6.4 **2 b** : **3 b** 13 : 1	0.7 **5 b**	1
BSA	VA	2.1 **3 b** : **4 b** : **2 b** 2.0 : 1.3 : 1.0	0	5
Ultraflo L	IPA	3.9 **3 b** : **4 b** : **2 b** 4.5 : 2.5 : 1.0	0	1

Conclusions

Our results proved Celluclast to be the best catalyst for acetylations of glucose and methyl α-D-glucopyranoside according to its selectivity, product yields, availability and price. Since Celluclast has significantly lower acetyl esterase activity comparing to the other enzymes tested in this study, the enzyme seems rather unique. The enzyme selectivity to position 3-O- gives a promiss of its use in two step enzymatic esterifications of saccharides, in which acetylation provides organic solvent-soluble sugar derivatives possessing free primary hydroxyls.

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L07 PREPARATION OF BIODIESEL FROM TALL OIL

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Introduction

It has known that after delignification kraft pulping process almost all extractable dissolved or emulsified substances of wood pass into black liquor. Evaporation of water from black liquor causes an increase in sodium salts content. Soaps are settled on all places, where liquor remains motionless. Tall soaps flowing on surface are separated in the form of foam in all tanks and their amount increases with density of black liquor[1–3]. Tall soap may be incinerated together with higher-density black liquor within chemicals recovery or separated and utilised to high value chemicals. The latter alternative is more cost effective[4]. Kraft tall soap (TS) may be converted by acidification to tall oil (TO) and individual components isolated from it by vacuum distilation.

TO is a dark brown, viscous and odorous liquid[5] containing mainly higher fatty acids (HFA), resin acids (RA), sterols and many other extractable substances[6].

Very interesting is HFA content ranging from 26 to 58 % wt.[7] and depending mainly on the sort of wood. Given the high amount of wood, which is delignificated in paper mill in Central-european region, it represents a cheap raw material for biodiesel production. Biodiesel is exclusively produced from expensive vegetable oil.

This work is focused on biodiesel production from tall oil and testing of selected biodiesel properties and their comparison with EN 14214[8]. A method based on esterification straight by methanol and subsequent vacuum distillation of methylesters HFA from mixture was chosen. The method seems to be more advantageous than that based on esterification of HFA fraction obtained preferentially by vacuum distillation of TO. Namely, boiling point of HFA methylesters is lower than that of HFA.

Our method is more efficient from the viewpoint of energy demands when compared with the method realised, e.g., by AltÂparmak D., Keskin A., Koca A., Gürü M., [9].

Experimental

Materials

Chemicals: sulphuric acid, 96 % wt., Microchem, SR,

Tall oil was prepared from aqueous solution of tall soap supplied by MONDI Packaging Paper Štětí, ČR.

Instruments, Facilities, Methods

Gas chromatography and mass spectrometry (GC/MS). Analysis of tall oil composition was performed by GC/MS with a Hewlett – Packard 5890A/5790B equipment.

GC conditions: column: PTE–5, 30 m, 0.25 mm ID, 0.25 μm film, carrier gas: helium, flow-rate 1 ml min^{-1}, pressure 50 kPa , purge off: 0 min (split), injection temperature: 275 °C, temperature of detector: 280 °C, temperature program: 170 °C (3 min), 5 °C min^{-1} – 270 °C (9 min), 15 °C min^{-1} – 300 °C (3 min)

MS conditions: mass range: 43–550 amu, EMV: 2400 V, threshold setting: 1000, SD: 3 min

IR spectroscopy. Infrared spectra of all specimens were obtained by Digilab Excalibur FTS 3000MX – FTIR method. Samples were measured by diffusion scatter method (DRIFT) in KBr powder (5 % wt.).

Vacuum (molecular) distillation. A laboratory molecular vacuum evaporator MO15[10] was used to isolate methylesters HFA and also to purify this product.

Other laboratory equipment: furnace, balance, centrifuge, vacuum rotary evaporator.

TO preparation from TS was carried out at following conditions:

Table I
Conditions of tall oil preparation from tall soap

Mass TS	~ 1,000 g
Heating time TS 25 → 95 °C	~ 15 min.
H_2SO_4 concentration	43.6 wt. %
Volume of added H_2SO_4 solution	1.5 dm^3
Reaction time	200 min. (3.3 h)
Agitation	intensive (3.3 h)

TO was consequently esterified at conditions:

Table II
Conditions of tall oil esterification by CH_3OH

Conc. sulphuric acid [H_2SO_4, 96 % wt.] of the mass of HFA in TO	5 % wt.
Temperature of reaction mixture	60 °C
Molar ratio of CH_3OH to HFA in TO	6 : 1
Reaction time	5.5 h
Intensive agitation of reaction mixture	5.5 h

Table III
Properties of tall soap and tall oil

Dry matter [% wt.]	65.56
Ash [% wt.]	11.36
TO in TS [% wt.]	84.00
Acid number of prepared TO [mg KOH g^{-1} TO]	154.70

Results and Discussion

Preparation of TO

TO was prepared by TS acidification by sulphuric acid at conditions given in Table I. Sulphuric acid was added in excess. Reaction mixture undergoes vigorous agitation during the process.

At the beginning, about 1,000 g TS (one batch) was heated in a 4,000 ml beaker in water bath. Temperature of water bath was kept in the range of 90–95 °C by adjustable cooker. After heating of TS at the water bath temperature, sulphuric acid was added and a blender activated. The blender ensured intensive agitation during the whole process. At TO preparation, the following reaction proceeded:

$$R—COONa + H_3O^+ \rightarrow R—COOH + H_2O + Na^+$$

Scheme 1
Conversion of saponified HFA to acid form

Volume of obtained tall oil after separation of aqueous and a lignin phase from one batch was 482 ml or 465 g, and the yield in % wt. was 70.9.

Having concluded the process, phase separation was performed. TO was separated from aqueous and lignin phases by separatory. Subsequently, warm water was used to wash TO, until pH of waste water reached ~ 6–7 due to necessity to eliminate H_2SO_4 residues from esterified oil. Finally, TO was dehydrated in a vacuum rotary evaporator.

Preparation of tall oil was performed using two apparatus simultaneously. For totally 3.3 h, 2 × 482 ml of oil after separation of aqueous and lignin phase was obtained. This process was repeated 6 times and a total amount of prepared oil was ~ 5,784 ml (5,580 g). A total amount of TS used in the process was ~ 7,867 g (besides moisture).

Average batch yield reached 70.93 % wt. Theoretical yield was 84.0 % wt., i.e. weight loss was 13.07 % wt. This amount included the matter remaining on the beaker and funnels walls owing to a decrease in temperature and increase in viscosity of tall oil. The inner surface of 4,000 ml beaker and 2,000 ml separatory funnel was significant.

Moreover, not all saponificed HFA and RA were converted to their acid form by the reaction shown in Scheme 1.

GC/MS Analysis of TO

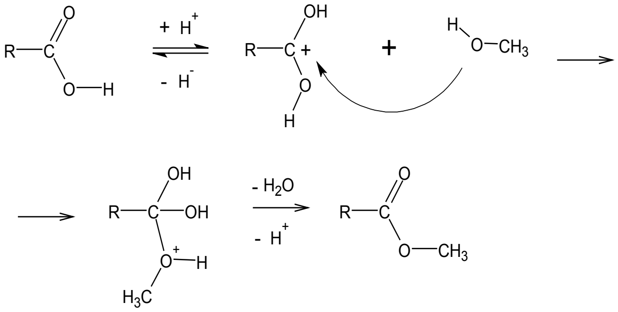
The prepared TO contained 30 % wt. HFA, 20 % wt. RA, ~ 18.5 % sterols. Non-analysed portion represented ~ 44.2 % wt. and contained higher fatty alcohols, hydrocarbons, lignin and cellulose fragments, non-methylated fatty and resin acids and inorganic compounds. These substances cannot be classified into the above three groups. In addition, these substances could be modified by the preparation process or its conditions.

Table IV
GC/MS analysis – composition of tall oil

Extractives	[% wt.]
Fatty acids	29.8
Resin acids	20.0
Sterols	6.0
Analysed portion	55.8
Non-analysed portion	44.2
Total	100.0

Esterification of Tall Oil – Preparation of Tall Oil Methylesters (TOME)

The prepared TO, after washing by water and dehydratation were subjected to esterification at optimal conditions based on Neaves D.E.[11].



Scheme 2
Mechanism of HFA esterification with methanol in acid media

Mechanism of acid catalysed esterification by methanol is proposed in Scheme 2. Conditions of esterification are summarized in Table II.

1,000 g TO with HFA content ~ 30 % wt. i.e. ~ 300 g was processed. As catalyst, suphuric acid ~ 15 g (8.2 ml at density 1.835 g cm^{-3}) was used. The total amount of methanol added to the reactor was ~ 204.8 ml (methanol was added in excess to HFA, in molar ratio 6 : 1)

Average molar mass of fatty acids was estimated as 281.55 g mol^{-1}. Stemming from the fact that optimal conditions (time 2–3 h) given by literature concern only the fraction HFA isolated from TO and not the fraction included in TO, reaction time was prolonged to 5.5 h to ensure maximum conversion.

The process was repeated 3 times and total volume of prepared TOME approached 3,000 ml (2,835 g).

Vacuum Distillation of TOME

Data on amounts of obtained distillates (D1, D2), residues (Z1, Z2) and weight losses (WL) from 1st and 2nd distillation stage are listed in Table V and VI.

Table V
Vacuum Distillation material balance: 1st stage

TOME	D1		Z1		WL
[g]	[g]	[wt. %]	[g]	[wt. %]	[wt. %]
2362	845	35.8	1419	60.0	4.2

Table VI
Vacuum distillation material balance: 2nd stage

TOME	D2		Z2		WL
[g]	[g]	[wt. %]	[g]	[wt. %]	[wt. %]
845.3	712	84.2	101.4	12.0	3.8

The prepared amount of TOME was heated and then let settle. Formed water and unreacted methanol were removed with a vacuum (molecular) evaporator.

Then 2.5 dm^3 TOME was withdrawn from 3 dm^3 volume of TOME and distillated at the folowing conditions: pressure 10–50 Pa and temperature 135–140 °C. 955 ml (~ 845 g) of distillate (D1) was obtained which represented ~ 38 % vol. (~ 36 % wt.). During this distillation D1 was polluted by RA.

Destillate D1 955 ml (~ 845 g) was consequently distillated at pressure 2–20 Pa and temperature 130–135 °C. Under these conditions, we obtained 835 ml (711.8 g) of distillate D2, i.e. ~ 87 % vol. from D1, (84 % wt.)

The total yield of these processes: from 2,500 ml TOME (2,363 g) we obtained 835 ml (711.8 g) which represented 33.4 % vol. (30.1 % wt.).

Acid number parameter of D2 (19.610 mg KOH g^{-1}) did not comply with European standard EN 14214 for biodiesel (stipulating less than 0.5 mg KOH g^{-1}), D2 had to undergo further treatment. D2 included some RA. They were neutralised and precipitated and D2 (tall oil fatty acids methyl esthers – TOFAME) after this treatment had acid number 0.3 mg KOH g^{-1}. This value fully meets EN 14214. However, total volume of the final product decreased from 835 to 630 ml (566.6 g) which represented – when comparing to the TOME amount – 25.2 % vol., (~ 24 % wt.).

The final product prepared in such a mode was subjected to analyses in laboratory of VÚRUP, which was accredited for analyses of biofuels. Results are assembled in Table VII.

Results Obtained by Fourier Transform Infrared Spectrometry (FTIR)

FTIR spectra of our product and a sample of soybean oil methylesters (SOME) were scanned and were found to comply with EN 14214. We observed very good similarity in absorption peaks of our product and SOME sample. Both spectra are almost identical. This may mean that both specimens have similar physical and chemical properties.

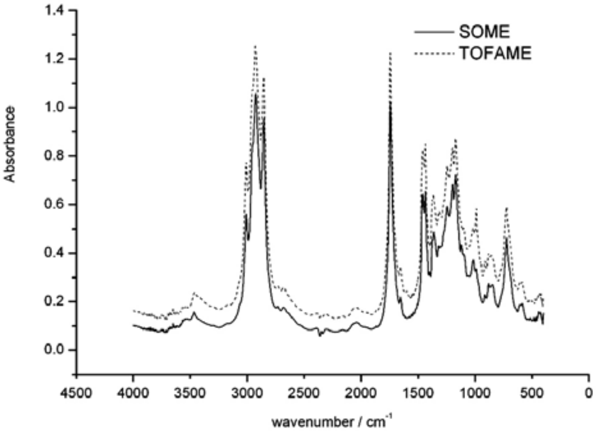


Fig. 1. **Comparison of FTIR spectra: TOFAME and SOME**

Test Results for Biodiesel from

Table VII
Values of prepared biodiesel compared with EN14214 standard values

Properties	Units	Determined value	EN 14214 value	Uncertainty
Density at 15 °C	$kg\,m^{-3}$	**899.3**	875–900	0.1 %
Carbon residue (10% distillation residue)	wt. %	**<0.010**	max. 0.3	10 %
Kinematic viscosity at 40 °C	$mm^2\,s^{-1}$	**3.932**	3.5–5	1.5 %
Cold filter plugging point	°C	**–12**	0 to –13 –13 to –20 (winter)	3 °C
Ester content	wt. %	**95.7**	min. 96.5	2.5
Linolenic acid methyl ester	wt. %	**0.7**	max. 12	–
Sulphur content	$mg\,kg^{-1}$	**1208**	max. 10	2 %

Accredited Laboratory

Conclusions

Prepared tall oil was esterified by methanol at optimal conditions in the presence of acid catalyst. Tall oil fatty acids methyl esters were isolated from TOME mixture by vacuum distillation.

Total yield of these processes: from 2,500 ml TOME (2,363 g), 630 ml (566.6 g) resulted which represents

25.2 % vol. (~ 24 % wt.). Biodiesel prepared in this procedure complies with EN 14214 except of chemicaly bonded sulphur.

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L08 ARTIFICIAL NEURAL NETWORKS IN FOOD ANALYSIS

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Introduction

During the last twenty years the chemists have get accustomed to the use of computers and consequently to the exploitation of various complex mathematical and statistical methods, by which they have been trying to explore multivariate correlations between the output and input variables more and more in detail. Input variables of different kind are often continuous and represent usually the results of instrumental measurements but also other observations, sometime discrete or categorical, are important for characterizing the investigated objects. Such kinds of input variables are for example the results of sensorial assessment of foodstuffs or simply some qualitative attributes like odour or colour (agreeable/disagreeable or clear/yellowish/yellow). The output variables are the targets of the corresponding study, which again can be represented by some continous variable or a categorical one with two or more levels. With the increasing complexity of analytical measurements, and the analysed sample itself, it becomes clear that all effects that are of interest cannot be described by a simple univariate relation but they are multivariate and often they are not linear. A set of methods, which allow study of multivariate and non-linear correlations that have recently found very intensive use among chemists are the artificial neural networks (ANNs for short)[1].

The ANNs are difficult to describe using a simple definition. Perhaps the closest description would be a comparison with a black box having multiple input and multiple output which operates using a large number of mostly parallel connected simple arithmetic units. The most important thing to characterize about all ANN methods is that they work best if they are dealing with non-linear dependence between the inputs and outputs. They have been applied for various purposes, e.g. optimisation[2,3] quantification of unresolved peaks[4,5], estimation of peak parameters, estimation of model parameters in the equilibria studies, etc. Pattern recognition and sample classification is also an important application area for the ANN[6–8], which is important and fully applicable in food chemistry.

The most widespread application areas of implementing the ANNs for solution of foodstuff problems are: *(i)* wine characterization and authentification, *(ii)* edible oil characterization and authentification, *(iii)* classification of dairy products and cheese, *(iv)* classification of soft drinks and fruit products, *(v)* classification of strong drinks. In this paper two examples are given, which exemplify the application of artificial neural networks for authentification of varietal wines and olive oils.

Theory

The theory of the ANN is well described in monographs[9–13] and scientific literature. Therefore only a short description of the principles needed for understanding the ANN application will be given here. The use of the ANN for data and knowledge processing can be characterized by analogy with biological neurons. The artificial neural network itself consists of neurons connected into networks. The neurons are sorted in an input layer, one or more hidden layer(s) and an output layer. The input neurons accept the input data characteristic for each observation, the output neurons provide predicted value or pattern of the studied objects, and the hidden neurons are interconnected with the neurons of two adjacent layers but neither receive inputs directly nor provide the output values directly. In most cases, the ANN architecture consists of the input layer and two active layers – one hidden and one output layer. The neurons of any two adjacent layers are mutually connected and the importance of each connection is expressed by weights.

The role of the ANN is to transform the input information into the output one. During the training process the weights are gradually corrected so as to produce the output values as close as possible to the desired (or target) values, which are known for all objects included into training set. The training procedure requires a pair of vectors, ***x*** and ***d***, which together create a training set. The vector ***x*** is the actual input into the network, and the corresponding target - the desired pre-specified answer, is the vector ***d***. The propagation of the signal through the network is determined by the connections between the neurons and by their associated weights, so these weights represent the synaptic strengths of the biological neurons. The goal of the training step is to correct the weights w_{ij} so that they will give a correct output vector ***y*** for the vector ***x*** from the training set. In other words, the output vector ***y*** should be as close as possible to the vector ***d***. After the training process has been completed successfully, it is hoped that the network, functioning as a black box, will give correct predictions for any new object x_n, which is not included in the training set.

The hidden x_i and the output y_i neuron activities are defined by the relations:

$$\xi_i = t(\xi_i) \tag{1}$$

$$\psi_i = t(\xi_i) \tag{2}$$

$$\xi_i = \sum_{j=1}^{p} w_{ij} x_j + \upsilon_i \tag{3}$$

where $j = 1, \ldots, p$ concern neurons x_j in the previous layer which precede the given neuron *i*. ξ_i is the net signal – the sum of the weighted inputs from the previous layer, υ_i is the bias (offset), w_{ij} is the weight and, finally, $t(\xi_i)$ is *transfer function,* expressed in various ways, usually as the threshold logic function, sigmoid function or hyperbolic tangent function. The most common sigmoid function is of the form

$$\pi(\xi_i) = \frac{1}{1+e^{-k\xi_i}} \tag{4}$$

where *k* is a constant. The aim of the neural network training is to *minimize the error E* by changing the weights and offsets

$$E = \sum_{i=1}^{r} E_i = \sum_{i=1}^{r} (y_i - d_i)^2 \tag{5}$$

where *r* is the number of the input-output vector pairs in the training set, d_i is the respective component of the required output vector and y_i is the response to the adequate component x_i of the input vector. The error *E* is minimized most often by the steepest descent method or another gradient method. The described theory is adequate mainly for the multilayer perceptron algorithms like Back Propagation, Quick Propagation and Quasi-Newton, with some differences in details.

The ANN calculations can be effectively made using several commercial software packages like Trajan[14], Statistica Neural Networks[15], SAS JMP[16] and others[17,18].

Experimental

Description of Wine Samples and Instrumentation

72 wine samples of 6 varieties originated from Small Carpathian region (Slovakia) and produced in West Slovakia in 2003 were quantitatively analysed by gas chromatography using headspace solid-phase microcolumn extraction. The set of samples contained 11 samples of Frankovka Blue (code FM), 12 samples of Chardonnay (Ch), 16 samples of Műller Thurgau (MT), 9 samples of Welsch Riesling (RV), 7 samples of Sauvignon (Sv) and 17 samples of Gruener Veltliner (VZ). Wine aroma compounds were extracted from the headspace into a microcolumn; the microcolumn was then transferred into a modified GC injection port for thermal desorption and the released compounds were analysed. Areas of chromatographic peaks of the same retention time corresponding to the selected 65 volatile aroma compounds were used in all samples. Wines were characterised by a set of identified compounds with corresponding relative abundances. Analyses were carried out on a GC 8000 Top Series, CE Instruments (Rodano-Milan, Italy) equipped with a modified split-splitless inlet and flame ionization detector. The inlet was modified so that it was possible to insert a glass microcolumn (1 mm i.d., packed with 5.0 mg of 60–80 mesh Tenax TA). The fused silica capillary column Omegawax 250, 30 m × 0.25 mm × 0.25 μm film thickness (Supelco, Bellefonte, Pennsylvania, USA) was used. The GC inlet and the detector temperatures were 250 °C and the initial column temperature was maintained at 25 °C. The thermal desorption was performed at 10 kPa pressure for 5 min, then the pressure was increased to 50 kPa and the column temperature was programmed at a rate of 4 °C min^{-1} up to 210 °C and maintained at 210 °C for 10 min. Helium was used as the carrier gas. A computer program Class-VP 7.2, SP1 (Shimadzu, Columbia, Maryland, USA) was used for data acquisition. Analyses of each wine sample were repeated twice.

Description of Drinking Water Samples and Instrumentation

93 water samples containing potable, spring and mineral waters, originated from Croatia (54 samples), Slovenia (30), the Czech Republic (6), and France (3) were studied. From each brand 3 specimens were sampled so that the analyses were finally made for 15 tap water samples, 51 spring water samples, of which 12 samples were carbonated, and 27 mineral water samples, of which 9 samples were carbonated. Experiments were performed using a high-resolution inductively-coupled plasma mass spectrometer (ICP MS) Element 2 (Thermo, Bremen, Germany) equipped by autosampler (ASX 510, Cetac Technologies, USA), the sample introduction kit with a conical nebulizer (Thermo, Bremen, Germany) and a Scott-type glass spray chamber (Thermo, Bremen, Germany) for transporting the analytes into the plasma of the ICP MS unit. The investigated water samples were analysed and characterized by thirty one continuous variables – nuclide concentrations determined by the ICP MS measurements: Ag107, Ag109, Al27, As75, B11, Ba138, Be9, Bi209, Cd111, Cd114, Co59, Cr52, Cu63, Fe56, Li7, Mn55, Mo95, Mo98, Ni60, Pb208, Sb121, Sb123, Se77, Sn118, Sn120, Sr86, Ti47, Tl205, U238, V51, and Zn66. The standard solutions and the blank solutions were prepared by adding of 1 % high purity nitric acid (Fluka, Steinheim, Switzerland) and 1 % high purity hydrochloric acid (Merck, Darmstadt, Germany).

Results

Classification of Varietal Wines

For quantitative analysis, based on the integrated peak area, 65 chromatographic peaks were selected. A complete assignment of the analysed compounds to the selected peaks was not necessary in the applied approach, however, for 19 peaks the corresponding species were identified. It is very important to note that the retention time order for all selected compounds was the same for all 72 samples and the way of chromatographic signal evaluation was identical. The obtained final data matrix suitable for chemometrical processing contained 72 rows (objects) and 65 columns (variables). Since the number of variables was too large compared to the number of objects, selection of the best variables, based on the F-test, was performed by stepwise feature selection. In this way, 30 best variables were chosen enabling best discrimination among the six studied wine varieties. For comparison purposes, the wine classification was performed not only using the ANNs but also several techniques of discriminant analysis were implemented.

The classification model was calculated using the training set of samples containing all samples but one when the leave-one-out validation was used or without three samples in case of using the leave-three-out validation. Thus, the remaining one sample or three samples were not included in the training procedure but were used for inspecting the quality of prediction whether the predicted variety of wine matches the real wine variety. The results achieved by several classification techniques and different software are summarized in Table I. The presented ANN results were achieved after optimising the neural network; the lowest error was obtained when using a three layer perceptron with 30 input neurons (areas of the selected best peaks), 3 hidden neurons and one six-level output neuron (representing the predicted wine variety).

All wine samples (100 %) were correctly classified into six classes by variety when the calculated multidimensional model is considered – no one sample was allocated to a wrong class. Considering the validation results, the performance in leave-one-out validation depend on the applied multivariate technique. The leave-three-out manual technique can be used also in the case when the automatic leave-one-out validation is not enabled for the given method and software. All validation results shown in Table I are above 90 %, which justifies very good ways of wine variety prediction enabling to confirm or reject wine authenticity.

Classification of Drinking Water

Three classification criteria were used for the classification of drinking waters: (1) by three types of water – potable, mineral and spring water, (2) by five types of water – potable, mineral, mineral carbonated, spring and spring carbonated water, (3) by three countries of origin – Slovenia, Croatia and Czechia (the category of French waters was not used due to a very low number of samples). Before the calculations new categorical variables were created, which correspond to the first, second and third classification criterion: WType 3, WType 5 and Country, resp. In addition, a special categorical variable Carbon was created in order to mark whether the sample is carbonated or not (c/n). When using the ANNs it is possible to utilize this variable at the input to provide some additional information about the sample. It is worth noting that the discriminant analysis techniques, except logistic regression, do not permit the use of non continuous input variables. The mentioned additional information cannot be of course used when the classification by the second criterion is used.

Table II shows the classification results for five cases using categorization by water type into 3 and 5 classes, the same categorization but with the help of additional categorical variable Carbon, and finally categorization by 3 countries of origin. Intelligent Problem Solver is an extremely useful module of Trajan software facilitating the selection of the optimal neural network. For the sake of place, Table II exhibits only five best networks, automatically selected by this module, but a good possibility is to make a choice among a larger nuber of networks. Moreover, the networks belonging to different ANN variants can be examined in this way (e.g. Radial Base ANN). In Table II, the ordinal number of the network is marked by No, the number of neurons in individual layers is marked by I (input), H (hidden), and O (output); Err. indicates the sum of squares error obtained both for the training and test sets. The most important results are

Table I
Success in prediction of wine variety using 72 varietal wines, 30 optimally selected chromatographic peaks, 5 classification and 2 validation techniques

Method	Classif. success	Leave-1-out	Leave-3-out	Software
LDA	Correct/all	69/72	69/72	SAS
	%	95.8	95.8	
QDA	correct/all	66/72	65/72	SAS
	%	91.7	90.3	
KNN	correct/all	72/72	67/72	SAS
	%	100.0	93.1	
LR	correct/all	–	69/72	SPSS
	%	–	95.8	
ANN	correct/all	–	68/72	JMP
	%	–	94.4	

Table II
ANN – selection of the best network for 5 different criteria of water classification using Intelligent Problem Solver of software Trajan 6.0

Categor variables	MLP networks Neurons				Train. set	Test set	Success [%]
	No	I	H	O	Err.	Err.	
Country	1	31	6	1(3)	0.009	3.759	77.3
	2	31	6	1(3)	0.023	4.706	81.8
	3	31	5	1(3)	0.023	2.801	77.3
	4	31	6	1(3)	0.017	3.464	81.8
	5	31	6	1(3)	0.0044	5.239	86.4
Country c/n	1	32	5	1(3)	0.0079	3.215	86.4
	2	32	6	1(3)	0.001	3.921	81.8
	3	32	6	1(3)	0.100	2.242	86.4
	4	32	6	1(3)	0.0032	1.829	86.4
	5	32	6	1(3)	0.144	0.480	90.9
WType3	6	31	6	1(3)	0.0054	0.0026	100.0
	7	31	6	1(3)	0.0008	1.602	86.4
	8	31	6	1(3)	0.0002	0.649	95.5
	9	31	6	1(3)	0.0010	0.204	95.5
	10	31	6	1(3)	0.0000	0.935	86.4
WType3 c/n	1	32	6	1(3)	1.4×10^{-5}	$2. \times 10^{-5}$	100.0
	2	32	6	1(3)	1.1×10^{-6}	5.8×10^{-6}	100.0
	3	32	5	1(3)	5.9×10^{-6}	0.0029	100.0
	4	32	6	1(3)	1.2×10^{-9}	8.2×10^{-6}	100.0
	5	32	6	1(3)	1.5×10^{-8}	9.1×10^{-3}	100.0
WType5	1	31	6	1(5)	0.2946	0.744	90.9
	2	31	6	1(5)	0.215	0.599	86.4
	3	31	6	1(5)	0.424	1.154	81.8
	4	31	6	1(5)	0.197	0.4498	59.1
	5	31	6	1(5)	0.480	0.934	90.9

in the last column, which expresses the ratio between the numbers of correct classifications to the total number in per cents. The results are excellent when categorization by the type of water is concerned. A bit less successful but still very good is classification by the country of origin, however, some difficulty here is cause by the fact that drinking waters cannot be strictly differentiated by the country borders but the geological factors can be more important.

Conclusions

Theoretical background and practical examples of exploitation of artificial neural networks were given.

Quantitative results facilitating characterization, classification and authentification of Slovak varietal wines using artificial neural networks and other multidimensional chemometrical techniques were acquired. Six studied wine varieties were correctly categorized on the basis of the areas of chromatographic peaks corresponding to the selected 65 volatile aroma compounds in all samples. In all studied cases the calculated classification performance was higher than 90 %. A higher classification performance was achieved when instead of all peaks 30 optimally selected peaks were used in the training procedure. Applied approach is suitable mainly when many analytical data and complex analytical signals are obtained.The established classification models are fully applicable for the prediction of the category of an unknown wine sample.

Using the nuclide concentration determined by the ICP MS method, five types of drinking water from different European countries were classified. The classification results corresponding to the selected classification criteria were very good – from 90 to 100 % for classification by the water type and about 85 % for classification by the country of origin. A detailed study may also enable to find the nuclides, which concentration level is characteristic for the given kind of the water sample. Some details concerning implementation of neural networks were also referred.

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L09 DETECTION OF SPICES' IRRADIATION BY MODERN SPECTROSCOPIC TECHNIQUES

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Introduction

Herbs and spices are daily used condiments, frequently undergoing the microbial contamination (average contamination by microorganisms and/or their spores can reach up 10^5–10^8 microorganisms per gram)[1]. Thus γ-irradiation treatment is accepted as one of the most effective sterilization technique.

Toxicological and nutritional tests proved the safety of γ-radiation doses below 10 kGy, which was accepted by Codex Alimentarius (CA) General Standard for irradiated foods as the maximum legal/allowed absorbed dose for dried aromatic herbs, spices and vegetable seasonings sterilisation, with the exception for cases when the higher dose application is necessary to achieve a legitimate technological purpose[2]. In contradiction to CA standards, the limitation of US Food and Drug Administration (FDA) set the maximum allowed dose for culinary herbs and spices to 30 kGy[3].

Besides the positive effects of γ-radiation, its negative impact on environment or even on human health forces the food control authorities to develop reliable and sensitive methods applicable for dosimetric purposes even long time after the radiation process.

It is well known, that γ-radiation of food samples results in the formation of free radical species. Thus, Electron Paramagnetic Resonance (EPR) spectroscopy represents a suitable tool to investigate the irradiated spice.[5–12] As follows from several recently published data, the application of EPR spectroscopy for dosimetric purposes is limited by several factors, mostly by limited lifetime and thermal stability of γ-radiation induced radicals.[5–13]

Our previous investigations were focused on the monitoring of radiation-induced changes e.g., in black pepper, oregano, allspice, ginger, or clove.[5,9–12]

The aim of the present study was to monitor the effect of γ-irradiation on the microbiological quality of powder samples of ground dry caraway seed (*Carum carvi*, L.) and ground dry laurel leaves (*Laurus nobilis*, L.). The influence of absorbed dose on the character of formed paramagnetic structures, as well as their life-time was investigated by means of EPR spectroscopy. Moreover, antioxidant properties of individual spice extracts were characterized using both EPR and UV-VIS spectrophotometer by means of 1,1-diphenyl-2-picrylhydrazyl (•DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+\bullet}$) radicals, ferric reducing power (FRP) and thiobarbituric acid reactive substances (TBARS) assays. Total contents of polyphenols (TPC) in each extract was also monitored and expressed as Gallic acid equivalent. In addition, multivariate statistical methods were used for the discrimination of native (non-irradiated) samples from that exposed to γ-radiation.

Experimental

Samples Characterisation

Samples of ground dry caraway seed (dry matter content, 92.1 %) originating from Austria and laurel leaves (dry matter content, 92.5 %) from Turkey were provided by Kotanyi, GmbH, Vienna, Austria. Spice samples were irradiated using ^{60}Co source at average doses of 5, 10, 20 and 30 kGy (dose rate, 2 kGy h^{-1}) according to commercial practices at Artim, Ltd. (Prague, Czech Republic). After the irradiation, all the samples were stored in closed bags in the darkness at ambient conditions.

Microbiological Analysis

Elementary microbiological analysis of all spice samples (total counts of microorganisms, presence of coliform bacteria, yeasts and moulds) was carried out following the relevant STN ISO standards two times: immediately after the irradiation and after 6 months of post-irradiation storage.[14–16]

EPR Experiments

EPR experiments with solid samples were performed identically as previously described elsewhere, using a portable X-band EPR spectrometer e-scan (Bruker, GmbH, Karlsruhe, Germany).[5,10–12] Spice sample (100 mg) was placed in the thin-wall EPR quartz tube (internal diameter, 3 mm) and cylindrically shaped column was formed (sample column heights: 2.1 ± 0.2 cm (caraway) and 2.0 ± 0.2 cm (laurel tree), respectively; and then inserted into the standard rectangular cavity of EPR spectrometer.

Ethanolic extracts of spice samples were prepared identically as described in our previously published papers, by mixing 0.4 g of respective spice sample with 8 ml ethanol of spectroscopic grade.[5,10,12] Their ability to terminate •DPPH and $ABTS^{\bullet+}$ radicals was monitored.[5,10,12,17,18]

Experimental EPR spectra were recorded at 298 K. The response and settings of EPR spectrometers were checked by means of solid DPPH and Strong pitch standards (Bruker) daily before the experiments, The obtained spectra were evaluated using WIN EPR and SimFonia software (Bruker) as described e.g. in.[5,9–11,19,20]

UV-VIS Experiments

Extracts used in UV-VIS experiments were prepared by mixing 2.0 g of respective spice sample with 50 ml methanol/water (80 %, v/v) solvent[9,11]. Double-beam UV-VIS spectrometer Specord M40 (Carl Zeiss, Jena, Germany) with accessories was used for the monitoring of antioxidant properties. All the experiments were carried out in the same square quartz UV-VIS transparent cells (path length, 1 cm). The monitoring of antioxidant ability of spices' extracts was performed identically as described in our previous papers, involving the DPPH, TBARS and FRP assays. Total phenolic compounds content of extracts was evaluated, as well. [9, 11]

Multivariate Statistical Analysis

Canonical discriminant analysis of all results obtained from UV-VIS experiments was performed using the Unistat® software in order to distinguish the native (non-irradiated) spice samples from that exposed to γ-radiation.

Results

Microbiological analysis performed immediately after the irradiation process proved, that as a result of γ-irradiation, the total count of microorganisms in caraway sample irradiated at dose of 5kGy decreased considerably from 2.8×10^4 colony forming unit (CFU) detected in reference sample, to less than 10 CFU g^{-1}. The same effect of γ-irradiation on laurel leaves was achieved using the dose of 10 kGy, still fulfilling the requirements of international standards on irradiation[3,4].

Table I
Microbiological analysis of ground caraway seeds (C) and laurel bay leaves (L) samples, γ-irradiated at doses of <0-30> kGy using 60Co-source performed one day after the γ-irradiation

Radiation dose [kGy]	Total count of microorganisms [CFU g^{-1}]		Coliform bacteria [CFU g^{-1}]		Moulds [CFU g^{-1}]	
	C	L	C	L	C	L
0	2.8×10^4	1.7×10^5	1.0×10^4	8.6×10^3	9.8×10^3	5.7×10^3
5	<10	5.7×10^3	<10	5.0×10^1	2.5×10^2	<10
10	<10	<10	<10	<10	<10	<10
20	<10	<10	<10	<10	<10	<10
30	<10	<10	<10	<10	<10	<10

As follows from data presented in Table I, the presence of oliform bacteria as well as of yields and moulds was effectively suppressed by the irradiation. Analysis performed 6 months of post-irradiation storage confirmed, that microbial status of both spices remained practically unchanged.

EPR spectrum of both reference (non-irradiated) samples represents broad singlet line with unresolved hyperfine splitting, attributable mostly to Mn^{2+} ions, upon which the additional sharp EPR line ($g_{eff} = 2.0022$, $\Delta B_{pp} \sim 1$ mT) is superimposed, previously assigned to stable semiquinone radicals produced by the oxidation of polyphenolic compounds present in plants. In addition, the presence of low-intensive EPR singlet line was noticed in caraway reference sample, attributable to radicals generated during the grinding process (Table II).[5,9–11]

EPR spectra of γ-radiation treated spices showed the formation of additional paramagnetic structures. As follows from detail simulation analysis of obtained spectra (Table II), different, mostly cellulosic and carbohydrate radical structures were identified.

Table II
Identification of radical structures found in reference and γ-irradiated samples of ground caraway and laurel leaves

EPR signal origin	g-value	Hyperfine splittings [mT]	ΔB_{pp} [mT]
	Reference samples		
Semiquinones	$g_\perp = 2.0042$	–	0.52
	$g_\parallel = 2.0030$		
Carbohydrate I	$g_\perp = 2.0041$	–	0.06
	$g_\parallel = 2.0028$		
	γ- irradiated samples		
Carbohydrate II	$g_\perp = 2.0041$	$A_\perp = 0.7$	0.45
	$g_\parallel = 2.0033$	$A_\parallel = 0.6$ (2H)	
Carbohydrate III	$g_\perp = 2.0032$	$A_\perp = 0.85$	0.67
	$g_\parallel = 2.0025$	$A_\parallel = 0.7$ (2H)	
Carbohydrate IV	$g_\perp = 2.0030$	$A_\perp = 0.45$	0.59
	$g_\parallel = 2.0038$	$A_\parallel = 0.40$ (1H)	
Cellulosic	$g_\perp = 2.0029$	$A_\perp = 3.00$	1.20
	$g_\parallel = 2.0014$	$A_\parallel = 1.70$ (2H)	

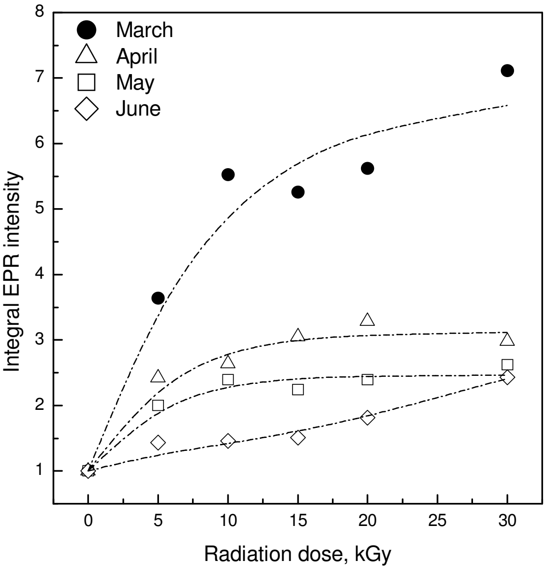

Fig. 1. **Dependence of integral EPR intensity of ground caraway seed on γ-radiation dose measured immediately after the irradiation (March) and during three month of post irradiation storage (April-June). EPR spectra were recorded using 0.633 mW microwave power at 298 K**

These radicals originate either from cleavage processes of cellulose matter (laurel leaves) and/or of other polysaccharides forming the skeleton of plant structures and their cells, as the cellulosic radicals were not detected in γ-irradiated caraway samples.

In accord with our previously published papers, the dose-dependent formation of radical structures' in γ-irradiated samples of both spices under study was found (Fig. 1.).

The obtained dependence can be effectively used as calibration curve enabling the estimation of previously absorbed dose.[5,9–11]

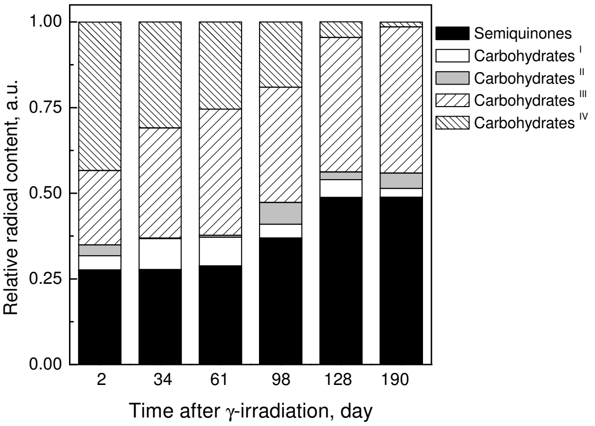

Fig. 2. **The dependence of relative radicals' content on time after the γ-irradiation obtained from the simulation analysis of experimental EPR spectra of caraway sample treated at dose of 30 kGy**

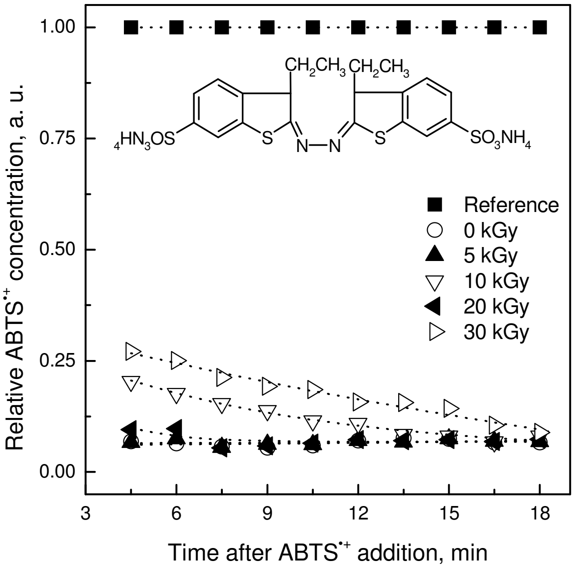

Fig. 3. **The dependence of relative ABTS$^{•+}$ concentration on time after its mixing with caraway ethanolic extracts prepared from samples exposed to different doses of γ-radiation. Inset represents the structural formula of ABTS. Extracts were prepared 2 days after the irradiation. Pure ethanol was used as reference**

As a result of irreversible decay of radical structures induced by γ-radiation, the integral EPR spectra intensity of irradiated samples decreased gradually as is clearly demonstrated on Fig. 1. and Fig. 2. for caraway samples. On the contrary, the signal intensity of both reference samples remains practically unchanged.

Detail evaluation of experimental spectra revealed the lowest stability of cellulosic radicals in laurel leaves (half life ~ 10 weeks) followed by carbohydrate radicals, which stability ranged from 20 up to 60 weeks. These results are in good agreement with our previously published papers.[5,9–11]

Ethanolic extracts of both reference spices revealed significant ability to terminate $^{•}$DPPH as well as ABTS$^{•+}$ radicals. As demonstrated on Fig. 3., it is only slightly influenced by the absorption of γ-radiation. Results obtained moreover showed, that radical-scavenging ability of laurel leaves' extracts is significantly higher than that of caraway; probably due to the differences in types and concentrations of polyphenolic compounds.

UV-VIS experiments proved, that radiation treatment resulted in significantly increased $^{•}$DPPH radical-scavenging activity of bay leaves methanolic extracts. In addition, the TBARS value of caraway extracts, representing a measure of oxidative products concentration, was slightly increased. As a result of post-irradiation storage, a minor increase of ferric reducing power, $^{•}$DPPH radical-scavenging ability as well as content of polyphenolic compounds of bay leaves extracts was noticed, whereas the TBARS values of both, caraway and bay leaves extracts, was somewhat reduced.

Discriminant analysis of all variables obtained from UV-VIS experiments was used to differentiate the studied spices according to the absorbed dose. As depicted on Fig. 4., the effective discrimination was achieved only when non-irradiated samples and samples treated by dose of 30 kGy were compared. Using this approach, 93% correctness of caraway classification was found, influenced mostly by TBARS values and TPC content. In the case of bay leaves, 73% correct differentiation of non-irradiated sample from sample γ-irradiated at 30 kGy was found.

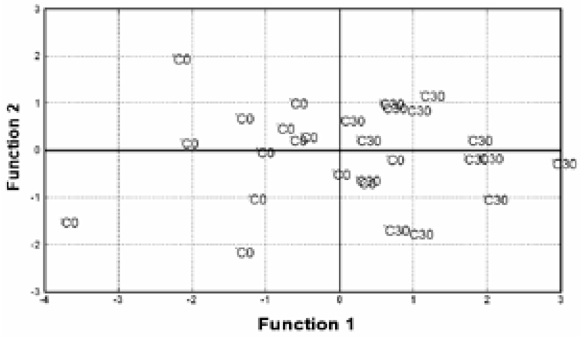

Fig. 4. **Canonical discriminant analysis of non-irradiated caraway samples (C0) and of samples γ-irradiated at 30 kGy (C30). Characteristics obtained from UV-VIS experiments ($^{•}$DPPH, TBARS, FRP, TPC) immediately after the irradiation and during the storage of samples at ambient conditions were used as variables**

Conclusions

It was proved, that γ-irradiation of caraway and bay leaves samples even at doses of 5 kGy and 10 kGy, respectively, is an efficient microbiological decontamination tool.

The consideration of γ-radiation impact on spices is a multi-component problem. As follows from results presented in this study, EPR spectroscopy represents a valuable dosimetric tool. In addition, the canonical discriminant analysis based on UV-VIS characteristics of spices' extracts can be effectively used for the differentiation of spices treated at higher doses of γ-radiation from the unaffected ones.

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L10 APPLICATION POTENTIAL OF NOVEL GAS CHROMATOGRAPHY HIGH THROUGHPUT TIME-OF-FLIGHT MASS SPECTROMETERY SYSTEM (TRU TOF) IN FOOD AND ENVIRONMENTAL ANALYSIS

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Introduction

In last years, analytical approaches employing gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF MS) proved to be a useful tool in assessment of quality and safety of food[1–4] and also environmental matrices[5]. But only recently, at the end of 2007, new time-of-flight mass spectrometric (TOF MS) detector specialy designed for high-throughput of samples, has been introduced. High throughput is the key to increased profitability of the analyses, while obtaining faster results and optimization. The need for selected ion monitoring (SIM) operation and low dynamic range associated with traditional quadrupoles and ion traps may take time and money away from laboratory's bottom-line. The assessed instrument (GC-HT TOF MS) is combining fast acquisition mass spectrometer (80 Hz) with specific data-mining algorithms. The aim of this benchtop instrumental set-up is to achieve the speed and resolution necessary to accomplish Time-Compressed Chromatography. Using such detector, sufficient data density is obtained to accurately characterize even the narrowest GC peaks produced under conditions of fast chromatography separation. The acquisition of the full mass spectral information of the sample with comparable sensitivity as obtained by selected ion monitoring (SIM) mode with quadrupole or ion trap instruments makes feasible the application of a deconvolution algorithm obtaining pure mass spectra even for coeluting compounds and achieving reliable confirmation. Consequently, trace level analysis of unknown sample components can be performed.

The schematic view of GC-HT TOF MS is shown in Figure 1. Within the mass spectrometer source, the filament continuously generates an electron beam. The GC effluent is introduced into the source through a heated transfer line. Electron ionization (EI) occurs as a result of interactions between an electron beam with an analyte molecule from the GC effluent. Chemical ionization (CI) occurs as a result of EI interactions between the electron beam with the CI reagent gas which creates charged reagent ions that ionize the analyte molecules from the GC effluent. Ions are pulsed from the orthogonal accelerator at a nominal frequency of 20 kHz. Each pulse of ions into the flight tube results in a mass spectrum which is referred to as a transient. The transients are then summed to provide mass spectral acquisition rates up to 80 spectra second^{-1}. The focusing optics are used to direct ions through the system and ensure a high recovery of signal at the detector. Deflection optics are used to prevent unwanted signals, such as ions from a solvent front or unwanted background ions generated by carrier gas or residual gas, and extend the life of the detector. The ions are pulsed into the flight tube with equal kinetic energies ($\text{K.E.} = 1/2\ mv^2$). Therefore, ions of varying mass-to-charge ratios will have different velocities as they move through the flight tube. Fragment ions with different velocities traveling over the same fixed distance will have different arrival times at the end of that distance (velocity = distance/time). Masses are resolved in time-of-flight mass spectrometers by the time each mass takes to reach the detector at the end of the flight path ($\text{time} = \text{constant} \times m^{1/2}$). For example, a mass of 100 mu will take approximately 15 microseconds to reach the detector while a mass of 1,000 mu will take 50 microseconds to travel the same distance.

In this work we aimed to evaluate new GC-HT TOF MS instrumentation in analysis of pesticides, pharmaceuticals, poly-chlorinated biphenyls (PCBs) and poly-brominated dibenzo ethers (PBDEs). The best GC-MS settings has been optimized in order to obtaine fast and reliable analytical methods for routine control of purified extracts of different food-stuffs, or sediment, and water. Appraisal of mentioned technique with respect to the cost and time demands was also done.

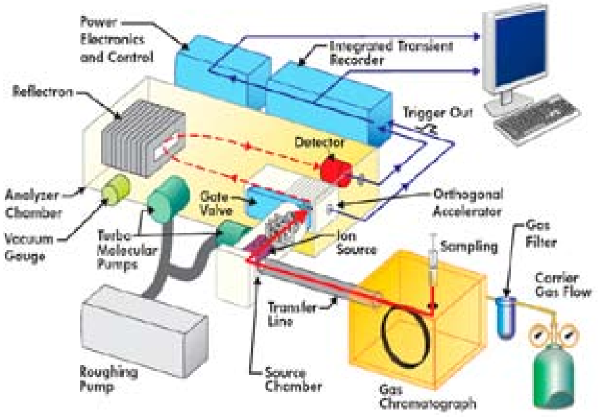

Fig. 1. **Schematic diagram of GC-HT TOF MS**

Experimental

Reagents and Material

Tested compounds (listed in Table II) with purity ranging from 95 to 99 % were purchased from Dr. Ehrenstorfer (Augsburg, Germany) in case of pesticides and PCBs. PBDEs and estrogenic pharmaceuticals were obtained from Cambridge Isotope Laboratories (CIL, UK). All solvents used within sample preparation (see Table I) were of analytical grade (Scharlau, Barcelona, Spain). Working solutions (concentration 1.25–250 $\mu\text{g dm}^{-3}$) were prepared by series of dilutions of the stock solutions (10 mg dm^{-3}) with appropriate solvent.

Table I
Sample preparation and GC-HT TOF MS instrumental set-up

Instrumental set-up	Analytes group			
	Pesticides (baby food)	PCBs (fat)	PBDEs (sewage water)	Pharmaceuticals (river sediment)
Sample preparation	Ethyl acetate extraction of baby food followed by HPGPC clean-up.[6]	Soxhlet extraction of adipose tissue using hexane:dichlormethane (1 : 1, v : v) followed by HPGPC clean-up.[7]	Filtration of water followed by microextraction in packed siringe (MEPS, C-18).[8]	Hexane:acetone (1 : 1, v : v) extraction followed by SPE clean-up.9
GC	Injection: splitless 1 µl, 250 °C Column: forte BPX-5 (40 m × 0.18 mm × 0.18 µm) Temperature programming: 70 °C (1 min), 45 °C min^{-1} to 300 °C (3 min) Column flow: 1 ml min^{-1}	Injection: pulse splitless 1 µl, 250 °C, 1.5 min at 50 p.s.i. Column: forte BPX-5 (30 m × 0.25 mm × 0.25 µm) Temperature programming: 80 °C (1.5 min) 45° C min^{-1} to 340 °C (6 min) Column flow: 1 ml min^{-1}	Injection: PTV, 10 µl, 50 °C (2 min) 400 °C min^{-1} to 300 °C Column: DB-XLB (15 m × 0.25 mm × 0.1 µm) Temperature programming: 80 °C (2 min) 50 °C min^{-1} to 320 °C (4 min) Column flow: 1.5 ml min^{-1}	Injection: pulse splitless 1 µl, 250 °C, 2 min at 90 p.s.i. Column: forte BPX-5 (30 m × 0.25 mm × 0.25 µm) Temperature programming: 80 °C (2 min) 55 °C min^{-1} to 330 °C (5 min) Column flow: 2 ml min^{-1}
TOF MS	Detector voltage: 1,750 V Time of analysis: 10 min	Detector voltage: 1,875 V Time of analysis: 14 min	Detector voltage: 1,800 V Time of analysis: 11 min	Detector voltage: 1,875 V Time of analysis: 12 min

For optimization and validation purposes blank matrix (pork fat, apple based baby food) was chosen. Sewage water was obtained from local water treatment facility (Prague) and sediment was sampled at Podoli site (river Vltava).

Sample Analysis

Different sample preparation procedures for each analytes group were applied.[6–9] Their brief overwiev can found in Table I. The GC-HT TOF MS system consisted of a HP 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with split-splitless injector (or Agilent PTV Inlet) and LECO TruTOF™ HT time-of-flight mass spectrometer (LECO, St Joseph, MI, USA). The detector operated in electron impact ionisation mode (EI) or positive chemical ionization mode (CI+). GC-TOF MS was performed using set-up and conditions summarized also in Table I.

Results

Fast Analysis and Spectral Deconvolution

To demonstrate the application potential of TruTOF HT system and ChromaTOF software for detection/identification, selected contaminats and residues were analyzed under optimized conditions shown in previous chapter. All of the analyzed sample has been processed by automated peak finding and deconvolution function. Within peak finding, the Chroma TOF software automatically detects peaks at all acquired single masses (*m/z* 35–650 in our work) above certain signal-to-noise level (S/N = 25 was used in the given cases). After that, deconvolution alghoritm mathematically separates mass spectra of chromatographically coeluted compounds. Deconvoluted spectra are then compared to NIST library and the identification is performed automatically. As the example Fig. 2. shows the Deconvoluted Total Ion Chromatogram (DTIC) for matrix matched standard (25 µg dm^{-3}). Due to rapid analysis (fast temperature programming rate 45 °C), several coelutions occured (see Fig. 2.A). Successful spectral deconvolution followed by positive identification of overlapping chromatographic peaks have been enabled by data acquisition rate of 15 Hz offered by the Tru TOF (see Fig 2.B–C). Althought, within all tested analytes groups and matrices certain coelutions occured, the quantification

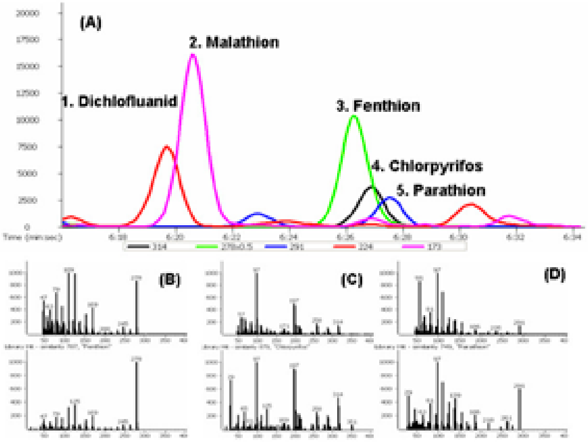

Fig. 2. **GC-HT TOF MS analysis of matrixe match standard of pesticides at 25 µg dm^{-3}(apple based baby food). (A) Deconvoluted TIC chromatogram. Measured mass spectrum (upper line) of Fenthion (B), Chlorpyrifos (C) and Parathion (D) along with respective NIST library hits (bottom line)**

of target compouds was possible using peak abundancies of deconvoluted peaks at their particular uniqu masses.

Methods Performance Characteristics

Relevant performance characteristics of the analytical methods (repeatability, LOD/LOQ and linearity) were tested on spiked samples in three replicates. As long as this work was mainly focused at the final determinative step of the whole analytical process, issues associated with sample preparation (such as recoveries) were not discussed. The results are summarized in Table II.

Table II
Method performance characteristics of selected analytes in various food matrices analyzed by GC-HT TOF MS. LOQs were estimated in purified matrix extracts or in water (for BPDEs). Repeatability (RSD) was measured at 25 μg dm^{-3}

Compound	Linearity (R2)	LOQ [μg dm^{-3}]	RSD (n =3 , [%])
Pesticides in baby food			
HCB	0.9991	2.5	2
DDT	0.9994	5	5
DDE	0.9981	5	7
DDD	0.9980	5	9
lindan	0.9995	2.5	4
endrin	0.9993	5	4
chlorpiryfos	0.9975	5	6
heptachlor	0.9972	5	8
PCBs in pork fat			
PCB 28	0.9983	2.5	5
PCB 52	0.9963	5	9
PCB 101	0.9955	5	6
PCB 118	0.9959	5	10
PCB 138	0.9960	5	5
PCB 153	0.9942	10	12
PBDEs in water			
BDE-28	0.9987	0.005	2
BDE-47	0.9954	0.001	4
BDE-66	0.9891	0.025	3
BDE-85	0.9930	0.010	4
BDE-99	0.9912	0.010	5
BDE-153	0.9854	0.025	7
BDE-183	0.9900	0.050	12
Pharmaceuticals in sediment			
Ethinylestradiol	0.9880	5	6
Dienestrol	0.9947	5	4
Diethylstilbestrol	0.9962	5	3

The limits of detection (LOD), were defined as the lowest detectable concentration (S/N≥3). The limits of quantification (LOQ) were estimated as a lowes calibration level (LCL).

Analysis of Real-life Samples

The ability of the presented technique to determine target analytes in real life samples was tested at various food extracts (for pesticides, PCBs) and water samples (for PBDEs).

As the example, case of PDBEs in sewage water is presented. It should be noted that the trace level target analysis as well as analysis of unknown sample components could be facilitated when working with TOF mass spectrometers due to their excellent confirmation power.[10] The identification of BDE-28 is documented in Fig. 3. Full mass spectrum obtained as a result of electron ionization (70 eV) was obtained after automatic spectral deconvolution. This fact proved, that coupling MEPS with PTV-GC TOF MS would express good potential for quantification of BFRs at their native concentration range (10^0–10^1 ng dm^{-3}) in releases of a waste water treatment plant effluent or sewage water.[11,12] Moreover, such results showed new possible concepts in rapid water analysis by employing microextraction in packed sirynge (MEPS) coupled to PTV-GC TOF MS. This approach minimizes sample handling and reduces time/cost of measurement.

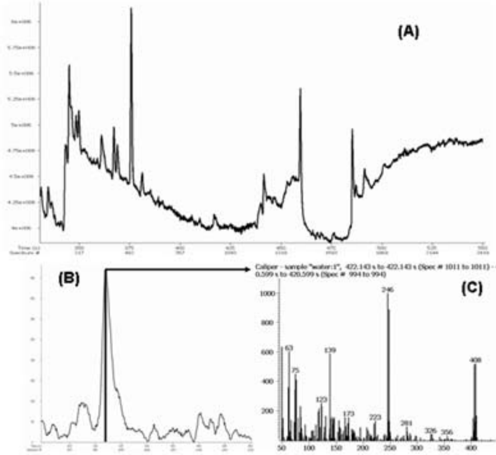

Fig. 3. **MEPS-GC TOF MS analysis of sewage water sample. (A) TIC chromatogram of sewage water. (B) Zoomed part of chromatogram – masses 406 + 408 are displayed.(C) Measured mass spectrum of natively present BDE-28**

Conclusions

This study briefly described a relatively fast separation for analysis of different microcontaminants in food and environmental samples. High-speed temperature programming significantly reduced the overall analysis time as compared to traditional methods. Detection by TOF MS gives all of the sensitivity needed in order to identify trace level components while at the same time providing the data density needed to define narrow GC peaks and deconvolute overlapping peaks.

Further tests will be facilitated in order to create ultra fast multimethod for simultaneous detection of wide spectrum of contaminants by GC-HT TOF MS. The hyphenation of this technique to microextraction sample preparations (MEPS, SPME) is the possible option to enlarge spectrum of HT TOF MS applications.

This work was realized with kind support of LECO Instrumente Plzeň s.r.o.

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L12 INTERACTIONS BETWEEN ORGANIC FOOD CONTAMINANTS AND PLASTIC PACKAGING MATERIALS

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Introduction

Polychlorinated biphenyls (PCBs) are a group of 209 man-made organic chemicals containing carbon, hydrogen and differing amounts of chlorine. The commercial production of PCBs started in 1929 but their use had been banned or severely restricted in many countries since the 1970s and 80s because of serious risks to human health and the environment. PCBs have been used in a wide range of products such as plastics, paints, and adhesives. Since PCBs are resistant to acids and bases as well as to heat, they have mainly been used in electric equipment as heat transfer fluids, or lubricants, respectively. Disposal of waste that contains PCBs in landfills or incinerators can lead to environmental contamination. PCBs were first detected in environmental samples in 1966[1]. PCBs can persist in the environment and accumulate in animals and along the food-chain. A human organism may be exposed to PCBs by ingestion of contaminated food and water, or inhaling contaminated air. PCBs have been shown to cause cancer and a number of serious non-cancer health effects in animals, including effects on the immune system, reproductive system, nervous system, endocrine system, more frequent infections, and changes of the skin, particularly rashes and chloracne. Their harmful effects to man and the environment are well documented in a number of reviews.[2–7] Therefore, the elimination of PCBs from foods and the environment is important. Adsorption of organic contaminants on plastic packaging materials is a promising way of decreasing levels of organic contaminants in foods.[8–13] For example, on the basis of interactions with plastic package, the concentrations of polycyclic aromatic hydrocarbons (PAHs) in a liquid smoke flavour stored in low density polyethylene (LDPE) bottles were lowered by two orders during 14 days[8]. The rate-limiting step was diffusion in the liquid media[14]. PAHs were primarily adsorbed on the LDPE surface, with migration into the bulk of the polymer, intensifing the effectiveness of the removal process[15]. This process is also effective for removal of PAHs from solid media, e.g. benzo[*a*]pyrene content in duck meat was lowered by 75 % after 24 hours of interaction with LDPE[16]. The ability of polyethylene terephtalate (PET) to decrease PAH concentrations in polar and non-polar liquid media has already been unambiguously proven[9]. However, the removal was limited only to surface adsorption which led to equilibrium between liquid and solid phase. Moreover, this process was also affected by other compounds (vitamins, synthetic antioxidants, fytosterols, etc.) present in such liquid media as vegetable oils[12]. The aim of this work was to study the possibilities of PCB removal from water and rapeseed oil by adsorption onto PET and polystyrene (PS) receptacles.

Experimental

PET Receptacles

In the experiment, pre-bubbled PET receptacles of cylindrical shape with i.d. of 21.4 mm and height 150 mm were used. The receptacles were provided by Palma-Tumys. The company uses them for oil and fruit syrup packaging after blowing to volume of 2 dm^3.

PS Receptacles

PS tubes with i.d. of 23 mm and height 120 mm were supplied by Čechvalab (Bratislava, Slovakia).

PCB

PCB congeners (PCB 28 – 2,4,4'-trichlorbiphenyl; PCB 52 – 2,2',5,5'-tetrachlorbiphenyl; PCB 101 – 2,2',4,5,5'-pentachlorbiphenyl; PCB 103 – 2,2',4,5',6-pentachlorbiphenyl; PCB 138 – 2,2',3,4,4',5'-hexachlorbiphenyl; PCB 153 – 2,2',4,4',5,5'-hexachlorbiphenyl; PCB 174 – 2,2',3,3',4,5,6'-heptachlorbiphenyl; PCB 180 – 2,2',3,4,4',5,5'-heptachlorbiphenyl) were purchased from LABSERVICE (Spišská Nová Ves, Slovakia) each with the concentration of 100 µg ml^{-1} in hexane.

Solvents

Hexane of analytical grade was purchased from Merck, Darmstadt, Germany. The solvents were rectified just before use in a distillation apparatus.

Other Chemicals and Materials

Anhydrous Na_2SO_4, was also purchased from Merck.

Experiment

Distilled water was spiked with six PCB congeners solutions diluted in acetone to obtain final concentration in water between 1–7 µg dm^{-3}. Then the water was heated to 40 °C for 1 hour and occasionally shaken to evaporate residual acetone. After cooling to 19 °C, the water was sampled for initial PCB concentration. Then the PET and PS receptacles were filled with the spiked water and placed into a polystyrene box to protect them from light and to keep constant temperature of 19.2 °C. The temperature was monitored and recorded by a thermometer (ThermoScan, BDV, mesto, Netherlands). The samples for analysis were taken after 1; 3; 5; 7; 11; 24; 72 h. To maintain the same conditions, a new set of receptacles was taken for each analysis.

Sample Preparation

5 ml of water were extracted with hexane four times. Hexane layers were combined, dried with anhydrous Na_2SO_4 and evaporated until nearly dry. Remaining solvent was evaporated at room temperature just before analysis. The residuum was dissolved in a known volume of standard solution of PCB-103 and analysed by GC.

GC Analysis

Analysis were performed on gas chromatograph Agilent Technologies 6890N equipped with electron capture detector using capillary GC column DB-5, 60 m × 0.25 mm (i.d.), 0.25 μm film thickness (purchased from J&W Scientific, Folsom, California, USA) using splitless technique with temperature program as follows: isothermal at 110 °C for 1.5 min., then temperature elevation to 200 °C at 30 °C min^{-1}, hold for 0.2 min, then temperature elevation to 300 °C at 2.5 °C min^{-1}. Helium was used as carrier gas with a constant flow rate of 0.8 ml min^{-1}.

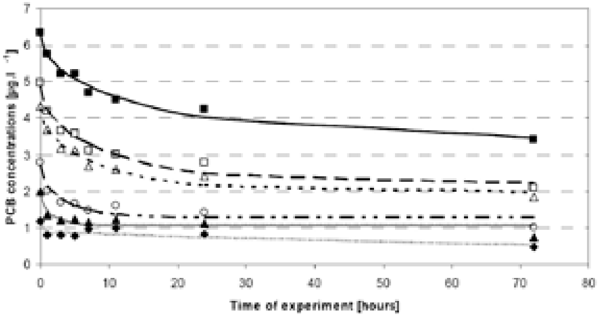

Fig. 1. **Changes in PCB concentration in water stored in PET receptacles (experimentally obtained data compared with data calculated using the kinetic equation of adsorption[1])**
(♦) – PCB 28 c_{exp}, (░) – PCB 28 c_{cald}, (▲) – PCB 52 c_{exp}, (▒) PCB 52 c_{cald}, (○) –PCB 101 c_{exp}, (———) – PCB 101 c_{cald}, (△) – PCB 138 c_{exp}, (-) – PCB 138 c_{cald}, (□) – PCB 153 c_{exp}, (— —) – PCB 153 c_{cald}, (■) – PCB 180 c_{exp}, (——) – PCB 180 c_{cald}

Results

At experiments the concentration of PCBs started to decrease immediately after filling the receptacles in both systems studied as seen from Figs. 1 and 2.

The observed dependences of PCBs concentration *vs.* time were modelled using the kinetic equation (1), which has been derived for the diffusion of PAHs in non-stirred liquids placed into cylindrically shaped receptacles[9]:

$$c_t = c_\infty + \left(c_0 - c_\infty\right)\sum_{n=1}^{\infty}\frac{4}{a^2\alpha_n^2}\exp\left[-D\alpha_n^2 t\right], \tag{1}$$

where c_0 is the initial concentration of PCBs in water, c_t is the concentration of PCBs in the medium at time t, and c_∞ is the concentration of PCBs corresponding to infinite time (equilibrium), a is the radius of the cylinder, α_n are the roots of the zero-order first-kind Bessel function and D is the diffusion coefficient of PCB in water. The parameters c_∞, $c_0 - c_\infty$ and D were identified by the non-linear least squares method by minimizing the sum of squares of differences between the PCB concentrations measured experimentally and those calculated by equation (1). The values of identified parameters are listed in Skláršová et al.[17].

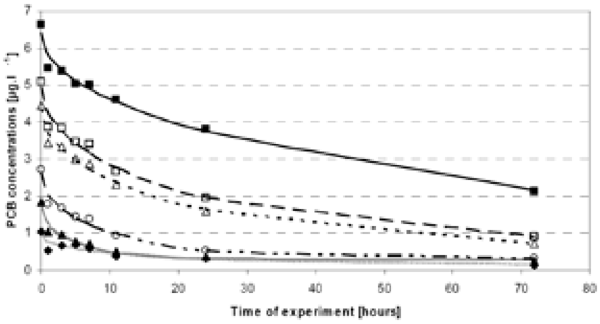

Fig. 2. **Changes in PCB concentration in water stored in PS receptacles (experimentally obtained data compared with data calculated using the kinetic equation of adsorption[1]).**
(♦) – PCB 28 c_{exp}, (░) – PCB 28 c_{cald}, (▲) – PCB 52 c_{exp}, (▒) PCB 52 c_{cald}, (○) –PCB 101 c_{exp}, (———) – PCB 101 c_{cald}, (△) – PCB 138 c_{exp}, (-) – PCB 138 c_{cald}, (□) – PCB 153 c_{exp}, (— —) – PCB 153 c_{cald}, (■) – PCB 180 c_{exp}, (——) – PCB 180 c_{cald}

The extent of PCBs removal can be characterized by the distribution coefficient expressed by the formula:

$$\beta = \frac{c_0 - c_\infty}{c_\infty}. \tag{2}$$

The higher is the value of β, the greater part of PCBs is removed from water after reaching the equilibrium. The values of β are listed in Skláršová et al.[17]. The values of β for PS are much higher than the corresponding values for PET. This implies a much higher affinity of PCBs to PS in comparison with PET.

For understanding the sorption process, it is useful to quantify the ratio of the"total PCBs area" to the contact area of the plastic receptacles. The surface areas S of the molecules of the congeners were calculated using the Savol program (Tripos, St. Louis, Missouri, USA) and are summarised in Skláršová et al.[17]. The number of PCB molecules adsorbed on the polymer surfaces was calculated from the differences between the initial and the equilibrium concentrations of PCBs in water. The following formula gives C, the coverage of the plastic surface by the PCB monolayer:

$$C = \frac{N_A a\left(c_0 - c_\infty\right)S}{2M}, \tag{3}$$

where M is the molar mass of the PCB, N_A is the Avogadro number. The results showed that the total area composed from partial PCB areas adsorbed onto PET is equal to 30.2 %

of the total PET area, while the total area adsorbed onto PS from water is equal to 50.7 %. These values indicate that the PCBs removal from water could be classified as monomolecular adsorption on the plastic surfaces. The values of PCB diffusion coefficients in water, calculated from equation (1) for both experimental systems are not very different which indicates that the same physicochemical processes take place in the PCB removal process from water. The curves of PCBs concentration *vs*. time are subject to measurement uncertainties that propagate into the values of D.

Conclusions

The results and findings of this work lead to the following conclusions:

- The PCB concentration in the water filled into PET and PS receptacles decreases due to adsoption of PCBs onto the surface of plastics.
- The interaction PCB – plastics can be classified as a monomolecular adsorption of PCBs on the polymer surface so that it can be expected that the adsorption equilibrium will obey the Langmuir isotherm.
- The values of diffusion coefficients obtained enable to predict the rate of PCB elimination at any time of the interaction; the values of the distribution coefficients characterize the extent of the PCBs removal.
- Although both polymers are suitable for PCB removal, PS is far more effective in comparison with PET.

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L13 ANALYSIS OF *FUSARIUM* MYCOTOXINS: A CRITICAL ASSESSMENT

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Introduction

Mycotoxins, secondary metabolites of microscopic filamentary fungi, are compounds generally considered to be very toxic. Their occurrence in agricultural commodities represents a major health concern for humans and animals. One of the most important fungi genera, that produce mycotoxins, is *Fusarium*. *Fusarium* species are able to produce lots of structurally different mycotoxins including trichothecenes, zearalenones and fumonisins. Trichothecenes are a group of tetracyclic seskviterpene alcohols and can be divided into two groups: type A and type B (they differ from each others in the existence of ketone at C8 in trichothecene type B molecule). The main representatives of type A trichothecenes include e.g. T-2 toxin, HT-2 toxin, diacetoscirpenol, 15-acetoscirpenol and neosolaniol. The most important naturally occurring type B trichothecenes is deoxynivalenol representing the most abundant trichothecene found in cereals, followed by 15 acetyldeoxynivalenol, 3-acetyldeoxynivalenol, nivalenol and fusarenone-X. Other mycotoxins belonging to the genus Fusarium are fumonisins (esters of 20-carbone backbone and two tricarballylic acids) and zearalenone (substituted lacton of resorcyclic acid).[1–3] Beside free mycotoxins, the existence of mycotoxin conjugates, toxins bound to more polar compounds like glucose, which originate as a result of mycotoxins metabolism by plants, was proved[4,5]. Deoxynivalenol-3-glucoside (DON-3-Glc) was found to be dominating[6].

Because of relatively high incidence of mycotoxins, there is an urgent need for effective monitoring of these compounds in foodstuffs. Over the past 15 years, a great progress in mycotoxins analysis area has been made. Sampling contributes to the largest variability in the analysis of mycotoxins , thus, obtaining of a representative sample represents a crucial step. Sampling strategies of agricultural commodities within mycotoxin analysis are defined by the European legislation EC/401/2006.[7–9]

There are various criteria typically considered when analytical method is developed. Not only the overall cost, but also other factors such as speed of the analysis, level of technical skills of an analyst, and the type of results provided by the method (qualitative or also quantitative) need to be balanced and the relative importance of each criterion evaluated. Especially for multidetection methods, when a large number of mycotoxins possessing a wide range of physicochemical properties considered, a compromise between the factors mentioned above is usually needed.[8–10]

The first step in multimycotoxin analysis includes the isolation of particular analytes. The extraction should perform a compromise between the solvent strength required for the transfer of analytes from the matrix to the solution and the compatibility of solvent with further analytical process. An acetonitrile – water (84 : 16, v/v) mixture is commonly used providing sufficient recoveries, and simultaneously minimises number of co-extracted matrix compounds. Additionally, this solvent mixture represents an azeotropic composition and thereby its evaporation within further sample preparation procedure is facilitated[11].

Once the sample extract is obtained, the clean-up step should be performed to remove impurities potentially interfering during the determination step. Beside this, the clean-up procedure helps to concentrate the mycotoxins prior to their analysis; especially immunoaffinity columns based on specific immunochemical reaction of analyte and antibody present in the cartridge are very convenient for this purpose. The specificity of immunoaffinity clean-up for only one target mycotoxin or class of mycotoxins can be in some respect a limiting factor, particularly in the case of multimycotoxin analysis[9,12]. However, multimycotoxin immunoaffinity clean-up cartridges specific to a wide range of analytes are already available[13,14]. Additionally, common SPE approaches representing by e.g. MycoSep columns are also widely used. The main advantage of MycoSep clean-up is the speed of procedure: no time-consuming rinsing steps as in the case of immunoaffinity and/or other SPE clean-up are required.[15–17]

The final step of the analytical procedure is the determinative step. Nowadays, reference methods for mycotoxins determination involve almost exclusively liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which provide both selectivity and sensitivity of the detection.[7–9] There are no limitations in sample preparation such as derivatisation step, which is needed within the gas chromatographic approaches GC-ECD or GC-MS (*Note*: derivatisation of hydroxyl groups in order to attain the volatility is typically performed using heptafluorobutylimidazole or trimethylsilylether).[18–20] A challenging option in the field of LC-MS analysis is the use of high-resolution system LC-TOFMS, which provides a feasible tool for non target masked mycotoxin screening.

Beside classical confirmatory chromatographic methods mentioned above, there are also methods based on immunological assays. Especially ELISA as rapid quantitative tools for mycotoxin analysis is very suitable. However, possible overestimation of results caused by cross-reactions with compounds similar to target analytes could be a limitation of this approach.[7– 9,17]

In the following text, three different analytical approaches are discussed. Firstly, performance characteristics of GC-ECD and LC-MS/MS methods are compared. Secondly, results obtained by means of two commercial ELISA assays and reference LC-MS/MS method are introduced. A proof of ELISA capability in terms of its cross-reaction potential is presented.

Experimental

Materials and Methods

Standards of mycotoxins nivalenol (NIV), deoxynivalenol (DON), deoxynivalenole-3-glucoside (DON-3-Glc), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenone-X (fus-X), T-2 toxin (T-2), HT-2 toxin (HT-2) and zearalenone (ZEA) were purchased from Biopure (Tulln, Austria). A Synergi Hydro RP column (Phenomenex, Torrance, CA, USA), a liquid chromatograph HP1100 Binary Series LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an ion trap mass analyser LCQ Deca (Finnigan, San Jose, CA, USA) were employed for LC separation and MS/MS detection. For GC–ECD method, a gas chromatograf HP 5890 Series II with an electron capture detector (^{63}Ni) and a capillary column HP-35 (30 m × 0,25 mm I.D. × 0,25 µm phase) with (35%-fenyl)-methylpolysiloxan stationary phase (Agilent Technologies, Palo Alto, CA, USA) were used. For ELISA analysis, two commercial available DON kits, i.e. Ridascreen® DON (R-Biopharm, Darmstadt, Germany) and AgraQuant® DON Assay 0.25/5.0 Test Kit (Romer Labs, Tulln, Austria), were purchased.

GC-ECD method: An amount of 10 g of homogenised ground sample was extracted by shaking with 100 mL of an acetonitrile–water mixture (84 : 16, v/v) for one hour and extract was filtered (Filtrak No. 390, VEB Freiberger, Berlin, Germany). The crude extract was passed through MycoSep™ #225 and four millilitres of cleaned extract was evaporated. Residue after evaporation was redissolved in methanol, transferred into the derivatisation vial, and after methanol evaporation using a gentle stream of nitrogen, derivatisation for 20 min at 60 °C using 100 µl trifluoracetanhydride with addition of 10 mg $NaHCO_3$ was performed. Further, 500 µl of isooctane, 1 ml of deionised water and 0.5 g of anhydrous sodium sulphate was added followed by removing of 300 µl of the organic layer and addition of 200 µl of isooctane to the vial for GC–ECD analysis.

LC-MS/MS method: An amount of 12.5 g of homogenised ground sample was extracted by shaking with 50 mL of an acetonitrile–water mixture (84 : 16, v/v) for one hour and the crude extract was filtered (Filtrak No. 390, VEB Freiberger, Berlin, Germany). Eight millilitres of filtered extract were passed through the MycoSep™ #226 column and four millilitres of filtered extract were evaporated to dryness and dissolved in 1 mL of a water–methanol mixture (50 : 50, v/v). Finally, the sample was passed through a 0.2 µm microfilter (Alltech, Deerfield, IL, USA) prior to analysis.

ELISA: In case of immunochemical assays, both sample preparation and the ELISA analysis itself were carried out strictly according to manufacturer recommendations. An amount of 20 g of ground sample was extracted with 100 ml of deionised water and vigorously shaken for 3 minutes. Further, the extract was filtered and an appropriate aliquot was placed into the microwell.

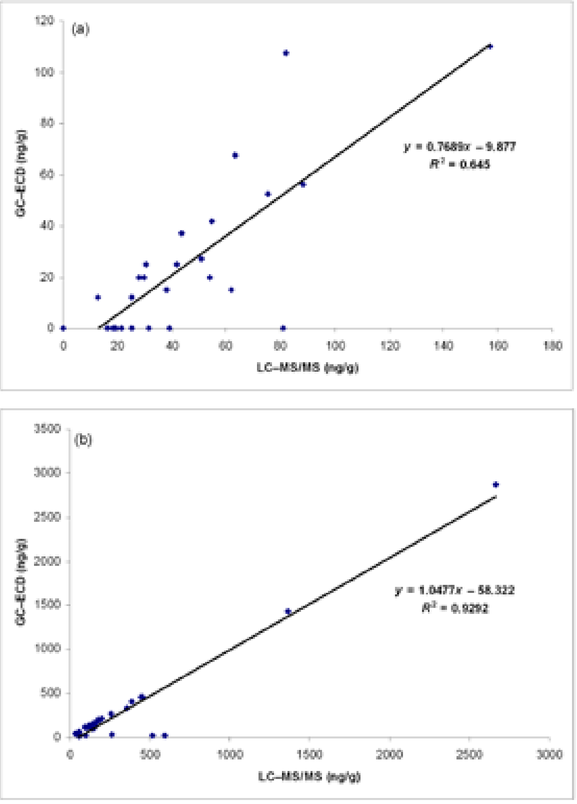

Fig. 1. **Correlation of the results of wheat samples obtained by LC–MS/MS and GC–ECD methods for a) NIV b) DON.**

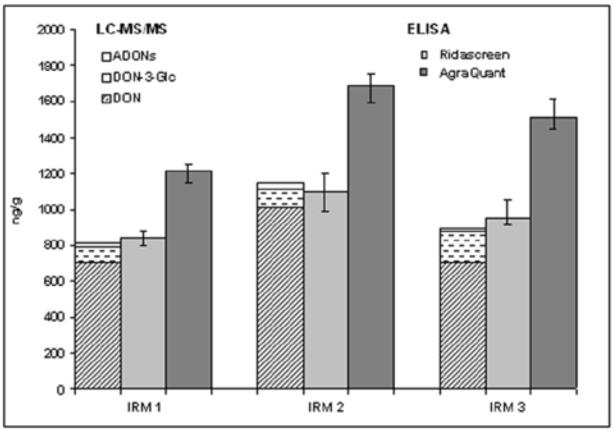

Fig. 2. **DON content in reference materials measured by LC–MS/MS and two types of commercial ELISA kits.**

GC-ECD *vs.* LC-MS/MS

Using the previously used GC–ECD method, NIV, DON, FUS-X, 3-ADON, 15-ADON, HT-2 and T-2 were analysed within a single run. Recoveries 68–97% and relative standard deviations (RSDs) ≤ 16 % were obtained for all of analytes analysed. As regards LC–MS/MS method, the scope of target analytes has been extended for ZEA and "masked" mycotoxin DON-3-Glc. Recoveries of LC-MS/MS ranged between 76–95 % with exception of NIV and DON-3-Glc, where recoveries were somewhat lower (approx. 65 and 43 %, respectively). The low recoveries of NIV and DON-3-Glc can be contributed to their partial adsorption onto the polar MycoSep™ #226 column cartridge. In spite of somewhat recoveries for NIV and DON-3-Glc, the RSDs of LC-MS/MS method were ≤ 10%.

The detection limits (LODs) were 2–10 times lower for LC-MS/MS compared to GC-ECD. In addition, for T-2, the LOD decreased even less (from 130 to 1 ng g^{-1}) by employing LC-MS/MS procedure.

With respect to our effort to increase the DON-3-Glc recovery, the LC-MS/MS analytical procedure was performed without MycoSep clean-up step. Although the LODs of LC-MS/MS method were approximately 5 times higher compared to LC-MS/MS method with MycoSep™ #226 clean-up. , the recovery of NIV and DON-3-Glc increased to 78 and 89 %, respectively. Since a higher amount of matrix components is introduced to the MS system (causing the ionisation suppression in ESI ion source), it is important to use for quantification purposes the matrix-matched standards to avoid quantification errors.

For comparison purposes, the data obtained by GC-ECD (with MycoSep™ #225 clean-up) and LC–MS/MS methods (with MycoSep™ #226 clean-up), a set of 25 naturally contaminated wheat samples has been analysed. With regard to low incidence of other mycotoxins than DON and NIV in the investigated samples, correlation of results was carried out for these two analytes only (see Fig. 1.). As shown in Fig. 1.a, GC-ECD method provided systematically underestimated results for NIV compared to LC-MS/MS. This phenomenon can be explained by decomposition of NIV during derivatisation[18]. For DON, acceptable correlation between GC-ECD and LC-MS/MS results was achieved (except of five outliers). Discrepancies between the results presumably lies also in the crucial derivatisation step[21,22].

ELISA *vs*. LC-MS/MS

Once the immunochemical assay was performed, comparison with results obtained by LC-MS/MS was accomplished. We should note that LC-MS/MS analyses were performed without MycoSep clean-up to avoid the discrimination of DON-3-Glc caused by its trapping on MycoSep cartridges). As shown in Fig. 2., the results obtained by ELISA (Ridascreen) are comparable to that of LC-MS/MS (*Note*: total DON content calculated from DON-3-Glc and acetylated DONs contribution is considered). Whereas AgraQuant kit provided overestimated results compared to LC-MS/MS (overestimation 49, 46 and 69 % for if total DON obtained by LC-MS/MS is considered 100 %). This trend could be explained by cross-reaction of kit antibodies with matrix components and/or with other DON conjugates. Similar phenomenon was observed during the analysis of beer samples by AgraQuant ELISA kit (overestimation compared to total DON determined by LC-MS/MS was up to 600 %)[23].

Conclusions

Different analytical strategies for sample preparation and instrumental analysis for the determination of mycotoxins were investigated. The choice of method used depends on various factors such as overall cost, detection level required, complexity of matrix and available instrumentation. Obtained results differed by method used, which was demonstrated especially for DON and partially also for NIV. For GC-ECD, derivatisation step was shown to the most critical part of the method; losses of analytes were demonstrated compare to LC-MS/MS approach. As regards ELISA assays (determination of DON), they tended to overestimate the results compare to LC-MS/MS method. We hypothesis that this can be partially explained by the cross-reactivity to DON conjugates (acetylated DONs and DON-3-Glc shown in our recent study)[23], however further research is needed to fully explain this phenomenon.

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3.2. Posters

P01 DETERMINATION OF Β-CAROTENE IN THE GELATIN CAPSULE

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Introduction

β-carotene is the most well known of the carotenoids, a phytonutrients family that represents one most of the widespread groups of naturally occurring pigments. Steenbock suggested that the plant pigment carotene was responsible for the vitamin A activity and that carotene could not be vitamin A, it may be converted metobolitically to the actual vitamin in the 1919[1]. β-carotene consists of a chain of 40 carbon atoms, with conjugated double bonds and a ring structure at each end of the chain. Depending on the positions of the molecular groups attached to the carbon chain, naturally occurring beta carotene may be: *cis-trans* and all *trans* isomers. The most common carotenoid in all green plants is β-carotene. Animal and our body cannot synthesis β-carotene and they depend on feed for their supply. There are many studies showing β-carotene can show role of antioxidant potency. Also several studies have been focused on the content of β-carotene in food products, food supplements and biological fluids.[2–4]

One of the vitamin supplement package products is gelatin capsule. Gelatin capsule has a very rapid dissolution property, usually 90 seconds in water at 37 °C. So, this protein is a very useful raw material for emulsifier in food, pharmaceutical and others products. Previous studies show that gelatin is used as a carrier, coating or separating agent for other substances. Supplemental intake of β-carotene probably should not exceed 3–15 mg per day. β-carotene supplements are found on the Generally Recognized as Safe (GRAS) list issued by the Food and Drug Administration.

The purpose of this study was to find new method for determination of β-carotene and isolation process, of course.

Experimental

High-performance liquid chromatography is generally accepted as the modern method of choice to separate, identify, and quantify caroteniods. Specially, it combined with ECD, UV-VIS and fluorescence detector are the common method for determination and identification of β-carotene in vegetables and other samples.

Chemicals and Materials

β-carotene was obtained from personal prepared tablet materials (at TBU in Zlin). Methanol (purity: 99.8 %), acetonitrile (purity: 99.9 %) were obtained from Merck KGa (Darmstadt, Germany). Phosphoric acid (purity: 85 %) was obtained from Chemapol (Prague, Czech Republic). All solvents were of HPLC grade. Deionized water, purified by Aqua osmotic system (Aquaosmotic, Tisnov, Czech Republic) was used for solve sample preparation stages. Hexan (purity: 99.9 %) was obtained from Penta (Prague, Czech Republic), was used in extraction of sample. Ethanol was obtained from Chemapol (Prague, Czech Republic), used to solve sample after evaporation.

Equipment and Chromatographic Conditions

HPLC separation was performed using HPLC with ECD detection (Coulochem III, ESA). Potentials of the cells for detector were 500 and 600 mV.

β-carotene was separated on the Supelcosil LC18 DB (250 × 4.6 mm,5 µm) with a mobile phase of methanol/acetonitrile/phosphoric acid (70 : 29.5 : 0.5, v/v/v) at a flow rate of 1.1 ml min^{-1}. The separation was carried out ambient temperature. In this experiment we used isocratic elution.

Sample Preparation

Filling from gelatin capsule was dissolved into 10 ml of deionized water and after that extracted using 10 ml of n-Hexan solution and extraction process was repeat six times. After extraction, sample solution was evaporated to dryness in rotary evaporator at 35–40 °C. After evaporation, the residue was dissolved in 10 ml of ethanol and was filtered trough 0.45 µm filter (Nylon) before injected into column. Sample (20 µl) was injected into a column that has been equilibrated with solvent mixture of mobile phase.

Results

The total carotenoid content of the tested gelatin capsule of vitamin supplement (β-carotene, extract from blueberries) was 6 mg (Table I).

Table I
β-carotene content in the gelatin capsules

Name	Weight [g]	β-Carotene [mg]	Extraction of blueberries [mg]
	0.3156	6	150

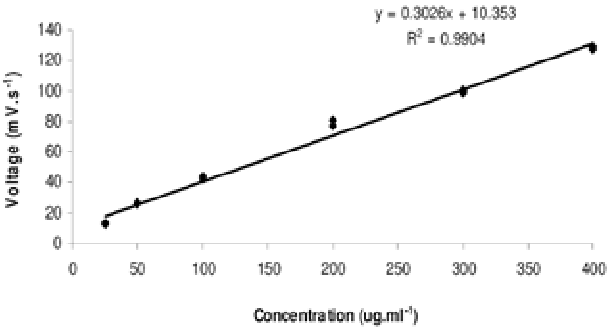

Fig. 1. **Calibration curve of β-Carotene**

Standard curve of β-carotene are shown in Fig. 1. We prepared standard solution from 25 μg ml^{-1} to 400 μg ml^{-1} for construction of a standard curve in our study.

We were interested to quantification of real value of β-Carotene in oil matrix gelatin capsule vitamin supplement. We were conducting extraction process at water bath at 30 °C for 2 min and at ambient temperature. The samples were completely extracted 6 times after 30 minute extraction using n-hexane.

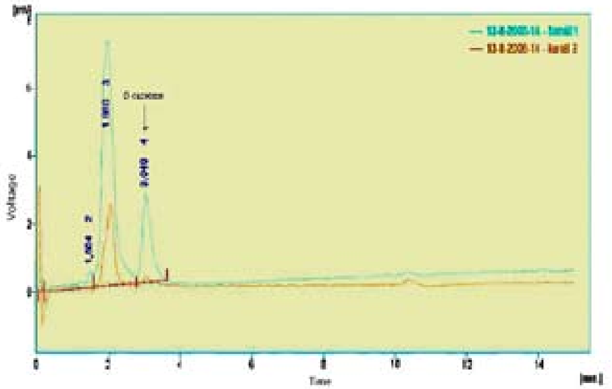

Fig. 2. **Chromatograms of a gelatin capsule vitamin supplements**

Peak 3 were positively identified like β-carotene. Each sample was measured five times (Table II).

Result of our study shows 5.83 ± 0.02 mg per capsule β-carotene in examined samples.

Table II
Content of β-carotene in a gelatin capsule vitamin supplement

Sample	Average peak area [mV s^{-1}]	Content of β-caroten in tablet [mg]
1	52.00	5.804
2	51.25	5.700
3	53.30	5.986
4	52.45	5.867
5	53.40	5.999
Average	52.48	5.83 ± 0.020

Conclusions

This study shows that the gelatin capsule vitamin supplement analyzed here contains 5.83 ± 0.02 mg of β-carotene. Producer declares 6.00 mg in each capsule. Must be checked it, if producer has a right vehicle for mixture of β-carotene and blueberry extract, because we have verified our method with standard addition. Finally we can conclude that HPLC with ECD method have shown to be acceptable for determination and quantification of β-carotene in a gelatin capsule vitamin supplements.

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P02 DETERMINATION OF B-CAROTENE IN TOMATO BY HIGH PREFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTOR

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Introduction

Commonly studied carotenoid in the human diet is β-carotene because of its antioxidative and provitamin A activities. Provitamin A carotenoids, particularly β-carotene in fruits and vegetables, are the major source of vitamin A for its deficiency is a serious health problem in many developing countries. The abundant sources of β-carotene are sweet potatoes, carrots, spinach, tomato and other vegetables and fruits. Since tomatoes are major use for human dietary in many countries, it is becoming a prevention of deficiency of antioxidant vitamins. Many studies have been focused on antioxidant vitamins content in tomatoes. Abdulnabi et al.[1] investigated the antioxidative vitamin (vitamin E, vitamin C and β-carotene) content in tomatoes cultivated in Hungary, using HPLC. This study suggests that the highest values of β-carotene were found in Gitana, Katinka and Delfino cultivars (3.13–3.79 $\mu g\,g^{-1}$) and the lowest levels of β-carotene were in Tampo and Selma cultivars. Therefore, β-carotene occurs in tomatoes and various tomato products in amount of 0.23–2.83 $mg\,100\,g^{-1}$(ref.[2]).

The objective of this work was to estimate, using modern analytical techniques (ESA Coulochem III Multi-Electrode Detector), and the β-carotene content of tomatoes.

Experimental

High performance liquid chromatography (HPLC) is the most commonly used method for the separation, quantitation, and identification of carotenoids found in vegetables samples.

Sample Preparation

Initially a 10 g sample of tomato was placed in a 50 ml flask and mixed with 26 ml of extraction solvent (acetone : hexane, 50 : 50, v/v). The mixture was shaken in a water bath at 35 °C for 20 min. The upper phase was collected and poured into a 50 ml flask. The lower phase was extracted again with same solvent and shaken for 30 min. The upper phase was also collected and poured into the same flask. After filtration through Filtrak No.390 filter paper. The filtrate was poured into a 250 ml flask and evaporated under vacuum at 30 °C and residues were redissolved in 5 ml of ethanol. The solution was filtrated through a 0.45 μm nylon filter and 20 μl was injected into HPLC system. HPLC separation was performed using ESA HPLC with ECD detection (Coulochem III) which is equipped with analytical column Supercosil LC18-DB (250 × 4.6mm, particle size 5 μm). Separation took place at 30 °C, flow rate of mobile phase (methanol : acetonitrile : phosphoric acid) was 1.0 $ml\,min^{-1}$. Potentials of the cells for detector were 500 and 600 mV.

β-carotene standard solution concentrations ranging from 25 to 400 $\mu l\,ml^{-1}$ were prepared for the standard curve.

Identification and Quantification of β-carotene in Tomato

The identification of β-carotene was carried out by the retention time. The equation from the calibration curve was used for the calculation of the amount of β-carotene in tomatoes. The regression equation and correlation coefficient (R^2) were obtained using Microsoft Excel 2003 software to calculate the quantity of β-carotene in tomatoes.

Results

Typical chromatogram depicting the separation of a β-carotene standard solution is shown in Fig. 1. We were interested in checking chromatographic condition and retention time of β-carotene using a selected method in this investigation.

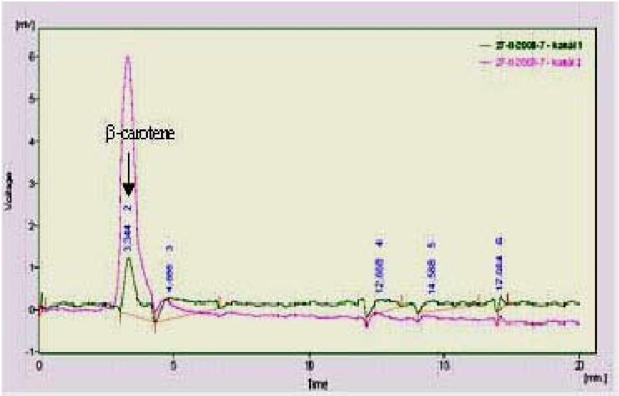

Fig. 1. **Chromatogram of standard β-carotene**

The predominant peak at approximately 3.3 min is β-carotene. This value is used for identification of β-carotene in samples. The calibration curve was measured with the

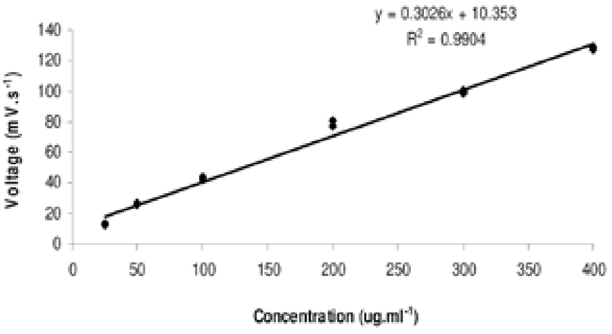

Fig. 2. **Calibration curve of β-Carotene**

standard of β-carotene that was dissolved in ethanol. Fig. 2. shows calibration curve of a standard β-carotene.

The regression equation was obtained from the calibration curve of β-carotene, $y = 0.3026\,x + 10.353$ ($R^2 = 0.9904$). This studied range was appropriate to calculate concentration of β-carotene in tomatoes.

The small peak showing the β-carotene on Fig. 3. (very closely at 2.912 min) has not been identified, because it is an admissive peak area that matches β-carotene's peak in chromatograms.

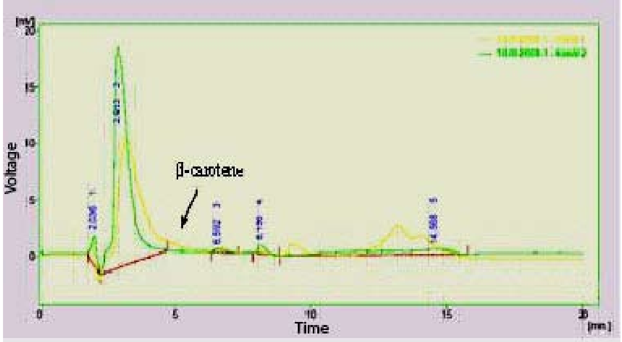

Fig. 3. **Chromatograms from tomato extract**

However, this does not pose any problem; usually tomatoes do not contain high concentration of β-carotene. Result of this chromatogram suggests the presence of other organic compounds in tomato. Many study indicated that lycopene and/or other common cartenoids's levels are higher than β-carotene in tomatoes.

Table I shows the results obtained from the analysis of β-carotene in different samples. The first sample was immediately extracted with extraction solution after purchased from supermarket. The second sample was stored 2 weeks in a refrigerator before extraction.

Table I
Tomato concentration of β-carotene

Sample	Weight [g]	Peak area [mV s^{-1}]	Amount of β-carotene [mg 100 g^{-1} of fresh mass]
1	10.0707	14.0	1.197
2	10.1726	1.6	0.024

The higher value of β-carotene (1.197 mg 100 g^{-1}) was obtained with the first sample. The values obtained in this study are very close to those reported by Olives Barba et al.[3], who reported a range of 0.6–1.2 mg per 100 g for β-carotene in 4 tomato varieties.

Bender (1993) has summarised the contradictory results of published studies designed to estimate the magnitude of vitamin loss during frozen storage of vegetables and fruits. Thus, we presumed that the content of β-carotene was reduced in second sample during storage period.

Conclusions

The present study shows that the measured levels of β-carotene (1.197 mg 100 g^{-1}) were low compared to the recommended intake. European epidemiological studies revealed 6–10 mg β-carotene as the recommended daily intake to provide good health and a reduction in risk of diseases (Lachance, 1998; National Research Council, 1989). Accordingly, one serving 100 g tested tomato contributes 11.97–19.95 % of the recommended daily intake of β-carotene.

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P03 PROTECTIVE EFFECT OF LYCOPENE FROM TOMATOES POWDER IN OXIDATIVE STRESS INDUCED BY ADMINISTRATION OF L-THYROXIN IN RATS

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Introduction

Accumulating evidence has suggested that the hyperthyroidism is associated with increases in radical oxygen species (ROS) production and lipid peroxidation products in some tissues of rats. The response of the antioxidant systems is unclear. The changes in the levels of antioxidant enzymes in various tissues were found to be imbalanced and often opposite[1]. Lycopene, the main carotenoids in tomatos, has been shown to be a potent antioxidant in vitro and in vivo. Recent interest in lycopene is due to the finding of an inverse association between dietary lycopene and risk of some types of cancer and cardiovascular disease[2].

There are few literature data, which present studies regarding the effect of nonenzymatic antioxidants administration on oxidative stress induced by thyroid hyper function. In the present work, effects of the ingestion of tomatoes powder, rich in lycopene, on the activities of antioxidant enzymes (superoxid dismutase – SOD, catalase, and glutathion peroxidase), the oxidation of lipids and on fatty acids composition on liver, thyroid and myocardium were examined in rats intraperitonial injected with L-thyroxin.

Experimental

Experimental Procedure

Experiments were performed on three groups of male white Wistar rats: control (C – nontreated animals, normal diet); H group (treated animals with thyroxin and normal diet) and HT group (animals treated with thyroxin and tomatoes powder enriched diet). We induced hyperthyroidism by intraperitoneal L-thyroxine administration, 10 µg(100 g bodyweight)$^{-1}$, daily for a week. Daily dietary for group HT diet was supplemented with a natural product, tomato powder, obtained by Kunding Food Company. These powders contain 62.5 µg lycopene (1 g powder)$^{-1}$. Each rats from HT group received 25 µg lycopene (100 g wet)$^{-1}$ day^{-1} corresponding to 0.4 g tomato powder (100 g wet)$^{-1}$ day^{-1} during 7 days. After 8 days the animals were sacrificed and thyroid, liver and myocardium tissue were collected.

Biochemical Determinations

Protein extracts was obtained using potassium phosphate buffer (pH = 7.35). The activities of SOD, catalase, glutathion peroxidase and lipids oxidation were estimated in this protein extract using photometric methods[3].

Lipids extracts from different tissues was obtained with methanol and chloroform, based on Folch procedure The fatty acids analysis was made on total lipids extracts using gas-chromatography (GC)[4].

Results

In all biological samples we can observed an increase of antioxidant enzymes activities in H – rats compared with control. In the case of rats treated with diet rich tomatoes powder it can be observed a diminution of antioxidant activities probably due the antioxidant properties of lycopene (Table I). The increase of antioxidant enzymes activity in thyroidian tissue could be explained due to stimulation effect of thyroid hormones on protein shynthesis.

Table I
Antioxidant enzymes activity

Tissue/ Experimental groups	Catalase Ncat [g wet tissue^{-1}]	Glutathone peroxidase U GPx [g wet tissue^{-1}]	Superoxid dismutase U SOD [g wet tissue^{-1}]
Liver			
C	5068.79	62.93	225.69
H	8499.99	40.30	243.61
HT	5891.08	61.64	206.23
Thyroid			
C	410.10	4.66	58.87
H	793.33	7.66	83.99
HT	424.99	5.16	100.96
Myocardium			
C	717.64	20.18	53.84
H	835.08	28.55	11.33
HT	690.62	23.67	95.14

Lipid peroxides levels were significantly increased compared with control group. Oral administration of lycopene resulted in a significant reduction of lipid peroxides levels in HT-group compared with rats treated only with thyroxin (Table II).

Table II
Lipids peroxides level (MDA µmoles g wet tissue^{-1})

Experimental groups	Liver	Thyroid	Myocardium
C	1239.44	4.98	912.11
H	1750.40	12.91	1201.92
HT	1294.74	7.96	988.24

The results of the analysis of fatty – saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) – are showed in detail by Table III.

In thyroid gland, compare with the control, we can observed a decrease of total fatty acids content in experimental group treated with L-thyroxin. The determination of content in PUFA and the ratio between UFA and SFA are the other methods that can be used in order to determinate the level of lipids peroxidation process. These ratio decrease in T group (so in this case appear a rise of lipids peroxidation level) but increase in group supplemented with lycopene.

Table III
Fatty acids contents in total lipids extract [mg g^{-1} wet tissue]

Tissue/FA	C group	H group	HT group
Liver			
SFA	1.56	3.59	10.07
MUFA	1.03	3.52	5.81
PUFA	1.36	5.70	18.15
Thyroid			
SFA	11.91	2.26	16.14
MUFA	5.85	1.20	7.95
PUFA	16.13	2.88	26.34
Myocardium			
SFA	4.02	2.23	5.32
MUFA	2.69	2.26	4.33
PUFA	4.35	3.08	8.68

Thyroid hormones stimulated the biosynthesis of FA in liver[5]. In the case of rats treated with L-thyroxin the FA content is higher compare with the control. The highest level apear in the case of rats from HT group because the administration of L-thyroxin as an effect the stimulation of FA synthesis and lycopene protect these acids against oxidation.

In myocardium fatty acids content decrease in T group compared with the control because in this tissue the lypogenic activities of thyroid hormones is lower compared with other tissue.

Conclusions

Tomatoes powder reduces the burden of oxidative stress in hyperthyroidism and led to reduction in lipid peroxidation and to increase in antioxidant level.

The administration of L-thyroxin has an effect a decrease of FA content in thyroid gland and myocardium. The content of FA in hepatic tissue prelevated from rats treated with L-thyroxin is higher compared with the control because this thyroid hormone stimulate the synthesis of these acids.

In all analyzed tissue the tomatoes powder administration has an effect an increase of FA content.

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P04 THERMOPHILIC BACTERIA APPLICATION TO WHEY BIODEGRADATION

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Introduction

In former times the whey was thought as valueless waste of dairy industry rising in cheese, cottage and casein production. Its application was in feed only. In these days, the whey meaning inceases simultaneously with new knowledges of nutrition, with sepative methods development, envirtonment protection endeavour and rapid progress of food and pharmaceutical industry. Environmental aspect is purity rivers maintenance and it means the prohibition of waste-water disposal (the whey too). It is possible to make the whey „liquidation“ by the help of microbial systems. Several microbial systems were used for these purposes but they are still pure described. Testing of new microbial systems for the degradation ot the whey is in progress. In this work, the testing of degradation of the whey medium using the mixture of thermophilic bacteria is described.

Experimental

Experiments were practised with thermophilic aerobic culture, the mixture of bacteria of the genus *Bacilus* and the genus *Thermus* (sludge from waste treatment plant Bystřice pod Hostýnem). The whey from cheese production (type ermine) was used as the medium. Casein (0.1 mol dm^{-3} H_2SO_4) and α,β-lactoglobulins (20 min at 85 °C) were taken out before cultivations. The whey was centrifuged and pH value was adjusted. All cultivations were practised in the laboratory fermentor BIOSTAT B (B. Braun Biotech.) with working volume 2 dm^3.

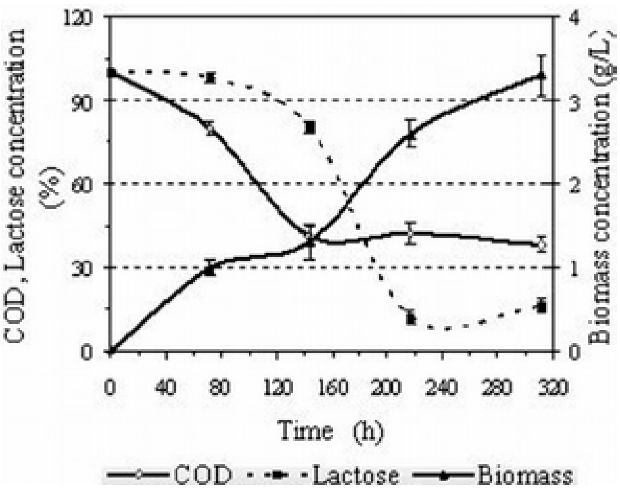

Fig. 1. **Batch cultivation progress in longer time (312 hours)**

Cultivation conditions: multiple turbo-stirrer 250 min^{-1}, aeration 10 dm^3 min^{-1}, pH adjusted on value 6.5, medium temperature 60 °C, vessel isolation Mirelon. The batch was selected as the cultivation method. The cultivation time was 66 h (1st experiments) and 312 h (2nd experiments). The process characteristics were determined direct by fermentor system (dissolved oxygen concentration) or by the help of samples taking and their analyses (biomass – turbidimeter, COD – spectrofotometer at 600 nm, lactose – HPLC after microfiltration, 100 % lactose ≈ 46 g.dm^{-3}. The statistical analyse was realized in the relevancy level 0.05.

Results

The experimental results are summarized in Fig. 1. & 2.

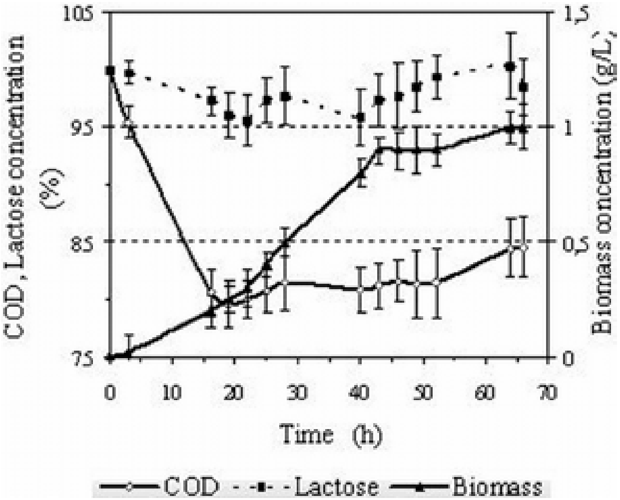

Fig. 2. **Batch cultivation progress in shorter time (66 hours)**

Hence it follows:

- Lactose was not used as primary substrate source (Fig. 2.), consequently the exponential growth phase in time 3–43 hours (specific growth rate $\mu = 0.05 \pm 0.01$ h^{-1}) is a result of simpler whey compound utilization probably, for example lactate.
- Lactose metabolisation was combined with the 2nd growth phase in time 120–260 hours (specific growth rate $\mu = 0.010 \pm 0.006$ h^{-1}) (Fig. 1.).
- The biomass accrument was very slow. It was probably an inhibition effect of concentrated substrate, especially in the 1st cultivation period (Fig. 1., Fig 2.). Total biomass concentration after 312 hours: (3.3 ± 0.3) g dm^{-3} at productivity 0.01 g.dm^{-3} h^{-1}.
- The oxygen limitation can contibute to slow biomass growth (primarily in the 1st growth phase).
- Maximum COD elimination was in the 1st half of every exponetial growth phase: 15 ± 3 % after the 1st growth phase and ± 4 % after the 2nd growth phase.
- The oxygen transfer coefficient was determined too, $k_L a = (270 \pm 21)$ h^{-1}.

Conclusions

It was achieved total COD elimination 62 % after 312 hours of the batch cultivation with thermophilic culture on waste whey. The biodegradation process rate is limited of lower biomass concentration and oxygen insufficiency. The correspondence between the time of exponential bacteria growth and the main COD elimination period was demonstrated. In conclusion, the mixture of termophilic bacteria population has significant whey biodegradation abilities in view of its application to dairy wastes.

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P05 GROWTH CURVES OF MIXED THERMOPHILIC BACTERIA

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Introduction

Temperature is one of the most important enviromental factors controlling the activities and evolution of organisms and is one of the easiest variables to measure. Not all temperatures are equally suitable for the growth and reproduction of living organisms. Most of animals, plants and eucaryotic microorganisms are not able to exist at temperatures above 50 °C. It contrast to this fact, some procaryotic microorganisms can grow at temperatures above 60 °C commonly. We call them thermophiles. The most thermophilic microorganisms live in the thermal zone of the Earth. Their industrial use is various, from food industry to waste-water treatment. The important knowladge for any application of these microorganisms is their basic growth characteristics. The aim of this work was testing of growth of the mixture of thermophilic bacteria with possible potential to be used in waste-water industry.

Experimental

Experiments were carried out using the mixture of thermophilic bacteria of the genus *Bacilus* and genus *Thermus* (sludge from waste treatment plant Bystřice pod Hostýnem). The substrates composition: C-source 4 g dm^{-3}, $MgSO_4 \cdot 7H_2O$, 1. g dm^{-3}, $(NH_4)_2SO_4$ 0.3 g dm^{-3}, $KH_2PO_4$7 g dm^{-3}, yeast extract 2.4 g dm^{-3}, Peptone 8.5 g dm^{-3}. Glucose, lactose, sacharose and maltose were tested as the main source of carbon in succesive steps. Inoculum was prepared in the same conditions as tested cultivations. Inoculation rate: 1 : 10. Cultivations were practised 50 hours in two different systems – in 1.5 ml vials (anaerobic conditions) and in 1.5 dm^3 laboratory fermentor (aerobic conditions).

The vials, with magnetic bar and tightly screw capped, were placed into autosampler plate thermostated at 60 °C. Flow injection analysis (FIA) was used for measurement of microorganism quantity. The amount of 10 µl of the sample was injected into water stream (0.2 ml min^{-1}) and the absorbance at 600 nm was measured. The area under the curve of chromatogram was used for microorganism quantification. The sample was drawn and analysed each 5 minutes for 50 hours. The HPLC system HP 1100 (Agilent Technologies, Palo Alto, USA) consisted of vacuum degasser unit (model G1322A), quaternary pump (G1311A), autosampler (G1313A) and quadrupole mass spectrometer (G1946VL) with electrospray ionization was used. The ChemStation software (Rev. A 10.02) controlled the chromatographic system and it was used for chromatogram evaluation. The vials with cultivation media were placed in thermostated plate KEVA (Ing. Pavel Krásenský, Brno, Czech Republic) and stirred using magnetic stirrer.

The cultivations in bioreactor were practised in a laboratory batch fermentor BIOSTAT B (B. Braun Biotech.) Cultivation conditions: multiple turbo-stirrer 250 min^{-1}, aeration 10 dm^3 min^{-1}, pH adjusted on value 6.5, medium temperature 60 °C, vessel isolation Mirelon. Biomass concentration was determined by the help of samples taking and their analyses in turbidimeter.

Results

The experimental results are summarized in Fig. 1. & 2.

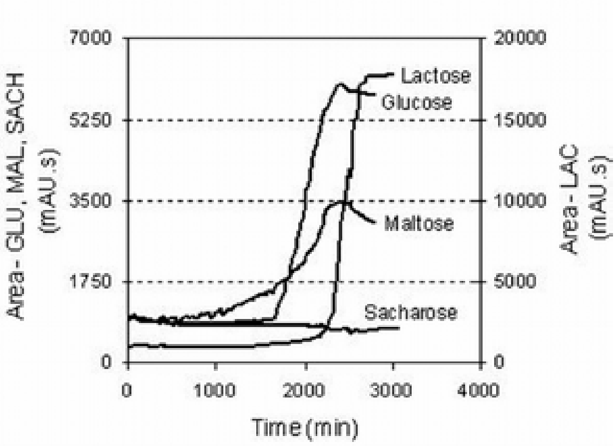

Fig. 1. **Growth curves on different substrates into vial (10,000 mAU.s ~ 1 g dm^{-3} (biomass concentration)**

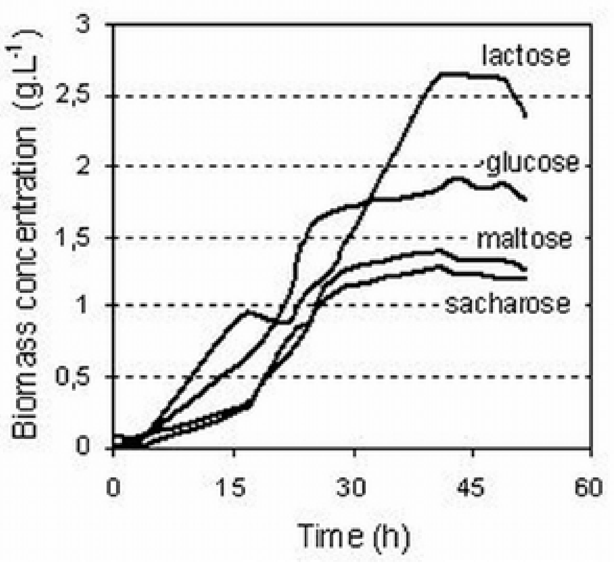

Fig. 2. **Growth curves on different substrates in fermentor**

Hence it follows (Fig. 1.):

- Sacharose was not utilised by mixed thermophilic bacteria in anaerobic system only. Enzyme decomposed sacharose (invertase) was probably not formed (Fig. 1.).

- In both systems, maximal cells growth was achieved on lactose (Fig. 1., 2.). Total biomass concentration after 50 hours: 1.7 ± 0,2 $g\,dm^{-3}$ in vials and 2.7 ± 0,4 $g\,dm^{-3}$ in the fermentor.
- Total biomass concentration on others substrates was as follows (Fig. 1., 2.): glucose 0.6 ± 0.2 $g\,dm^{-3}$ (vials) and 1.9 ± 0.5 $g\,dm^{-3}$ (fermentor), maltose 0.3 ± 0.1 $g\,dm^{-3}$ (vials) and 1.4 ± 0.3 $g\,dm^{-3}$ (fermentor), sacharose 0,1 $g\,dm^{-3}$ (vials) and 1.3 ± 0.2 $g\,dm^{-3}$ (fermentor).
- The difference between total biomass concentration in the vials and in the fermentor is statistically significant on statistic level 0.05. It follows from these results that tested culture has mainly aerobic character.
- The biggest biomass productivity was reached in cultivation in fermentor, on lactose: 0.054 ± 0.009 $g\,dm^{-3}\,h^{-1}$.
- Lag-phase period (Fig. 1., Fig 2.) was the shortest in cultivations in the aerobic fermentor sytems (~ 3h on all substrates). In anaerobic vials was this phase very long: on maltose ~8h, on lactose (~25h). (Sacharose is not comparised).
- Specific growth rates: glucose 0.19 ± 0.05 h^{-1} (vials) and 0.10 ± 0.05 h^{-1}, maltose: 0,05 ± 0.01 h^{-1} (vials) and 0.06 ± 0.02 h^{-1} (fermentor), sacharose 0.06 ± 0.02 h^{-1} (fermentor only). The difference between specific growth rates in the vials and in the fermentor is not statistically significant on statistic level 0.05.
- It is possible to differ two exponential growth phases (I: 3–15 h., specific growth rate 0.25 ± 0.03 h^{-1} and II: 22–38 h. specific growth rate 0.12 ±0.02 h^{-1}) in the bioreactor cultivation on lactose. Lactose served as the main carbon source in the first phase of cultivation, in the second phase lactose was consumed immedately and the growth proceeds on organics acids by metabolic way generated (Fig. 2.).
- In vials on lactose was observed different situation, there was one exponential growth phase with specific growth rate: 0.31 ± 0.01 h^{-1}.(Fig. 1.).
- It follows from our experiments that the biggest growth rate was achieved in bacteria cultivation on lactose (in both systems – in the fermentor and in the vials).

Conclusions

The dramatically differences between growth rate and total biomass concentration in cultivations on lactose and others substrates were detected. Lactose was the most suitable substrate in growth aspect in both aerobic and anaerobic conditions. With regard to better growth results in the fermentor cultivations, the main population of the mixture of bacteria tested is aerobic.

Maltose and glucose were utilised by thermophilic microorganisms too, but growth was slower. Sacharose was not utilised in anarobic vials, thermophilic bacteria was not able to decompose this substrate.

In conclusion, this work described growth and growth characteristics of tested microorganisms and contributed to better knowledge of thermophilic mixed bacteria cultures.

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P06 MONITORING OF ACRYLAMIDE LEVELS USING LC-MS/MS: MALTS AND BEERS

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Introduction

Acrylamide (AA), a hazardous chemical, is classified as a probable human carcinogen (IARC, 1994)[1]. AA is formed during Maillard reaction in starch-rich foods. Its occurrence in human diet was for the first time reported by Swedish scientists in 2002[2].

Alike many other heat processed foods prepared from cereals, malts may contain AA, originated from sugar and asparagin contained in barley. Besides of other factors, AA levels depend on the temperature and the time employed for treatment.

In our study, various types of malts, commonly used as additives (colorants, aroma donors etc.) in bakery and/or in brewing industry were examined for AA levels using LC-MS/MS.

In the next phase of our experiments the transfer of this processing contaminant into the beer was studied. The analytical procedure used for the analysis consists of cleanup step employing solid-phase extraction (SPE) followed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). A wide range of bottled beers obtained at the Czech retail market was examined within this monitoring study.

Experimental

Chemichals and Materials

AA (purity 99,5 %) was from Sigma/Aldrich/Fluka (Switzerland). $^{13}C_3$-AA (isotopic purity≥99 %) was purchased from CIL (USA). Magnesium sulphate was from Sigma/Aldrich/Fluka (Switzerland). Sodium chloride was from Penta (Czech Republic). Aluminium oxide (basic) was from Merck (Germany). All organic solvents were of HPLC grade quality.

Calibration standard solutions were prepared in water by diluting stock standard solutions in concentration range

1–250 ng ml^{-1} with fixed amount of $^{13}C_3$–AA–100 ng ml^{-1}.

Isolute Multimode® (6 ml, 500 mg) and Isolute ENV+® (6 ml, 500 mg) SPE cartridges were purchased from IST (U.K.).

Samples

Czech special roasted malts (4 from barley and 1 from wheat) were employed for the study. In addition to the malt samples, intermediates collected during processing (that was conducted under different time and temperature conditions) were examined.

The German set of samples included 12 malts (brew, special, caramel, wheat and rye), used in beer industry.

Thirty beer samples representing various typical brands were purchased from the Czech retail market.

Malt Sample Preparation

The key step of this method is the transfer of AA from the primary aqueous extract into acetonitrile forced by added salts ($MgSO_4$ and NaCl); separation of aqueous and organic phases is induced[3]. Most matrix interferences are then removed from organic phase by dispersive solid phase extraction ($MgSO_4$ and basic Al_2O_3 are used for this purpose). Acetonitrile is evaporated under a gentle stream of nitrogen and solvent is exchanged to water.

Beer Sample Preparation

After decarbonization the beer sample undergoes clean-up with two subsequent SPE cartridges. Final extract is in 60% methanol in water (v/v solution). The methanol is evaporated by a gentle stream of nitrogen[4].

Analysis of AA by LC-MS/MS

LC-MS/MS measurements were accomplished with the use of a separation module Alliance 2695 (Waters, USA) interfaced to a Quattro Premier XE mass spectrometer (Micromass, UK). Chromatographic separation was carried out using an Atlantis T3 column (3 mm × 150 mm × 3 μm). Isocratic elution of AA was achieved with mobile phase composed of acetonitrile and water (3 : 97, v/v).

The mass spectrometer, equipped with pneumatically assisted electrospray interface (ESI), was operated in a positive ionization mode.

LOD´s and LOQ´s for malt samples were 10 μg kg^{-1} and 30 μg kg^{-1}, respectively. LOD´s and LOQ´s for beer samples were 2 μg dm^{-3} and 5 μg dm^{-3}, respectively.

Results

AA levels in malt corresponded to the temperatures employed for their production. In dark malts AA content was relatively high, clearly due to advanced Maillard reaction induced by heating process.

Table I
Czech malts

Malt	AA (μg kg^{-1})
Semi-finished light caramel	35
Light caramel	37
Semi-finished dark caramel	404
Dark caramel	243
Coloured	111
Czech	25
Wheat	< 10

The overview of this processing contaminant in various Czech and German malts is summarized in Table I and II.

The AA levels as high as 900 µg kg^{-1} were found in German caramel rye malt used for the production of the speciality beers. Generally, only low AA levels were found in pale malts.

Table II
German malts

Malt	AA (µg kg^{-1})
Rye	<30
Chocolate wheat	49
Wheat malt	<10
Munich dark	150
Red caramel	81
Caramel rye	909
Light caramel	<30
Caramel aromatic	409
Melanoidin	105
Smoked	<30
Pilsner	<30
Munich	67

Some transfer of AA into beer was shown by analysis of beers collected at the Czech detail market (Table III). About 30 % of examined samples contained levels above limit of quantification of the LC-MS/MS method. The range of AA level was between 5–10 µg dm^{-3}. The measured mean concetratin was 7 µg dm^{-3}. Two samples of non-alkoholic beer contained AA. In the 1 of 2 semi-dark beers was determined AA. Only 2 from the all (16) light beers were positive for AA. The most percentage of positive samples were in the group of dark beers. 4 from 5 dark beers contained acrylamide in mean level 9 µg dm^{-3}. No AA was detected in two special beers.

Conclusions

The levels of AA in malts vary in a wide range. Generally, the highest level of this proccesing contaminant occurred in caramel rye malt used for production of special beers.

The examination of semi-finished malts indicates possible degradation of AA during roasting of malts. After reaching a maximum, the levels successively decrease.

Predictably, the highest levels of AA were found in dark beers. In a few of light beer samples was AA detected too.

Table III
Czech beers

Beer	AA µg dm^{-3}
Light	6
Light, non-alcoholic	6
Light	<5
Light, lager	<5
Special lager	<2
Light	<2
Light, lager	<2
Light, low sugar content	<2
Dark, lager	10
Light	<2
Light, lager	<5
Light, lager	<5
Light, lager	<5
Light, non-alcoholic	<2
Light	<5
Light, lager	<5
24% Special, dark	<2
Light, non-alcoholic	6
16% Special,	<2
Dark, lager	10
Light, lager	<5
Dark, lager	8
Light, lager	<2
Light	<5
Dark, lager	<5
Semi-dark, lager	<5
Semi-dark, lager	5
Light	<2
Light, lager	7
Dark, lager	8

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P07 CONTENT OF POLYCYCLIC AROMATIC HYDROCARBONS IN HONEYS FROM THE CZECH REPUBLIC

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) belong to the family of persistent organic pollutants having many properties with a negative impact on human organism (carcinogenicity, mutagenicity).

In addition to the inhaled air the main exposition source of polycyclic aromatic hydrocarbons for the most of population are foodstuffs. PAHs were found in many foodstuffs both of animal origin (mainly as a result of culinary treatment) and plant origin (due to atmospheric deposition and growing in the contaminated soil). The data about PAHs content in honey are rare[1]. In the study dealing with the PAHs content in honey considerably high concentrations are reported[2], especially for the honeydew honey, not only for those PAHs with 3–4 aromatic rings (acenaphten 187 $\mu g\,kg^{-1}$, fluoren 163 $\mu g\,kg^{-1}$, phenanthren 625 $\mu g\,kg^{-1}$, anthracen 635 $\mu g\,kg^{-1}$) predominantly occurring in foodstuffs ant materials of plant origin[3], but also for the toxic PAHs (benzo-k-fluoranthen 58 $\mu g\,kg^{-1}$, benzo-a-pyren 126 $\mu g\,kg^{-1}$).

The aim of this study was to estimate content of PAHs in Czech honeys whose high quality is known (absence of antibiotics, minimal content of hydroxymethylfurfural). The content of 15 priority pollutants according to the US EPA was monitored, namely naphtalen (NAPT), acenaphten (ACENAPT), fluoren (FLU), anthracen (ANT), fluoranthen (FLT), pyren (PY), benzo-a-anthracen (BaA), chrysen (CHRY) benzo-b-fluoranthen (BbF), benzo-k-fluoranthen (BkF), benzo-a-pyren (BaP), dibenzo-a,h-anthracen (DBahA), benzo-g,h,i-perylen (BghiPE) and indeno-1,2,3-cd-pyren (IPY).

Experimental

Instruments

The Waters 2695 Alliance chromatographic system was equipped with the Waters 2475 fluorescence detector. A column PAH C18 (250 mm × 4.6 mm I.D. 5 μm (Waters, Germany) was used. The column was held at 30 °C with a column heater. Mobile phase A: water, B: acetonitrile, flow 1.4 $ml\,min^{-1}$, linear gradient were used. PAHs were detected by the fluorescent detector using an excitation and emission wavelength program (λ_{exc} ranged from 232 nm to 300 nm and λ_{em} from 330 nm to 500 nm). The HPLC system was controlled and the data were processed by Waters Empower 2 software.

Chemicals and Reagents

Acetonitrile (Merck KGaA), dichloromethane (Merck KGaA) and hexane (Scharlau), the PAHs standard mixture (PAH mix 9, Dr. Ehrenstorfer), sodium sulphate anhydrous, sodium chloride, HPLC water. All solvents were HPLC grade or for residual analysis, other chemicals p.a. quality at least.

Samples

10 samples of honey obtained directly from Czech bee-keepers and 10 samples of honey from shops with the Czech Republic declared as a country of origin were analysed. Each sample was analysed in at least duplicates, blank samples in each series were performed.

Procedure

Three kinds of procedures of sample preparation were used.

The first procedure: 10 g sample of honey was dissolved in 100 ml of deionised water, 1 g NaCl and 10 ml hexane added and thoroughly shaken for 30 min. By means of separatory adapter an aliquot of organic layer was taken and evaporated to dryness. The residue was dissolved in 1 ml of acetonitrile, filtered through nylon membrane filter (0.45 μm) and analysed by HPLC.

The second procedure: 10 g of honey sample was mixed with anhydrous sodium sulphate, added 40 ml of dichloromethane and extracted by means of ultrasonic bath and Ultra Turrax. After filtration the solvent was evaporated and next process was the same as in the first procedure.

The third procedure: 10 g of sample was dissolved in 100 ml of deionised water and extracted by means of solid phase of SPE cartridges. The next process was as in the first procedure.

Results

Fig. 1. represents typical chromatogram obtained with honey sample. The average concentrations of each PAHs ranged from 0,02 to 2.22 $\mu g\,kg^{-1}$ (Table I). Recoveries, which were calculated by using observed and spiked concentrations for PAHs, ranged from 60 to 90 %. Limit of quanti-

Table I
Concentrations of PAHs [$\mu g.kg^{-1}$] in samples of honey

honey from	NAPT	ACENAP	FLU	PHE
bee-keepers	0.29	0.20	0.66	0.32
shops	0.82	0.45	0.49	2.22
	ANT	FLT	PY	BaA
bee-keepers	0.07	0.02	0.15	0.55
shops	0.07	0.07	0.05	0.16
	CHRY	BbF	BkF	BaPY
bee-keepers	0.15	0.04	0.08	0.05
shops	0.02	0.02	0.02	0.02
	DBahA	BghiPE	IPY	
bee-keepers	0.26	0.10	0.07	
shops	0.09	0.0	0.02	

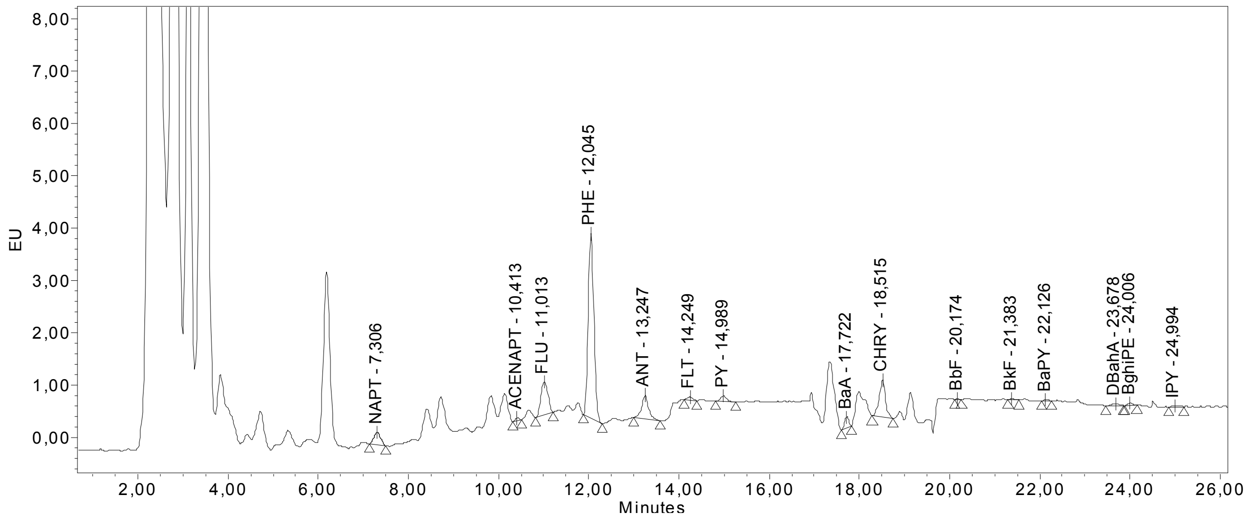

Fig. 1. **Chromatogram of PAHs in honey from shops. The conditions of analysis are shown in Section 2**

fication, which was obtained from blank experiments, was 0.01 μg kg^{-1}. RSD ranged from 0.01 to 20 %. These results were similar for each type of procedures.

Conclusions

The content of polycyclic aromatic hydrocarbons in analysed samples were very low, they ranged between 0.02 to 2.22 μg kg^{-1}for individual PAHs. These values are usually found in uncontaminated foodstuffs. In conclusion we can say that Czech honeys proved their traditional safety and high quality.

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P08 QUANTITATIVE DETERMINATION OF SULFONAMIDE RESIDUES IN CHICKEN MEAT BY A NEW SOLID PHASE EXTRACTION AND HPLC-UV FOR DETECTION

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Introduction

Sulfonamides (SAs) are a group of synthetic antibiotics that have been used in food-producing animals for therapeutic and prophylactic purposes[1]. There is a risk of occurrence of unwanted residues in animal products if these antimicrobials have been improperly administered or if the proper withdrawal period has not been observed. To safeguard human health, the European Community has adopted for SAs safe maximum residue limits (MRLs) at the total level of 100 μg kg^{-1} in food of animal origin[2].

Many methods such as LC and LC-MS, GC and GC-MS, ELISA, biosensor immunoassay (BIA) and high performance capillary electrophoresis (HPCE) have been developed for the determination of SA residues in food[3]. Solid phase extraction (SPE) was used as clean-up or enrichment method for SAs in tissues. The main sorbents used for the extraction of SAs are C18, aluminium oxide, strong cation-exchange[4].

Multi-walled carbon nanotubes (MWCNTs) are a novel carbon material used as an effective solid phase adsorbent for organic compounds (including SAs)[5].

In this paper, a sensitive method was developed for the determination of six SAs in chicken meat using MWCNTs and aluminium oxide as solid phase adsorbents followed by HPLC with UV detector.

Experimental

Materials and Reagents

Chicken muscle tissues were purchased from local food market and deep frozen until analysis. Organic solvents such as acetonitrile, acetic acid and 1- propanol were all pesticide residue grade , commercially available from Merck . Anhydrous sodium sulfate was analytical grade (Bucarest, Romania). Deionized and redistilled water was prepared from Milli-Q Plus (Millipore). Sodium acetate trihydrate (Merck) was used as buffer for HPLC mobile phase. Sulfadiazin (SDZ), sulfamerazine (SMR), sulfapyridine (SPY), sulfisoxazole (SIO), sulfamethoxazole (SMO) and sulfadimethoxine (SDM) were purchased from Sigma.

MWCNTs were purchased from Institute for Research and Development of Isotopic and Molecular Technologies Cluj-Napoca, Romania.

Standard stock solutions were prepared by accurately dissolving approximately 10 mg of SAs in 10 ml of acetonitrile LC grade and stored at 4 °C. Working standards were prepared weekly by appropriate dilution in acetate buffer at pH 4.5.

Chromatografic System and Conditions

All experiments were carried out by using Shimadzu VP Series liquid chromatograph equipped with a UV-VIS detector.
The chromatographic separation was accomplished in 30 min with gradient elution on a C 18 (250 mm × 4.6 mm, 5 μm) analytical column (Alltima) with a mobile phase 0.01M acetate buffer pH 4.5 (A) and acetonitrile (B). Flow 1 ml min^{-1} was used for the separation of analytes at the following program: 20 % B to 50 % within 22 min, back to 20 % in 3 min, equilibration for 5 min. The injection volume was 20μl and the detection of SAs was conducted at 266 nm.

Sample Preparation and Sample Clean-up

Ten grams of minced chicken tissue was placed into a 50 ml polypropylene tube. 20 ml acetonitrile and 5 grams baked anhydrous sodium sulfate was then added. The sample was homogenized with an Ultraturax for about 1 min., and then centrifuged at 5,000 rpm for 5 min.The residue was extracted by sonication with 20 ml acetonitrile and then centrifuged at 5000 rpm for 5 min.The extracts were combined and the solvent was concentrated to 5 ml.The solution was passed through the Alumina N SPE cartridge preconditioned by 10 ml acetonitrile.The analytes were eluted with 5 ml acetonitrile : water (80 : 20, v/v).The eluent and loading solvents were combined and transferred to concentration bottle.Then 5 ml 1-propanol was added and the solution was evaporated to near dryness. The residue was dissolved by ultrasonication for 1 min with 30 ml acetate buffer (pH 5). A second SPE cartridge (200 mg MWCNTs) was utilized to remove potential interferences. The SPE was conditioned with acetonitrile (5 ml) and water (5 ml) prior to loading the sample. The analytes were eluted with a mixture of 2 ml acetate buffer (pH 4.5) and 4 ml acetonitrile. The eluate was evaporated to 1 ml under nitrogen stream in a 40 °C block heater and filtered through a 0.45 μm disposable syringe filter.

Results

The present procedure uses two SPE cartridges for clean-up because acetonitrile extracted a lot of endogenous compounds from meat sample.The first one, Sep-Pak Alumina N, is a polar sorbent SPE cartridge. The second SPE cartridge (MWCNTs) is a non-polar sorbent and was utilized to further cleanse the extract.

The calibration graphs obtained by plotting peak area versus drug concentration in 0.1–10 μg ml^{-1} range are reported in Table I.

Table I
Retention time (t_R) and linearity of SAs

Sulfonamide	t_R [min]	b^a	a^b
SDA	7.51	127301	–3582
SPY	8.82	108274	–24580
SMR	9.65	121645	–9957
SIO	12.88	97992	2987
SMO	13.37	112048	18902
SDM	20.79	83942	–14029

b^a: Slope
a^b: Intercept

The linear correlation coefficient (r^2) are all above 0.999.

Chromatograms of a blank sample and of a spiked sample of chicken muscle are shown in Fig. 1. and 2.

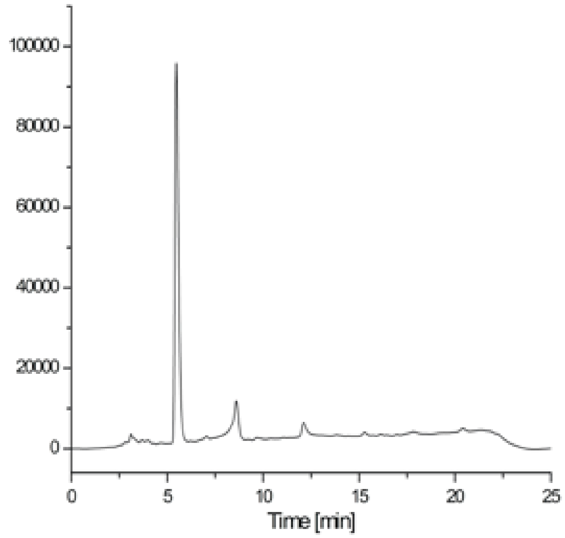

Fig. 1. **The chromatogram of blank chicken muscle**

Table II
Recoveries of SAs in chicken meat tissue (n = 3)

Sulfonamides	Spiked [$\mu g\,kg^{-1}$]	Recovery [%]
SDA	50	78. ± 2.3
	100	80.4 ± 4.7
SPY	50	72.6 ± 6.8
	100	75.3 ± 7.2
SMR	50	65.6 ± 4.3
	100	67.9 ± 2.5
SIO	50	77.8 ± 1.2
	100	79.4 ± 3.4
SMO	50	74.7 ± 5.6
	100	76.5 ± 6.4
SDM	50	76.3 ± 7.6
	100	78.7 ± 6.9

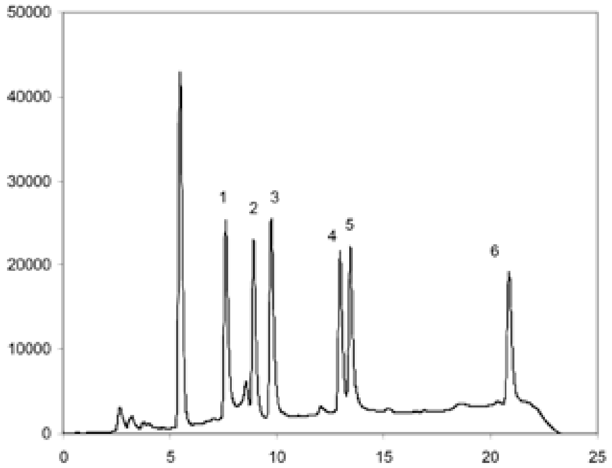

Fig. 2. **The chromatogram of the spiked sample with 50 $\mu g\,kg^{-1}$ of the following sulfonamides: 1:SDA, 2: SPY, 3: SMR, 4: SIO, 5: SMO, 6:SDM**

Under this extraction and pre-treatment procedure , the sulfonamide fortified chicken sample did not induce peaks interfering with SAs peaks. The recoveries of analytes were evaluated by spiking 0.5 and 1 μg of each standard analyte to 10.0 g tissues. The results are listed in Table II.

Good recoveries ranging from 65.6 to 80.4 % were determined, which indicated that sample preparation procedure was suitable for the analysis of SAs in chicken meat sample.

As an application, 30 samples of commercial chicken meat purchased from local food market were analysed using the present method. No residue sulfonamide was detected in all meat sample. All HPLC chromatograms were free from interferences.

Conclusion

In this study , an analytical method to determine six SAs in chicken meat was developed utilizing two SPE cartridges for sample clean-up and pre-concentration of analytical components prior to HPLC analysis. The newly developed sample pre-treatment procedure effectively removed the potential matrix interferences from endogenous compounds of meat. Average recoveries of analytes from spiked meat ranging from 65.6 to 80.4 % of six SAs were determined.

This work has been supported by the Natio-nal Authority for Scientific Research of Romania, CEEX project no. 62/2006.

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P09 MONITORING OF SENSORIALLY ACTIVE SULPHUR SUBSTANCES IN MALT AND BEER

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Introduction

Recently, a great deal of research effort has been devoted to sensorially active substances affecting beer quality. Quality of brewing materials, unhopped wort and hopped wort production technology, fermentation technology, beer maturation and beer ageing contribute significantly to analytical and sensorial beer characteristics.

Heterocyclic and sulphur compounds, some of them with high sensorial activity even in extremely low concentrations, belong to sensorially active substances affecting beer quality principally[1]. Trace amounts of these compounds commonly detectable in food contribute to their flavor, therefore this effect can be generally assessed as favourable. In malt and beer, however, it is true to a limited extend only and the presence of heterocyclic and sulphur substances is evaluated rather negatively[2]. Sulphur compounds get into beer either with initial materials (malt, hop) or they are formed in the course of chemical or enzymatical reaction during the respective production phases (mashing, brewing, fermentation, ageing). Content of sulphur compounds in barley and hop depends not only on a variety but on a growing locality, course of weather and growing technology employed as well. In malt, content of sulphur substances depends first of all on malting technology and possible contamination with undesirable microorganisms[3]. Most of the sulphur compounds present in barley, malt and beer are non-volatile substances (amino acids, proteins, inorganic sulphates). These substances do not directly account for unfavourable beer flavours and odours but under certain conditions they may be important precursors of sensorially active substances. These substances are, in majority of cases, volatile and their amount is usually less than 1 % of the total amount of the sulphur containing substances in beer, i.e. actual amounts of substances responsible for sulphur odours are extremely low[4].

Experimental

Sensorically Active Sulphur Substance

Following volatile sulphur substances were monitored: dimethylsulphide [75-18-3], dimethyl disulphide [624-92-0], dimethyltrisulphide [3658-80-8], carbondisulphide [75-15-0], ethylsulphide [75-08-1], diethyldisulphide [111-81-6], methionol [505-10-2], 3-methylthiophen [616-44-4], ethylthioacetate [625-60-5], 2-methyl-1-buthanthiol [1878-18-8].

Selection of the Analyzed Samples

Sensorially active sulphur substances were determined in beers bought in retail stores. Six pale dispensed beers, 4 dark, 6 lager and 3 non alcoholic beers were selected for the analyses.

The analyzed malt samples were produced from different barley varieties (Bojos, Jersay, Malz, Prestige, Tolar, Xanadu), from 3 growing stations (Branišovice, Věrovany, Vysoká).

Determination of Volatile Sulphur Substances

Direct analysis of sulphur sensorially active substances is not easily applicable regarding their very low concentrations ($\mu g\,kg^{-1}, dm^{-3}$ – $ng\,kg^{-1}, dm^{-3}$) in the analyzed matrixes (malt, beer)[5,6]. Prior to the analysis, the analytes must be extracted from the matrix and concentrated. The HS-SPME method was used for extraction and concentration[7,8]. The CAR/PDMS fibre was chosen as the optimum fibre for this technique. The gas chromatographic analysis with flame photometric detection was employed to determinate sensorially active sulphur substances.

HS-SPME/ GC/ FPD conditions:

Sample:	2 ml of beer (vial 4 ml)
	6 ml of malt extract (vial 50 ml)
Sample temperature:	45 °C, 10 min
SPME fiber:	85 µm CAR™/PDMS
Extraction:	HS-SPME, 30 min, 45 °C
Desorption:	3 min, 250 °C

Chromatographic conditions:

Capillary column:	GS-Gaspro (30 m × 0.32 mm)
Thermal program:	from 40 °C to 235 °C
Flow of carrying gas He:	1.5 ml min^{-1}
PTV injector:	250 °C, splitless (3 min)
FPD detector:	T detector – 150 °C,
	T base – 250 °C

Results and Discussion

Two types of fibres (PEG, CAR/PDMS) were tested for the HS-SPME analysis. Fig. 1 shows higher affinity to the CAR/PDMS fiber for the analytes studied. This fibre was chosen for the analysis of sulphur substances, sorption time was optimized at 45 °C. Optimum sorption time was 30 minutes.

Validation parameters for the relevant analytes are given in Table I.

Carbon disulphide, methionol, dimethylsulphide, 3-methylthiophen and diethyldisulphide were detected in beers. Table II shows results of beer sample analyses.

In malt samples only dimethylsulphide was determined. Results of malt analyses are given in Fig. 2.

Table I
Validation parameters of the analyzed sulphur substances

Analyte	Range of calibration concentrations [μg dm^{-3}]	R^2	LOQ [μg dm^{-3}]	Extended relative uncertainty [%]
carbon disulphide	0.04–0.15	0.9809	0.01	22
methionol	2.4–10.0	0.9308	0.5	25
ethylsulphide	0.15–0.70	0.9845	0.01	30
dimethylsulphide	0.10–0.50	0.9898	0.005	3
3-methylthiophen	0.05–0.20	0.9945	0.001	4
dimethyldisulphide	0.03–0.15	0.9918	0.01	13
2-methyl -1- buthanthiol	0.10–0.55	0.9768	0.05	29
diethyldisulphide	0.2–0.8	0.9782	0.05	28
dimethyltrisulphide	0.03–0.15	0.9956	0.005	25
ethylthioacetate	0.03–0.15	0.9867	0.01	15

Table II
Contents of sulphur substances in beer

[μg dm^{-3}]	Pale dispensed beers (n = 6)	Pale lager beer (n = 6)	Dark beer (n = 4)	Nonalcoholic beer (n = 3)
carbon disulphide	0.02–0.05	< 0.01	0.05	< 0.01
methionol	4.30–6.20	2.0–3.34	2.03–4.01	< 0.5
ethylsulphide	< 0.01	< 0.01	< 0.01	< 0.01
dimethylsulphide	0.85–1.45	0.29–0.67	0.9–1.29	1.0
3-methylthiophen	0.10–0.16	< 0.001	0.18	< 0.001
dimethyldisulphide	< 0.01	< 0.01	< 0.01	< 0.01
2-methyl -1-buthanthiol	< 0.05	< 0.05	< 0.05	< 0.05
diethyldisulphide	0.08–0.20	0.05–0.25	0.12–0.19	0.36
dimethyltrisulphide	< 0.005	< 0.005	< 0.005	< 0.005
ethylthioacetate	< 0.01	< 0.01	< 0.01	< 0.01

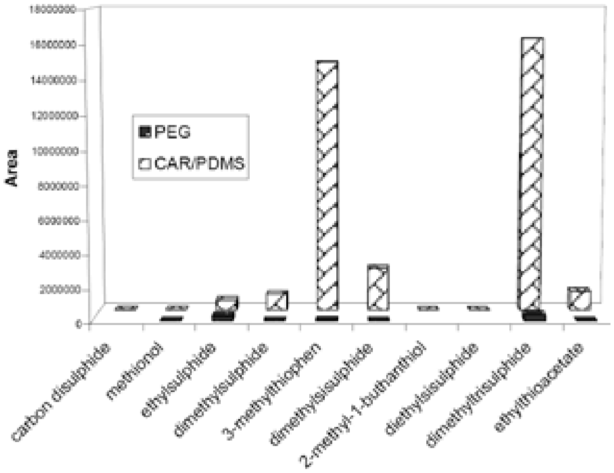

Fig. 1. **Comparison of suitability of SPME fibres for the analysis of sulphur substances**

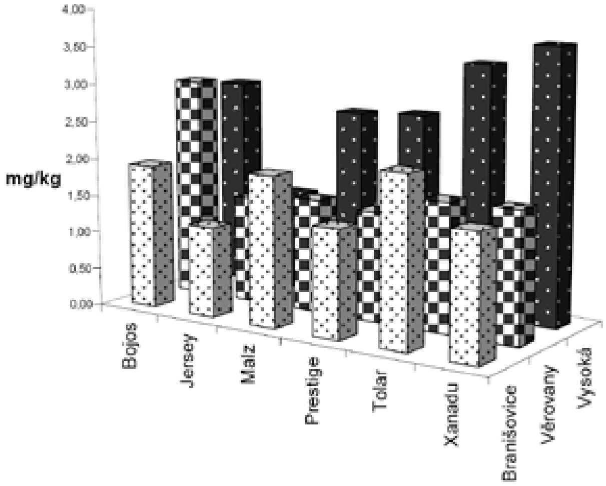

Fig. 2. **DMS content in the analyzed malts**

Conclusions

The HS-SPME/GC/FPD method was optimized and validated for the assay of sulphur volatile substances in malt and beer. This method determined following volatile sulphur substances in the beer sample: carbon disulphide, carbon disulphide, and dimethylsulphide in the sample of malt.

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P10 MONITORING OF FERULIC ACID CONTENT DURING MALT PRODUCTION

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Introduction

Antioxidant activity of ferulic acid plays an important role in a biochemical process of beer production. Currently, causes of wort haze have been investigated and relationships between wort haze, filterability and beer quality have been studied. Information on ferulic acid may help elucidate causes of haze formation[1].

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) belongs to phenolic acids derived from cinnamic acid. It is a natural antioxidants and inhibitors of enzymatic browning. In plant cell walls it protects cells from the effect of pathogens, controls expansibility of cells, growth and affects biological digestibility. Ferulic acid is frequently a part of fibre where it is bound by ester linkage to hemicelluloses.

Generally, ferulic acid plays a principal role in breakdown of barley caryopsis cell walls. High content of ferulic acid in walls can serve as a protection against premature hydrolysis. The presence of ferulic acid protects cell walls against microbial attack. It stops lipid peroxidation and is effective at suppression of hydroxyl radicals[2,3].

The aim of our project was to determine the dependence of ferulic acid content on a barley variety and malting technology.

Experimental

Ferulic acid was determined in samples of eight barley varieties (Jersey, Maltz, Bojos, Radegast, Sebastian, Prestige, Tolar, and Xanadu). Barleys were from harvest 2007 and they came from two different growing localities – Krásné Údolí (forage area, worse growing conditions for spring malting barley) and Věrovany (sugar-beet area, good growing conditions for spring malting barley). Malts were prepared in the micro malting plant of the Malting Institute of the RIBM in Brno using the method with short steep and sucking off CO_2. Wort was made by a so-called congress method, i.e. standard mashing procedure with finely ground malt.

Alcalic hydrolysis with 2 M of natrium hydroxide was used to release ferulic acid from compact homogenized samples. After pH adjustment, the extract was purified using the SPE (Solid Phase Extraction).

The purified extract was microfiltered and transferred to a vial. In liquid samples extraction with alcalic hydrolysis was omitted, following procedure remains the same.

The samples were then analyzed using gradient elution on the UPLC WATERS ACQUITY liquid chromatograph equipped with WATERS 2996 PDA detector. Mobile phase consisted of 0.05M phosphate buffer (pH 3.5) and acetonitrile. Column used was ACQUITY UPLC BEH C18 1.7 µm 2.1 × 50 mm.

Results

Content of total ferulic acid was determined in a series barley – malt – wort. Results are presented in the Table I.

Conclusions

The results of measurement prove the dependence of content of total ferulic acid on a variety and growing locality in the series barley – malt – wort.

Concentration of total ferulic acid in barley samples varied in the range of 650–950 mg kg^{-1} in dependence on a variety and growing locality.

Concentration of total ferulic acid in malt samples varied in the range of 900–1,740 mg kg^{-1} in dependence on a variety and growing locality.

Concentration of total ferulic acid in wort samples varied in the range of 6–9 mg dm^{-3} in dependence on a variety and growing locality.

Results were obtained in the framework of solution of projects: VZ MSM 6019369701 and MŠM VC 1M0570

Table I
Content of total ferulic acid in a barley – malt – wort chain from the localities Krásné Údolí and Věrovany

	Krásné údolí			Věrovany		
	Barley [mg kg^{-1}]	Malt [mg kg^{-1}]	Wort [mg dm^{-3}]	Barley [mg kg^{-1}]	Malt [mg kg^{-1}]	Wort [mg dm^{-3}]
Jersey	746.1	1,012.6	7.99	825.1	1,537.0	8.09
Maltz	693.7	1,044.6	7.42	803.1	1,613.2	8.01
Bojos	672.9	970.5	6.14	682.0	1,392.1	5.52
Radegast	806.7	1,067.0	6.05	844.1	1,361.6	5.91
Sebastian	712.2	948.7	8.87	832.9	1,737.4	6.96
Prestige	733.5	1,113.9	7.90	911.0	1,428.6	7.23
Tolar	734.3	1,153.7	6.11	825.4	1,563.2	5.57
Xanadu	648.8	905.9	6.44	692.4	1,263.0	8.45

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P11 CONTENT OF STROBILURIN FUNGICIDES IN BARLEY, MALT AND BEER

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Introduction

Strobilurin pesticides represent an important group of fungicides with a broad efficiency spectrum. These fungicides began to be used commercially in 1996 and they were produced by the companies Syngenta (azoxystrobin) and BASF (kresoxim-methyl)[1].

Natural strobilurins (*Strobilurus* and *Oudemansiella*) were isolated from wood-rotting bracket fungi (Basidiomycetes). These wood decay fungi produce fungicidially acting substances in defense against lower fungi. The name strobilurine comes from *Strobilurus tanacetus*, one of the first fungi from which they were isolated (strobilurin A)[1].

Strobilurins represent an important group of efficient fungicides and together with oxazoline-diones (famoxadone) and imidazolinones (fenamidone) they are Qo inhibitors (Quinone outside Inhibitors). Their action is based on inhibition of mitochondrial respiration, they bind Qo instead of cytochrom b, which is a part of cytochromal complex bc1 localized in the inner mitochondrial membrane of fungi. Binding blocks the transfer of electrons between cytochrom b and cytochrom c1, inhibiting thus production of ATP and disrupting the energy cycle of fungi. Originally, strobilurins are natural substances produced by higher fungi, e.g. *Strobilurus tanacetus* (strobilurin A), *Oudimansiella mucida* (oudemansin A). Chemically they belong to methoxyacrylates (e.g. azoxystrobin and picoxystrobin), methoxycarbamates (pyraclostrobin), oximino acetates (kresoxim – methyl, trifloxystrobin), ozimino-acetamides (dimoxystrobin, etc.), and dihydro-dioxazinones (fluoxastrobin). Their characters, including efficiency spectrum, are very variable. They affect mainly preventively, but also curatively and some even have eradicative effects. All of them act in contact, some of them in depth or translaminarily or systemically (azoxystrobin and picoxystrobin). They move acropetally in a plant. Their efficacy is very wide, they are active against sac fungi (Ascomycetes), bracket fungi, imperfect fungi and oomycetes. They are highly imperiled by the origin of resistance (cross resistance within the QoI fungicides)[1,2].

Resistance was proved in more pathogenic fungi. In cereals, powdery mildew (*Blumeria graminis* f.sp. *tritici*, *Blumeria graminis* f.sp. *hordei*), septoria blotch (*Mycosphaerella graminicola*), net blotch (*Pyrenophora teres*), helminthosporioses in wheat (*Pyrenophora tritici – repentis*), and in the other crops, e.g. in apple, scab (*Venturia inaequalis*), grapevine downy mildew (*Plasmopara viticola*), and grapevine powdery mildew (*Erysiphe necator*). When applying strobilurins, observance of all provisions is highly recommended to avoid the development of resistance[1,2,3].

Following fungicides are used against fungal diseases of barley in the Czech Republic azoxystrobin (Amistar), picoxystrobin (Acanto, Acanto Prima), trifloxystrobin (Sfera 267.5 EC), fluoxystrobin (Fandango 200 EC), and kresoxim-methyl (Juwel, Juwel Top). Azoxystrobin (Ortiva) is registered against fungal diseases of hop in the CR.

Considering the increase in the extent of strobilurin use in the protection of barley and hop and their stability in a plant, it is necessary to introduce monitoring of their residues, intermediaries and final product.

Experimental

25 samples of beer, 50 samples of malt and 50 samples of barley were analyzed.

60 ml of methanol/acetone mixture was added to 20 g of ground matrix. Disintegration of the mixture was performed in an ultrasound bath for 30 minutes. The disintegrated mixture was centrifuged at 6,500 rev min^{-1} for 15 min at 10–15 °C. The obtained solutions were transferred to boiling flasks (250 ml) and evaporated to dryness on a vacuum evaporator. The obtained dry residue was dissolved with 20 ml of distilled water in the ultrasound bath for 1 minute. Mixture was purified through the SPE column ENVI™CarbII/PSA.

pH of the beer sample (100 ml) is adjusted to pH 6 by adding NaOH solution and the sample is then purified through the LiChrolut column.

The analyses of samples were performed on a gas chromatograph (Trace GC Ultra, Thermo Finnigan) connected to a mass detector (Trace DSQ, Thermo Finnigan). To separate the analyzed substances a DB5-MS capillary column (30 m × 0.25 mm i.d., 0.25 mm) with following thermal program was used: initial temperature 70 °C for 1 min., increase in temperature 10 °C min^{-1} to 280 °C, maintained for 5 min.

Table I
Barley, malt (SPE-EnviCarbII/PSA)

	SPE recovery [%]	LOQ [mg kg^{-1}]	RSD [%]
azoxystrobin	83–88	0.003	8–12
kresoxym - methyl	80–84	0.002	5–7
picoxystrobin	85–90	0.002	5–7
trifloxistrobin	82–87	0.002	7–10

Table II
Beer (SPE-LiChrolut)

	SPE recovery [%]	LOQ [mg kg^{-1}]	RSD [%]
azoxystrobin	88–90	0.003	< 8
kresoxym – methyl	87–90	0.002	< 5
picoxystrobin	90–95	0.002	< 5
trifloxistrobin	87–91	0.002	< 5

Table III
Content of strobilurin residues in the analyzed samples

	Number of analyzed samples	azoxystrobin	kresoxym – methyl	picoxystrobin	trifloxistrobin
barley	50	<LOQ	<LOQ	<LOQ	<LOQ
malt	50	<LOQ	<LOQ	<LOQ	<LOQ
beer	25	<LOQ	<LOQ	<LOQ	<LOQ

Programmed flow of carrying gas He was from 1.5 ml min^{-1} to 3 ml min^{-1}. Temperature of PTV injector 280 °C, splitless regime for 0.8 min. Temperature in the transfer line between GC and MSD was 200 °C. Mass spectrometer was adjusted in SCAN (50–450 m/z) and SIM (Selected Ion Monitoring) mod (EI+ – positive electron ionisation) and selected values (m/z) for relevant analytes were as follows:

- azoxystrobin – 344, 388 (m/z)
- trifloxistrobin – 116, 131, 222 (m/z)
- picoxystrobin – 145, 335 (m/z)
- kresoxym – methyl – 116, 131, 222 (m/z)

Results

The method for determination of strobilurins in barley, malt and beer was optimized. Validation parameters for the individual matrices and SPE columns are given in Table I and Table II. Contents of strobilurins in all the analyzed samples were below the limit of quantification (Table III).

This work has been supported by project MSM 6019369701 of Czech Ministry of Education.

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P12 THE EFFECT OF FERMENTATION CONDITIONS ON THE YEAST AROMA PROFILE

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Introduction

Several factors influence the aroma profile during the alcoholic fermentation of the grape must. One of the widely discussed topics is the using of apiculate microflora and its contribution to the aroma of wine. Several authors have studied[1–3] the behaviour of various yeast genera – *Kloeckera, Pichia, Bretanomyces* and their contribution to the wine aroma, considering the apiculate microflora as spoilage. Our previous results[4] confirmed the role of apiculate microflora in the wine aroma production under semiaerobic conditions and support of the original character of wine by apiculate microflora.

The aim of this work was to determine the effect of changes in external conditions – oxygen, temperature or substrate composition on the sensorial expression of genera *Rhodotorula*, *Sporobolomyces*, *Pichia*, *Hansenula Issat-chenkia,* and *Torulospora* under the winemaking process. All above mentioned factors influence positively or negatively the final wine product. Multivariate analysis of variance was used to determine the overall weight of each one factor.

Table I
Sensorial evaluation of fermented grape must by various yeast strains under various fermentation conditions

	Anaerobic conditions grape must				Anaerobic conditions synthetic medium			
Yeast strain	10°C		25°C		10°C		25°C	
	aroma	percept	aroma	percept	aroma	percept	aroma	percept
C. intermediata	yeast	+	sulphuric	–	grape	+	acetic	–
R.mucilaginosa	socks smelly	–	yeast	+	yeast	+	socks smelly	–
T.delbruecki	yeast	+	acetic	–	sweety	+	bread	–
S. cerevisiae	acidic	–	fruity	+	sulphuric	–	sulphuric	–
I. orientalis	autolyses	–	acethone	–	yeast	–	Not recognised	–
Pichia anomala	acethone	–	acidic	–	fruity	+	acethone	–
S.cerevisiae	yeast	–	sulphuric	–	fruity	+	acethone	–
P. membranofaciens	autolyses	–	sulphuric	–	socks smelly	–	Not recognised	–
R.mucilaginosa	autolyses	–	acidic	–	corny	+	sweet	–
P. anomala	acethone	–	fruity– apple	+	candy floss	+	Not recognised	–
Sp.pararoseus	Not recognised	+	fruity	+	socks smelly	–	acethone	–
S.cerevisiae	acidic	–	sulphuric	–	rubber smell	–	autolyses	–
S.cerevisiae	fruity	+	sulphuric	–	acidic	+	autolyses	–

	Semiaerobic conditions grape must				Semiaerobic conditions synthetic medium			
Yeast strain	10°C		25°C		10°C		25°C	
	aroma	percept	aroma	percept	aroma	percept	aroma	percept
C. intermediata	acidic	–	fruity	+	floral	+	fruity	+
R. mucilaginosa	acethone	–	fruity	+	frowstilly	–	vanilla	+
T.delbruecki	vanilla	+	vanilla	+	honey	+	fruity	+
S. cerevisiae	autolyses	–	grape	+	sulphuric	–	acetic	–
I. orientalis	acethone	–	fruity	+	autolyses	–	autolyses	–
P. anomala	honey	+	fruity	+	fruity	+	fruity	+
S. cerevisiae	honey	+	acetic	–	yeast	+	floral	+
P. membranofaciens	yeast	+	acetic	–	medical	–	yeast	+
R.. mucilaginosa	honey	+	fruity	+	sulphuric	–	Not recognise	–
P. anomala	honey	+	fruity	–	sweety	+	yeast	+
S. pararoseus	yeast	+	fruity	+	sweety	+	Not recognise	+
S. cerevisiae	yeast	+	yeast	+	sweety	+	Not recognised	+
S. cerevisiae	yeast	+	fruity	–	Not recognised	+	Not recognised	+
S. cerevisiae	yeast	+	fruity	–	autolyses	–	Not recognised	+

Experimental

Following yeast strains were isolated from the grape must: *Rhodotorula mucilaginosa* (2 strains), *Sporobolomyces pararoseus, Pichia membranefaciens, Pichia anomala* (2 strains), *Candida intermediata, Torulospora delbruecki,* and *Issatchenkia orientalis*. For comparison 4 *Saccharomyces cerevisiae* yeast strains were also used. Two of them were isolated from the grape must; two of them were commercial active dry yeast strains (Lallemand).

Fermentation media: grape must or semisynthetic medium (10 g dm^{-3} glucose, 5 g dm^{-3} $(NH_4)_2SO_4$, 2 g dm^{-3} KH_2PO_4, 1 g dm^{-3} $MgSO_4{\cdot}7H_2O$, 0.1 g dm^{-3} $CaCl_2{\cdot}2H_2O$, 0.1 g dm^{-3} NaCl, 3 g dm^{-3} yeast autolysate, were used. The fermentation processed under semiaerobic and anaerobic conditions at the temperature 10 and 25 °C, respectively.

At the end of fermentation process the samples were sensorially evaluated by a group of degustators.

The identical samples were subsequently analysed by gas chromatography for the aroma compounds production. Each sample was analysed on the GC MS (Shimadzu QP 2010) equipment and also on the GC FID equipment (GC 8000 CE Instruments).

Two methods of sample preparation were done:

- Samples (20 ml) were extracted by ether (2 ml), and centrifuged prior to analysis. The etheric extract was used for analysis. (liquid – liquid extraction). This method was used for higher alcohols (propanol, isoamylacohol, ethyl esterand esters determination.
- Samples were extracted by Tenaq (solid phase microextraction) and than 10 min sampled in the GC according to[5].

The same column and the same conditions were used by both analysis: Column: DB WAX 30 m, 0.25 × 0.25, temperature programme: 30 °C, hold 2 min, increase by 4 °C min^{-1} up to 230 °C, hold 10 min, 1 ml of sample was injected to injection port at 200 °C, detector temperature 220 °C, carrier gas: helium, injektion mode: split 1 : 100, flow control mode: pressure 70 kPa

Results

MANOVA test was used to explain the variation in the aroma compounds produced by various yeast strains under aerobic/anaerobic conditions. The aerobic conditions influenced the variance in several compounds like ethylacetate, izoamylacete, and propanol.

The other important factor was the temperature. When the temperature increased more compounds were produced by all yeast strains, but the effect of higher temperature was negative. From the statistical view point the less important factor was evaluated by the media composition, interpreting only 5 % of data variance.

Table I shows the sensorial evaluation of fermented grape must by various yeast strains. There were differences in the specific aroma in several cases. For example Rhodotorula mucilaginosa was recognized as fruity aroma in fermenting grape must, and as vanillic aroma fermenting the synthetic media under semiaerobic conditions, however socks smelly and yeasty under anaerobic ones. Similar results were obtained by Torulospora delbruecki, fermenting semiaerobically grape must, however “only” honey and fruity aroma fermenting synthetic medium.

Conclusions

Using statistical data, we confirmed that the aroma compounds production is strongly influenced by the presence of oxygen. The temperature affected the production of aroma compounds to the lower extend and the media composition had the minor impact.

The changes in the sensorial evaluation of aroma are more intensive and important than changes in the chemical composition.

Under defined conditions the apiculate microflora can produce aromas which directly contribute to the variety or origin character and highly improve them.

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P13 BIOGENIC AMINES REDUCTION BY PROBIOTIC *L. CASEI* DURING RIPENING OF FERMENTED SAUSAGES

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Introduction

Fermentation technology is one of the oldest food technology applications. The use of starter cultures in the field of meat fermentation has been reviewed several times[1–3]. Starter cultures have been used in the sausage production to reduce fermentation time, ensure low residual nitrate and nitrite contents in the end product and standardize the organoleptic characteristics[2]. Starter microorganisms can also improve safety and stability of the product, extending the shelf life and provides diversity resulting in new sensory properties. Metabolic activities of the microorganisms used as starters are essential for the desirable changes determining the particular characteristics of the fermented meat product. Lactic acid bacteria represent the most important group of starter microorganisms as they are well adapted to the meat environment and are involved in all changes occuring during fermentation and ripening[3].

In the last years, the adding of probiotics into fermented sausages became more and more popular. It has confronted new challenge in the area of functional food. Probiotics are defined by the FAO/WHO as live microorganisms which when administered in adequate amounts confer a health benefit on the host. When ingested in sufficient numbers regularly, probiotics are believed to play an important role in the control of host intestinal microbiota and maintenance of its normal state[4]. The presence of adequate number of live probiotic cells in fermented sausages in the time of consumption represents the first challenge for the development of such probiotic product, taking the recommended dose of at least 10^8 cells a day[1].

Biogenic amines are organic molecules which occur in wide variety foods, mainly fermented foods like meat and dairy products[5–8]. They are formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones[9]. When high amounts of biogenic amines are consumed or when normal methabolic pathways of amine catabolism are inhibited, various negative effects like hypertension or hypotension, nausea, headache, cardiac palpitation or other complications can occur[10]. When applied into fermented foods, some probiotic were described to decrease biogenic amines formation (Sacco, Italy). To evaluate this theory, this study was carried out.

Experimental

The aim of this work was to study the effect of probiotic bacteria *L. casei* in the mixture with two different starter cultures (1 – *Staphylococcus carnosus*, *Lactobacillus curvatus*; 2 – *Pediococcus acidilactici*) on biogenic amine formation during fermentation, ripening and storage of typical Czech fermented sausages „Herkules". Other parameters like pH, total counts of microorganisms, LAB, counts of probiotic *L. casei* and the amounts of biogenic amines tyramine, histamine, putrescine and kadaverine were determined.

Bacterial Cultures

Probiotic culture *L. casei* 431 (Sacco, Italy) was obtained in lyofilized form. Two different starter cultures *(i) Lactobacillus curvatus*, *Staphylococcus carnosus* and *(ii) Pediococcus acidilactici* were used for production of fermented sausages.

Sausages Production

Two batches of sausages called „Herkules" were made using as follows: mixture of frozen pork and pork fat (80 %), beef (20 %), sugars (0.6 % dextrose), 2.5 % nitrate salt (with 0.9 % nitrite), E 250, E 316 and spices were added according to traditional recepture. Furthermore, 0.025 % starter (1 and 2) and the same amount of probiotic bacteria (*L. casei*) were added. Two control batches (starters 1 and 2) with no probiotic bacteria were included. The sausages were smoked for 6 days at 26–24 °C and processed in climate chamber in accordance with a traditional four weeks programme (11–13 °C, 75 % relative humidity). After fermentation, sausages were ripened at 15 °C for further 21 days.

pH Measurement

pH was measured directly from the sausages as the mean value of three measurements in the day of production and after 3, 5, 7, 14, 21, 28 and 49 days of fermentation and ripening.

Microbiology Analysis of Sausages

Three sausages from each batch were analyzed at the day of production and after 14, 28 and 49 days after production to observe probiotic counts. Ten grams of each sample were homogenized in 90 ml of Ringer solution (Noack, France) using Stomacher. 1 ml of appropriate dilution was plated on: *(i)* Plate count agar (PCA, Noack, France) to determine total microbial count; *(ii)* MRS agar (Merck, Germany) to count lactic acid bacteria (LAB); *(iii)* MRS agar (Noack, France) containing 112 $mg\,dm^{-3}$ moxalactam (Sigma-Aldrich, Germany) for selective enumeration of *L. casei*. Plates for enumeration of total microbial count were incubated at 30 °C for 72 hours. All other plates were incubated anaerobically for 3 days at 37 °C.

Biogenic Amines Determination

The analytical determinations of biogenic amines were performed in triplicate. The amount of 10 g of each sausage sample was weighted into the plastic test tube. A volume of 0.5 ml of an internal standard, 1,7-diaminoheptane, with concentration 1 mg ml^{-1} was added to the sample and it was extracted with 20 ml of 5% trichloroacetic acid for two minutes using a disintegrator Heidolph Diax 900 (Heidolph Instruments, Germany). Suspension was then centrifuged for 10 minutes (3,000 rpm, 4 °C). The supernatant was filtered through paper filter (Filtrak no. 390) and the solid residue was extracted for the second time (using method described above). Both extracts were collected into one tube, filled in 50 ml with 5% trichloroacetic acid and filtered through the nylon membrane filter (13 mm, 0.45 μm, Chromatography research Supplies, Edison, USA). An aliquot 1 ml of an extract was mixed with 0.5 ml of saturated sodium carbonate solution (Sigma-Aldrich, s.r.o, Prague, Czech Republic) and derivatized with 1 ml of acetonic solution of dansyl chloride (5 mg ml^{-1}) for 1 hour at 40 °C. The excess of unreacted derivatisation agent was removed by reaction with 250 μl 10mM ammonia solution. Derivatized biogenic amines were then extracted into diethyl ether (3×1 ml); etheric extract was evaporated to dryness and dissolved in 500 μl of acetonitrile. The same procedure was applied on standard solution of biogenic amines (Sigma-Aldrich). Biogenic amines were determined as described in Burdychova and Komprda[11] and Burdychova and Dohnal[12] by high performance liquid chromatography with UV detection at 254 nm. The formation of biogenic amines by starter and probiotic cultures was screened as described by Burdychova and Dohnal[12].

Results

Microbial Counts

A number of health benefits have been claimed for probiotic bacteria such as *Lactobacillus casei*. Because of the potential health benefits, this organism is increasingly incorporated into fermented foods. However, studies have shown low viability of this probiotic in market preparations[13].

In both bathes with probiotic bacteria, *L. casei* was detected at concentration of 10^4 CFU g^{-1} during ripening period and stayed at this level during the whole storage period. The changes in probiotic flora during ripening and storage of sausages are shown in Fig. 1. Initial LAB counts in probiotic batches were significantly higher than in the control samples without probiotics ($p < 0.05$), due to the inoculation of starter strains. During first 14 days of ripening LAB numbers reached levels up to 10^7 CFU g^{-1} in all batches; thereafter, during next 14 days of ripening, the counts of LAB significantly decreased in all batches to the level of 10^6 CFU g^{-1}. The total microbial count reached levels up to 10^8 CFU g^{-1} in all batches during first 14 days of ripening and their counts gradually decreased to the level of 10^7 CFU g^{-1} during next 14 days of ripening and 21 days of storage (data not shown).

To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 CFU g^{-1} of a product[13] and daily consummation of 100 g of such product is recommended. However, the minimal dose is depended on several factors such as individual person, probiotic strain and type of food product[14]. On the other hand, dry sausages are products, which may be suitable carriers for probiotics into the human gastrointestinal tract. The question still is, if those types of meat products can be called functional foods and consumed daily.

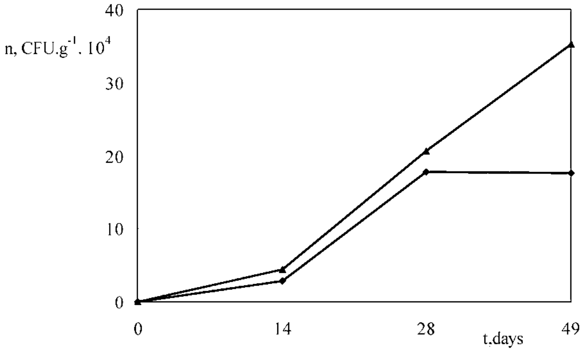

Fig. 1. **Changes of probiotic *L. casei* during ripening (0–28) and storage (28–49) of sausages „Herkules“, ▲ starter + probiotic *L. casei*, ♦ starter 2 + probiotic *L. casei***

Biogenic Amines Formation

Biogenic amines are formed by the microbial decarboxylation of free amino acids in food[15] and they are generally present in dry sausages as reviewed by Maijala[16] and Eerola et al.[17] As described by Buckenhüskes[18], the absence of biogenic amines formation should be a selection criterion for strains used as meat starter cultures. In our study was confirmed that neither starter nor probiotic cultures formed biogenic amines (results not shown). All sausages were analyzed for biogenic amines by HPLC. The concentrations of BA in the raw material were low but during ripening, fermentation and storage period a sharp rise occurred in both control and probiotic batches. The only BA which were at low levels during the whole ripening and storage periods was histamine, which concentrations varied from 0.5 to 2.5 mg kg^{-1} in all batches (data not shown). Nout[19] pointed out that histamine contents should be in the range of 50–100 mg kg^{-1} in sausages processed according to „Good Manufacturing Practice“, the amount of histamine measured in this study is in agreement with this rule.

Putrescine was the main amine formed in both control batches, followed by cadaverine and tyramine. The concentration of putrescine increased from 1.9 to 101.2 mg kg^{-1} (control batch 1) and from 4.6 to 350.5 mg kg^{-1} (control batch 2). The amount of cadaverine increased from 2.0 to 39.9 mg kg^{-1} (control batch 1) and from 11.7 to 81.6 mg kg^{-1} (control batch 2). The level of tyramine increased from 6.3 to 204.4 mg kg^{-1} (control batch 1) and from 10.5 to 300.4 mg kg^{-1} (control batch 2), respectively. In this study, putrescine, cadaverine and tyramine accumulation was significantly inhibited in probiotic sausages when compared with controls without probiotic *L. casei* culture. Putrescine concentration increased gradually during ripening and storage period, reaching levels 53.7 mg kg^{-1} (probiotic batch 1) and 163.5 mg kg^{-1} (probio-

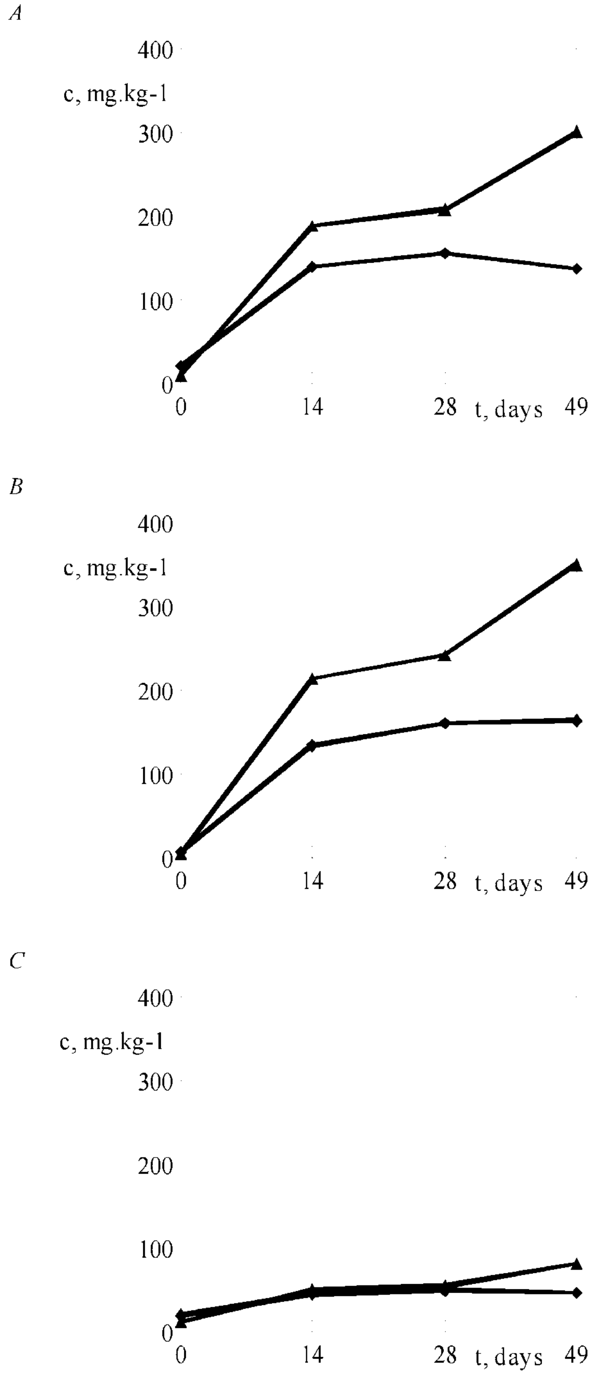

Fig. 2. **Changes of BA during ripening (0–28) and storage (28–49) of sausages „Herkules“. (A) tyramine concentrations, (B) putrescine concentrations, (C) cadaverine concentrations. ▲ starter 1, ♦ starter 1+probiotic *L. casei* (control batch 1)**

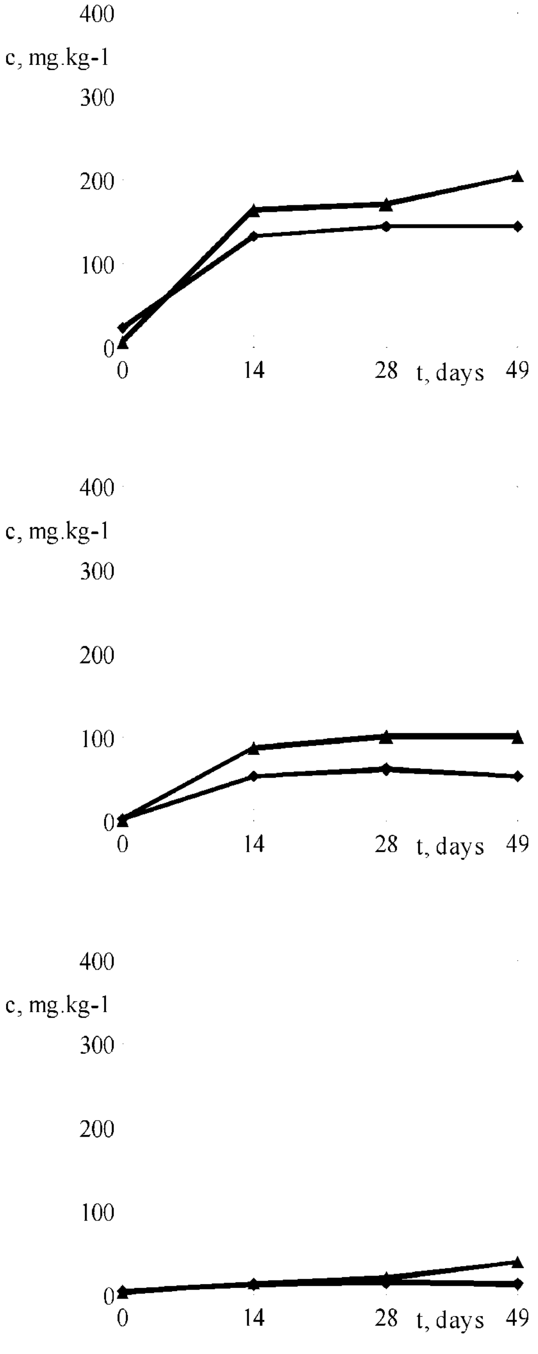

Fig. 3. **Changes of BA during ripening (0–28) and storage (28–49) of sausages „Herkules“. (A) tyramine concentrations, (B) putrescine concentrations, (C) cadaverine concentrations. ▲ starter 2, ♦ starter +probiotic *L. casei* (control batch 2)**

tic batch 2) and at the end of storage. Cadaverine amounts increased gradually during ripening period, during storage period level down to 13.2 mgkg^{-1} (probiotic batch 1) and 46.3 mgkg^{-1} (probiotic batch 2). The levels of tyramine followed the same trend reaching concentrations 144.6 mgkg^{-1} (probiotic batch 1) and 137.5 mgkg^{-1} (probiotic batch 2) at the end of storage period. The results are shown in Fig. 2. and Fig. 3.

The allowable maximum level of tyramine in foods is 100–800 mg kg^{-1} and 1,080 mg kg^{-1} is toxic[20]. The tyramine contents were lower than these values in all cases. Nout[19] concluded that for „Good Manufacturing Practice“, the amount of tyramine should be in the range of 100–800 mg kg^{-1}. I was described[21–23] that with occurrence and accumulation of biogenic amines putrescine and cadaverine largely contributes the contaminant microbial population (such as enterobacteria). Probiotic *L. casei* has evidently inhibitory effect on the growth of this bacteria or formed metabolites which influence the production of BA.

p H

pH profiles of control and probiotic batches were almost the same (data not shown). During the first 3 days of ripening, the pH of all sausages decreased ($P < 0.05$) to about 4.1–4.5 due to production of lactic acid and other organic acids by lactic acid bacteria as described by Lücke[3]. After that, pH increased ($P < 0.05$) due to decomposition of acids to become almost constant. Some authors have reported that the main reason for low levels of biogenic amines is the low pH during r ipening period[6]. In this study, no correlation between pH and biogenic amines formation was found.

Conclusions

Some authors published that the inoculation of competitive and decarboxylase-negative starter cultures has been shown to be a useful tool to inhibit spontaneous aminogenic microflora and thus considerably reduce aminogenesis[24,22]. It seems that the lowering of BA concentrations by probiotic *Lactobacillus casei* described in this study is in agreement with these statements. Evidently, *L. casei* 01 (Sacco, Italy) was well adapted to meat fermentation and could have inhibitory effect on microorganisms producing BA.

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P14 TESTING OF DIFFERENT *SACHAROMYCES* SPECIES FOR THE ABILITY TO SORB DEOXYNIVALENOL

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Introduction

Mycotoxin contamination of food and feed is a high risk to human and animal health[2]. The majority of the causal organisms are producers of mycotoxins such as highly toxic trichothecenes[4]. Four types of trichothecenes are described, type A and type B, which differ in the presence or absence of a keto group at C-8 of the trichothecene skeleton, type C with additional epoxydic group and macrocyclic type D. The most common trichothecene in cereals is type B trichothecene deoxynivalenol (DON).

Deoxynivalenol, also called vomitoxin, is produced by *Fusarium fungi*, such as *F. graminearum* or *F. culmorum*. It has negative effect on animal growth and health. DON inhibits the synthesis of DNA, RNA and proteins at the ribosomal level. High doses causes the vomiting in pigs, lower concentrations in the diet reduces feed intake and animal growth[5]. Different physical and chemical methods have been recommended for detoxification of mycotoxin-contaminated food and feed. Nevertheless, only a few of them have been accepted for practical use. Thermal degradation of trichothecenes is not so effective, because they are relatively stable and they decomposes at 210 °C within 30–40 min[10]. From physical methods are the most frequently used feed additives on sorbent basis, such as bentonite or active charcoil. The disadvantages of adsorbents are their relatively high dosage and sorption of biologically active compounds, e.g. vitamins or trace metals. In addition, the sorbents can bind to only a limited group of mycotoxins and in some cases does not provide required effect[7]. Biological decontamination of mycotoxins using microorganisms is one of the well-known strategies for the management of mycotoxins in food and feed. Biological decontamination of mycotoxins by different microorganisms was reviewed several times.[1,8,11,15] There are two ways of action – sorption on cell walls or enzymatic degradation, for example using epoxydase. De-epoxylated form is less toxic than original DON. Among the different potential decontaminating microorganisms, the genus Saccharomyces represent the unique group, which is widely used in food fermentation and preservation. The aim of this study was screening of ability of different Saccharomyces species to remove deoxynivalenol from liquid medium.

Experimental

Microbial Cultures and Culture Conditions

All cultures were obtained from Culture Collection of Yeast (Bratislava, Slovakia). The cultures were *Saccharomyces cerevisiae* 20_1; isolated from loaf of hornbeam, *Saccharomyces bayanus* 21-31-12; isolated from mushrooms, *Saccharomyces paradoxus* 2 isolated from needles of spruce, *Saccharomyces paradoxus* 21-53-2 isolated from soil and *Saccharomyces paradoxus* isolated from the loaf of locust. All cultures were cultivated in Plate Count Broth (PCB; Merck, Germany) at 30 °C for 48 hours and then subcultured by transfering 4 ml of the culture to the cultivation test tube. Three replicates per sample of inoculum were used at each measuring. On the base of O. D. (600 nm) values that have been taken during the cultivation of yeasts with deoxinyvalenol the analysis of their counts has been done.

Preparation of Yeast Cultures for Testing of DON Sorbtion

All used chemicals were analytical or gradient grade. Standard of deoxynivalenol (DON) was obtained from Sigma-Aldrich, s.r.o. (Czech Republic). The stock solution of DON was prepared by dissolution of 1 mg of DON in 5 ml of acetonitrile to give solution with concentration 0.2 mg ml^{-1}. The solution was stored at –18 °C. Working solutions for calibration curve measurement were prepared by dilution of stock solution.

The amount of 0.5 μg of DON was transferred to test tube and evaporated to dryness. In the next step, 4 ml of cultivation media (PCB) with yeasts were added to the test tube and the sample was cultivated in thermostated box at 30 °C for 4 hours. The concentration of free DON was measured at the beginning of cultivation and after 4 hours of cultivation.

Prior the DON determination it was necessary to remove yeasts from culture medium using ultrafiltration through polytetrafluoroethylene membrane filter (SMI-LabHut Ltd., UK) with pore size 0.20 μm. After this step, the filtrate was diluted with acetonitrile (in ratio 16 : 84). Next, the clean-up with MycoSep® 225 Trich was applied. Briefly, 5 ml of solution was transferred to the glass tube and pushed through the MycoSep® 225 Trich column. 2 ml of this eluate were evaporated to dryness and redissolved in 400 μl of HPLC mobile phase. Mobile phase consisted of 1mM formic acid/acetonitrile (90 : 10, v:v) with flow rate 1 ml min^{-1}.

Chromatographic Determination of DON

The HPLC system HP 1100 (Agilent Technologies, Palo Alto, USA) consisted of vacuum degasser unit (model G1322A), quaternary pump (G1311A), autosampler (G1313A) and quadrupole mass spectrometer (G1946VL) with electrospray ionization was used. The ChemStation software (Rev. A 10.02) controllig chromatographic system and was used for chromatogram evaluation.

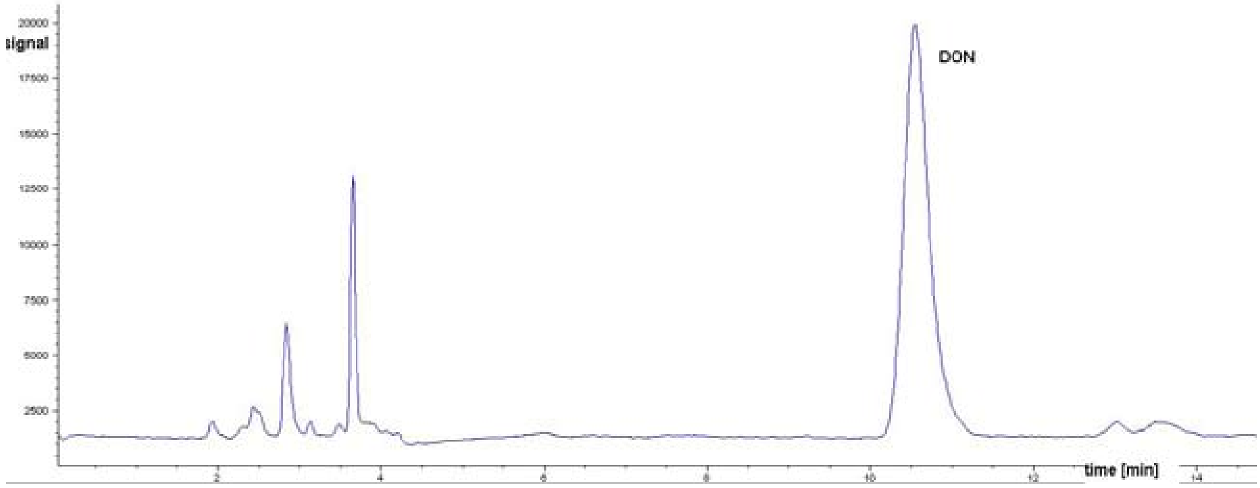

Fig. 1. **Elution of DON in mobile phase**

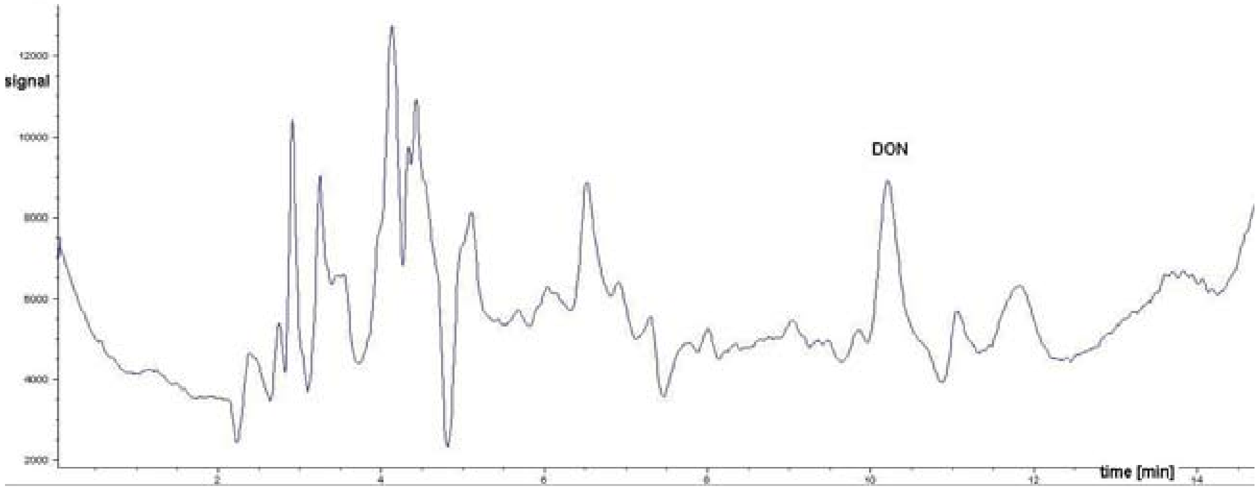

Fig. 2. **Elution of DON in culture medium (Plate Count Broth)**

Analytical determination of DON was performed on reverse-phase chromatographic column LUNA (250 × 4.6 mm, particle size 5 μm; Phenomenex, USA). DON was detected on mass spectrometric detector (MS) as positively charged ions: $[DON+H]^+$, m/z = 297 and $[DON+Na]^+$, m/z = 319). The time of analysis was 15 min, DON was eluted at 10.9 minutes. The separation was performed at laboratory temperature.

The calibration curve was measured using standard addition method. The appropriate amount of DON was added into pure cultivation media solution and processed in the same way as the real samples.

Results

Knowledge on interaction of yeasts with mycotoxins goes back more than three decades[6,12].

The yeast of the genus *Saccharomyces* were described as capable to bind different types of mycotoxins, like aflatoxins[14], ochratoxin A[13], T2 toxin and zearalonon[9].

In this study, isolates of yeasts belonging to different species of the genus *Saccharomyces* were tested for deoxinyvalenol binding.

First of all, the detection of DON in culture medium was tested. It was observed that the detector signal is much lower when analyzed DON in culture medium (PCB) than when analyzed it in pure mobile phase (see Fig. 1. and 2.).

The reason of this phenomenon could be the lowering of deoxynivalenol inonization caused by interfering compounds in culture medium.

The detector signal of initial concentration of deoxynivalenol in culture medium was taken as 100 % for futher calculations.

In all culture batches there was observed decrease in the concentration of DON, which was measured using HPLC.

Of the 5 *Saccharomyces* isolates, all of them were able to sorbe DON.

The percentages of lowering of DON concetrations after cultivation with all *Saccharomyces* isolates are shown in Table I.

Table I
Lowering of DON concentration [%] in culture media with different *Saccharomyses* strains

Yeast strains	Incubation time [hours] 0	 4	 8
	Concentration [%]		
S. cerevisiae	67.71	71.12	78.15
S. bayanus	67.75	68.07	72.05
S. paradoxus	66.04	67.92	75.65
S. paradoxus	5.83	12.67	18.95
S. paradoxus	57.77	62.61	76.25

The maximum sorbtion of deoxinyvalenol was observed in all yeasts strains immediately after DON addition to culture medium with cultured yeasts.

The next cultivation did not significantly influenced further lowering of DON concentration.

Most of the yeast strains bound more than 70 % (w/w) of deoxinyvalenol. The only isolate from soil (*Saccharomyces paradoxus* 21-53-2) bound less than 20 % (w/w) of the added toxin in PCB.

It follows from Table II that the most significant decrease of live yeast cells was detected at isolate of *S. bayanus* (isolated from muschrooms).

This strain was the most sensitive to deoxynivalenol. On the other hand, the most resistant to deoxynivalol was *S. paradoxus* (isolated from the loaf of locust).

From results obtained in this study it is clear that the ability to sorb deoxynivalenol by several strains of the genus *Saccharomyces* was demonstrated. Commertially, the yeast cell walls are applied as feed additives (Mycosorb). Their detoxifying activity is based on complex formation between glucomannan and mycotoxins.

Tabulka II
The yeasts concentration in cultivation media after addition of DON

Yeast strains	Incubation time (hours) 0	 4	 8
	Yeast concentration [CFU ml^{-1}]		
S. cerevisiae	1.4×10^7	1.3×10^6	1.1×10^5
S. bayanus	1.9×10^7	1.6×10^5	1.5×10^4
S. paradoxus	1.7×10^6	1.6×10^5	1.3×10^5
S. paradoxus	9.9×10^4	4.0×10^4	5.5×10^3
S. paradoxus	4.5×10^5	1.5×10^5	1.1×10^5

Conclusions

It seems that, according to results of experiments realized till present time, microorganisms are the main living organisms applicable of mycotoxin biodegradation. Further screening of microorganisms for their ability to sorbe deoxynivalenol may lead to detection of more efficient and better applicable yeasts and bacteria.

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P15 SEPARATION OF COMPLEX OLIGOSACCHARIDES FROM WORT AND BEER USING HPLC

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Introduction

Oligosaccharides (OS) including maltooligosacharides (MOS) are essential non-volatile compounds in fermentation processes appeared in a wide range of food products and beverage production involving beer. Specifically, monitoring of changes in carbohydrate pattern before, during, and after the malting is of great fundamental and practical significance for brewing technologies and the sensory characteristics of the beer.[1–3]

The intent of the study was to optimize analytical conditions for separation and quantification of the OS in the congress wort, hopped worth, green beer, and lager beer.

Experimental

Materials and Methods

Standards of MOS (Sigma-Aldrich, St. Louis, USA) with degree of polymerization (DP) from maltotriose (MA3) up to maltoheptaose (MA7) were used for the primal identification and column calibration. Separation was achieved on 1100 Series HPLC system equipped with refractive index (RI) detector (Agilent Technologies) on Prevail Carbohydrate ES analytical column (250 × 4.6 mm, 5 μm; Alltech) using a mixture of acetonitrile (ACN)/water 3 : 2 (v/v) with a flow rate of 1 ml min^{-1} and 10 μl injection loop.

Samples

Eleven defined varieties of wort samples (congress wort, hopped worth, and green beer) were harvested during the year 2006 in different regions all around the Europe. For comparison one sample of home-made lager beer with 5.0 % (v/v) of ethanol was used. Samples were sonicated for 10 minutes, centrifuged and filtered with 0.22 μm PVDF Millipore filter (Millipore, Bedford, USA) before analysis.

Results

Standards of non-derivatized MOS from MA3 up to MA7 were separated using different ratio of ACN/water to find optimal separation conditions. The ratio of 3 : 2 (v/v) enabled simultaneous and sufficiently selective separation in 20 minutes duration. Then, calibration dependencies were determined. Standard solutions of MOS in the concentration range of 0.25–10.0 mg ml^{-1} for MA3, 0.25–5.0 mg ml^{-1} for MA4, and 0.2–2.0 mg ml^{-1} for MA5, MA6, and MA7 were prepared by dissolving in mixture of ACN/water 1 : 1 (v/v). Triple injections were used for each standard solution and the peak areas were plotted against the corresponding concentration. The concentration dependencies were linear with correlation coefficients about 0.99 for all standards.

After optimization, samples of eleven different barley varieties of congress wort, hopped worth, green beer, and the lager beer were separated using HPLC according to their DP. Elution time below 45 minutes for all wort samples was observed (Fig. 1.).

Individual peaks in chromatogram (Fig. 1.) were labeled by DP in their increasing order. In general, the retention times

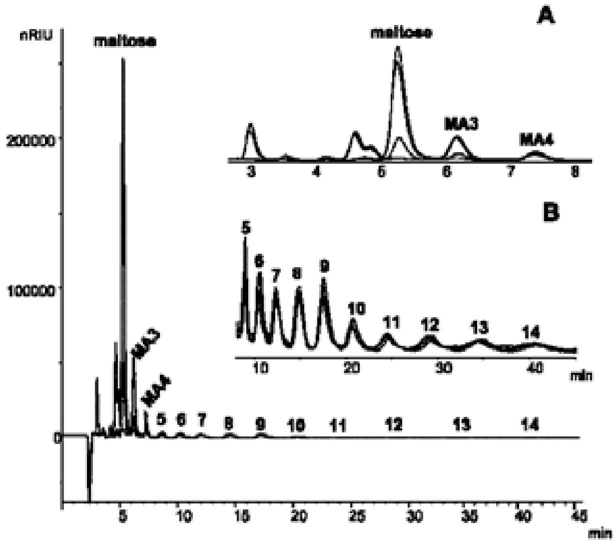

Fig. 1. **Separation of congress wort (full line), hopped worth (dotted line), green beer (dashed line) of the same variety and lager beer (broken line) using HPLC. Detail of separation in the low-weight range (A) and higher oligosaccharides (B). Peak numbers correspond to the DP**

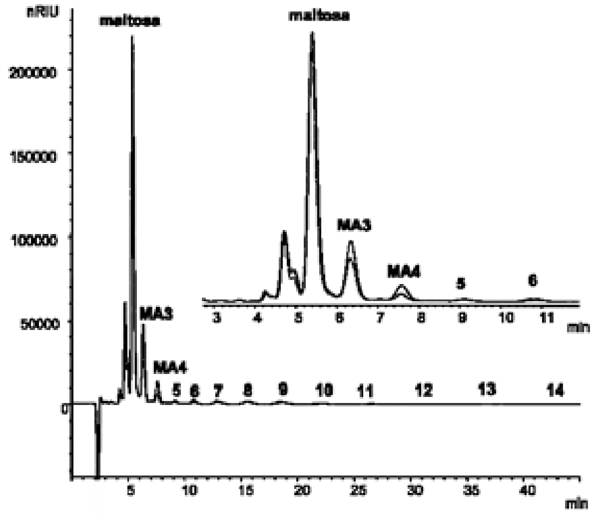

Fig. 2. **Differences in concentration profiles of OS in the congress wort prepared from variety Maltasia (full line) and Quench (dashed line) using HPLC. Detail of separation for low-weight OS. Peak numbers same as in Fig. 1.**

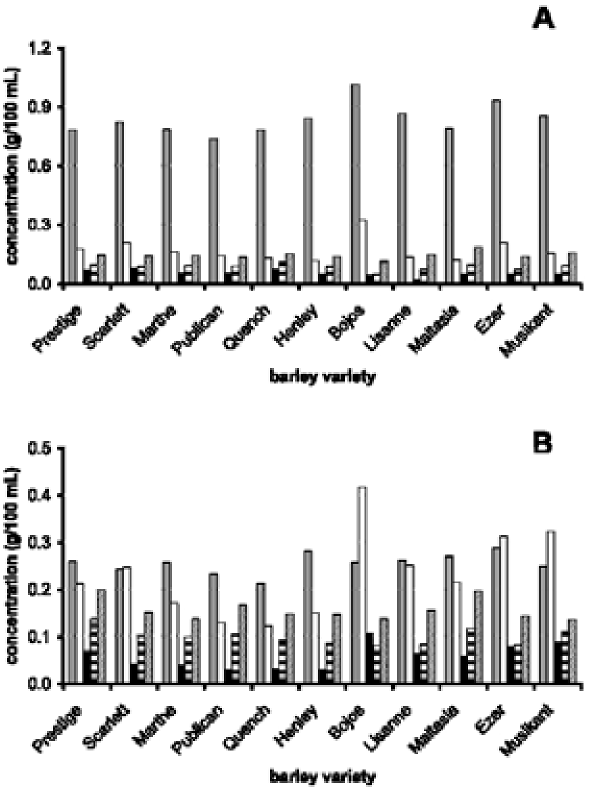

Fig. 3. **Concentration profile of individual OS in the congress wort (A) and green beer (B) for different barley varieties. Symbols: DP 3 (vertical lines), DP 4 (white), DP 5 (black), DP 6 (horizontal lines), and DP 7 (transversal lines).**

of eluted OS with DP<4 corresponded exactly to the retention times of standards of MA3, MA4 respectively, while small shifts in the retention times for OS with DP>4 compared to the standards of MOS were observed. It seems that OS with DP<4 are mostly consisted of linear α-(1 → 4)-linked glucose chains, while higher OS can be substituted by maltose/maltotriose residues of α-(1 → 6)-linked chains to form hardly fermentable saccharides so called dextrins. To identify the actual chain position, combination of HPLC with mass spectrometry has to be applied.

Beside the quality differences, noticeable changes in the quantity of OS for multiple DP were observed. There was high concentration of mono-, di-, and trisaccharides in the congress wort and hopped worth, while green beer and beer revealed significant decrease of these saccharides as expected after fermentation process. Especially maltose – the most abundant saccharide in the congress wort – was mostly fermented, so its concentration significantly decreased in the green beer (Fig. 1.A). In comparison, the concentration of OS with DP>4 did not show any significant changes neither in the wort nor in the beer samples. Their concentration remained on the same levels in all wort samples (Fig. 1.B).

Beside concentration changes in the wort samples of the same variety, significant changes in the content of low-weight saccharides (up to DP 4) in the congress wort of different barley varieties were found (Fig. 2.).

Obtained calibration data were used for quantification of OS corresponded to those with DP 3 to DP 7. The content of individual OS from different barley varieties is shown in Fig. 3.

The highest concentration of MA3 and MA4 was found in the Bojos variety, while Maltasia revealed the lowest concentration. Observed changes in the saccharide concentration can serve as the indicator of the quality and the usefulness of individual barley varieties; therefore, is essential in the development and production of new sort of beer.

Conclusions

ACN/water 3 : 2 (v/v) with flow rate of 1 ml min^{-1} provided for good separation of oligosaccharides up to tetradecasaccharides in 45 minutes duration. Significant changes in the quantity of low oligosaccharides in eleven different wort samples were found. The changes in the concentration and pattern of OS are important knowledge for development of the new brewing technologies.

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P17 EXTENSION OF ASPARAGINASE APPLICATION TO ACRYLAMIDE MINIMIZATION FROM POTATO TO CEREAL PRODUCTS

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Introduction

Acrylamide is a suspected human carcinogen, formed in fried and baked carbohydrate-rich foodstuffs such as potatoes and cereals. The free amino acid asparagine and reducing sugars are considered as the main precurosrs[1].

A number of raw material pre-treatments were investigated which could mitigate acrylamide formation. Unfortunately, most of them may also have an impact on the product sensory quality. For example, the acidification may result in a sour product taste, the addition of amino acids may generate unpleasant off-flavours upon heating or in the case of calcium chloride addition, product texture might be improved, but on the other hand it causes a bitter aftertaste[2]. However, the application of L-asparaginase enzyme before heat treatment results in a sufficient decrease of acrylamide amount with no undesirable impact on sensory quality of final products[3].

The aim of this work was an extension of enzymatic way of acrylamide elimination in potato products[3] to cereal of *rosquillas* type in collaboration with CSIC Institute del Frio in Spain. In addition, the impact on the sensory quality of products prepared with L-asparaginase was evaluated.

Experimental

L-asparaginase (Novozymes, Denmark) produced by *Aspergillus oryzae* was applied in simulated potato and cereal matrices and food products on potato and cereal base such as potato pancakes and typical Spanish cereal product named *rosquillas* (Spanish doughnuts), respectively. Following analytical parameters were determined:

- Saccharides using HPLC/RI according to ref.[4]
- Amino acids using LC/MS/MS according to ref.[5]
- Acrylamide using LC/MS/MS according to ref.[6]

The content of main acrylamide precursors (monosaccharides and amino acid asparagine) were analysed in raw material. The conversion of asparagine to aspartic acid and final acrylamide content after heat treatment was observed.

In potato pancakes the preliminary sensory evaluation was done using a dual method. An appearance, colour, texture and selected descriptors of taste and aroma were determined using 0–5 point scale. Five trained assessors payed an extraordinary attention to offlavour detection.

Potato Matrix

Before the enzyme application in food products, the appropriate conditions of L-asparaginase incubation (time and temperature) were tested in simulated matrices. Potato matrix consisted of the main acrylamide precursors (asparagine and glucose), potato starch and water in typical proportions: asparagine and glucose were used in equimolar ratio and content of water was 80 %. Potato starch was dried before the application to matrix in the ratio of 1 : 0.2 with mixture of asparagine and glucose.

Cereal Matrix

Cereal matrix simulated Spanish cereal products recipe for *rosquillas* preparation and was composed of asparagine, glucose, fructose, wheat starch and water. Glucose and fructose were used in equimolar ratio and in 1 : 4 ratio with starch in a final mixture. Potency of 3 levels of asparagine (0.1, 0.5 and 1.0 %) were compared. Content of water in cereal matrix was 50 %.

Asparaginase Application

L-asparaginase was applied in concentrations of 2 and 10 U g^{-1} to simulated potato matrix or 0.1, 0.5 and 1.0 U g^{-1} to simulated cereal matrix. The enzyme was incubated at 20, 37, 50, 60 and 70 °C for 5, 10, 20, 30 and 60 min in thermostat (Fried Electric, Haifa, Israel). Subsequently L-asparaginase was applied to different potato varieties (*Marabel* and *Bellarosa*) purchased from local Slovak market in concentrations of 1 and 2 U g^{-1}, respectively, to potato pancakes (potato-cereal food product) in concentration of 1 U g^{-1}, and to cereal product rosquillas in concentrations of 100 and 500 U kg^{-1} of flour. L-asparaginase in food samples was incubated at 37 °C for 10 and 15 min, respectively.

Thermal Treatment

For potato matrix 0.2 g of glucose and asparagine mixture, 1 g of potato starch was weighed into the vial and 4 ml of water (control sample) or 4 ml of enzyme solution with required concetration was added and in next step, after enzyme incubation, treated at 180 °C for 20 min in thermoblock (Liebisch Labortechnik, Bielefeld, Germany).

For cereal matrix 0.24 g of glucose and fructose mixture and 0.76 g of wheat starch was weighed into the vial, 0.76 ml of asparagine solution (concentration of 0.1, 0.5 and 1.0 g in per 100 ml) in control sample or 0.38 ml of asparagine solution (concentration 0.2, 1.0 and 2.0 g per 100 ml) and 0.38 ml of enzyme solution with demanded concentration was added. Vials were incubated and heated at 190 °C for 15 min in thermoblock.

Then, samples were cooled to room temperature and final content of acrylamide was determined.

Potato Pancakes Preparation

Potato-cereal powder for pancakes preparation was purchased from local market (Bramborák, Amylon, Czech Republic). Dough was prepared with water (control sample) and L-asparaginase solution (1 U g^{-1}). 4 pancakes with weight of 35 ± 1 g were fried on teflon pan (Tescoma, Czech Republic) in one run in microwave MW 800 HW 25 (Orava, Slovak Republic) using convection programme (170, 185 and 200 °C), 10 min for each side of pancake.

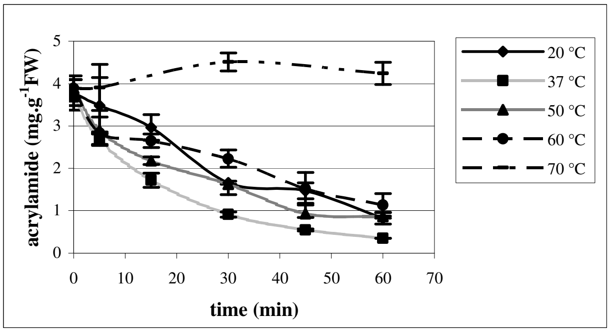

Fig. 1. **L-asparaginase efficiency to acrylamide mitigation in dependence on the incubation temperature (20, 37, 50, 60 and 70 °C) in a model system**

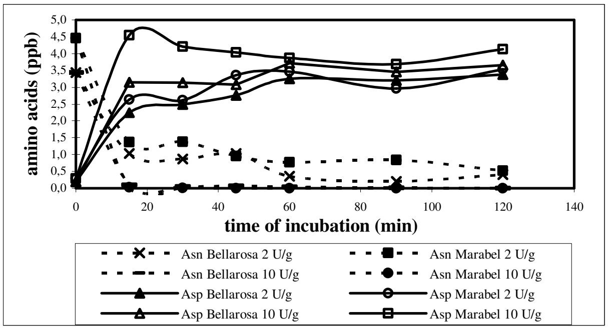

Fig. 2. **Conversion of asparagine to aspartic acid by L-asparaginase application (2 and 10 U g^{-1}, 37 °C) in 2 varieties of potatoes (Marabel, Bellarosa)**

Cereal Products Preparation

Typical Spanish cereal products *rosquillas* were preapared from dough according to three different and simplified recipes consisted of flour, sugar or equimolar mixture of glucose and fructose, in water (flour : sugar : water was in 4 : 1 : 2 ratio). In one recipe also sodium bicarbonate was added. From the dough samples of 10.0 ± 0.5 g weight and stick shape were formed and fried in sunflower oil in a fryer (Taurus, Spain). 4 samples in one run and also in duplicate were prepared for each temperature (180 and 200 °C), time (4, 6, 8 min) for each recipe (control and two levels of L-asparaginase: 100 and U kg^{-1} of flour). The dough with enzyme was incubated at 37 °C for 15 min in thermostat (Memmert, Germany).

Results

The content of asparagine in potato varies in the range from 2.3 to 39.4 mg g^{-1} of dry weight[7] that was comprised in a simulated potato matrix construction. Using different concentration of enzyme (2, 10 and 20 U g^{-1}), time and temperature of enzyme incubation (at 20, 37, 50, 60 and 70 °C for 5–60 min) the efficiency of L-asparaginase activity was compared. It was found out that temperature of 37 °C was the most suitable for sufficient acrylamide content elimination (Fig. 1.). In the case of potato matrix the time of enzyme incubation was reckoned as a limiting factor. After 30 min at 37 °C acrylamide reduction in final heated samples in levels of 8, 25 and 46 % was achieved and 91 % using 60 min of enzyme incubation.

The content of asparagine in the used potato samples was determined on 4.42 mg g^{-1} of fresh weight for variety *Marabel* and 3.52 mg g^{-1} of fresh weight for variety *Bellarosa.* Two levels of L-asparaginase (2 and 10 U g^{-1}) were applied and incubated at 37 °C. In potatoes the conversion of asparagine to aspartic acid was observed to be very fast (Fig. 2.) resulting in a sufficient acrylamide elimination (98–99 % in *Marabel* and 84–86 % in *Bellarosa*) after 10 min of enzyme incubation.

The content of asparagine in potato pancakes was 2.2 mg g^{-1} of powder. Using 1 U g^{-1} of L-asparaginase and 15 min of enzyme incubation at 37 °C, the 86% reduction of acrylamide content in a final product was achieved. No differences in sensory properties caused by enzyme were observed in the appearance (colour, texture) as well as in the taste and the aroma.

The asparaginase application in the simulated cereal matrix resulted in 18, 90 and 96% reduction of acrylamide using enzyme concentration of 0.1, 0.5 and 1.0 U g^{-1}, respectively, which was followed by enzyme treatment of cereal products. This time the determination of final acrylamide reduction in these products is in progress.

Conclusions

L-asparaginase application is proved to be an effective way of acrylamide reduction in potato and cereal products. In the presented study the 80–98 % of acrylamide elimination was achieved in dependence on matrix composition. Unambiguously, the distinctive advantage of L-asparaginase application is the avoiding undesirable changes in organoleptic properties of final products.

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P18 AMINO ACIDS PROFILE OF SELECTED WHOLEGRAINS IMPORTANT TO ACRYLAMIDE FORMATION IN CEREAL-BASED PRODUCTS

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Introduction

Acrylamide as a suspected carcinogen attracts great attention due to its widespread occurrence in many staple foods of daily usage[1] as well as due to the recommendation of the European Commission since 2005 to minimize its level[2]. It is known that acrylamide arises from naturally occurred compounds in plants such as reducing saccharides and amino acids during the commonly used process of heat treatment of foods[3,4]. Acrylamide is preferably formed from amino acid L-asparagine, only in a less extent from aspartic acid, glutamine, and glutamic acid in the presence of saccharides during baking[5]. It is well established that the amount of reducing sugars is more important than free asparagine for the formation of acrylamide in potato-based products[6]. However, in cereal foods including bread, the main determinant of acrylamide formation during baking is free asparagine amount in raw material and ingredients, in particular cereal flour.

The concentration of free asparagine have been studied in different commercial milling fractions of wheat and rye[7]. Whole grain flours showed higher amounts of asparagine (for wheat and rye 0.5 $\mathrm{g\,kg^{-1}}$ and 1.1 $\mathrm{g\,kg^{-1}}$, respectively) versus sifted flours. Of the wheat fractions, wheat germs was reported to have the highest level of asparagine (4.9 $\mathrm{g\,kg^{-1}}$).

However, agronomical factors (e.g. crop variety, climatic conditions fertilizer regimes) may significantly impact the amount of free asparagine in cereal crops[8] and consequently the concentration of acrylamide in the final product. Regional differences may account for levels that vary by more than fivefold, and today the scientific data that may explain this variability is lacking.

Based on this knowledge it can be said that in the cereal sector the main way of acrylamide reduction is through amino acids control responsible for acrylamide formation. From the point of view of acrylamide formation, the selected 10 sorts of wholegrains appointed for human usage in bread and cereal breakfast production which are bred in Slovakia as well as 6 kinds of wheat flours from different milles in Slovakia were assorted according to profile of amino acids asparagine, aspartic acid, glutamine, and glutamic acid.

Experimental

Raw Materials

Wheat grains of 5 varieties (PS-3/05, PS-11, PS-9/06, PS-27/06, PS-51/06) and oat grains of 5 registrated varieties (Vendelin, Valentin, Zvolen, Atego, Detvan) were obtained from the Research and Breeding Station at Vígľaš-Pstruša. Wheat flours originated from Slovak milles (PMD Bratislava, Kolárovo, Sládkovičovo, Šurany) were purchased from local markets.

Reagents

Asparagine (Asn) standard (99.5%) was supplied by Fluka (Steinheim, Germany) and aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln) standards (99%) were supplied by Merck (Darmstadt, Germany). D3-glutamic acid (d3-Glu) standard (97%) was supplied by Cambridge Isotope Laboratories (Andover, USA). Acetic acid (glacial) was HPLC reagent grade and obtained from Fisher Scientific (Loughborough, UK). Perfluorooctanoic acid (PFOA) (96%) and HPLC gradient grade acetonitrile were obtained from Sigma-Aldrich (Steinheim, Germany). Deionized water from a PURITE Select system (Oxon, UK) was used for preparation of amino acid and ion-pairing reagent solution.

Instrumentation

The LC/ESI-MS-MS apparatus for quantification of 4 free amino acids were performed by Agilent 1200 HPLC system (Waldbronn, Germany) consisting of a binary pump, an autosampler and a temperature controlled column oven, coupled to an Agilent 6410 Triple Quad detector equipped with ESI interface. The analytical separation was performed on a Purospher® STAR RP-8ec column (150 mm × 4.6 mm, 3 μm) using isocratic mixture of 100 ml of acetonitrile and 900 ml of aqueous solution of PFOA (0.05mM) at a flow rate 0.5 $\mathrm{ml\,min^{-1}}$ at room temperature. All parameters of the ESI-MS-MS system were based on in-source generation of the protonated molecular ions of the 4 amino acids measured and the internal standard (d3-Glu), as well as collision-induced production of amino acid-specific fragment ions for multiple reaction monitoring (MRM) experiments.

Sample Preparation

Stock solution of amino acids 1,000 $\mathrm{\mu g\,ml^{-1}}$ were prepared by dissolving 25 mg of each in 25 ml of deionized water. Working standards were prepared by diluting the stock solution of amino acids to concentrations of 0.05–2.00 $\mathrm{\mu g\,ml^{-1}}$. Each working standard solutions consist of 0.5 $\mathrm{\mu g\,ml^{-1}}$ of internal standard (d3-Glu). Finely ground or homogenized sample (1 g) was extracted by 10 ml of 0.2mM acetic acid and after mixing in a vortex mixer for 2 min the mixture was centrifuged at 5,000 rpm for 10 min at –5 °C and filtered through 0.45 μm nylon syringe filter prior to LC/MS analysis[9].

Results

In this study, 5 varieties of wheat grains, and 5 varieties of oat grains as well as 6 kinds of wheat flours were used for determination of amino acids profile. Since particularly free amino acid L-asparagine (Asn) and in a less extent also L-aspartic acid (Asp), glutamine (Gln) and glutamic acid (Glu) are responsible for the formation of acrylamide in cereal products during baking, the amino acids mentioned above were determined in the samples of wheat flours (Table I), wheat grains (Table II), and oat grains (Table III).

Table I
The amount of asparagine, aspartic acid, glutamine and glutamic acid in the samples of wheat flours

Wheat flours	Asn [mg kg^{-1}]	Asp [mg kg^{-1}]	Gln [mg kg^{-1}]	Glu [mg kg^{-1}]
A	78.51	130.86	25.01	45.53
B	115.22	154.14	39.12	77.82
C	56.14	106.85	22.28	51.68
D	92.56	133.03	32.38	67.73
E	88.37	117.11	24.62	56.20
F	81.75	121.87	31.03	59.59

Table II
The amount of asparagine, aspartic acid, glutamine and glutamic acid in the samples of wheat grains

Wheat grains	Asn [mg kg^{-1}]	Asp [mg kg^{-1}]	Gln [mg kg^{-1}]	Glu [mg kg^{-1}]
PS-51/06	221.71	79.46	24.74	76.41
PS-11	228.88	218.43	44.08	126.72
PS-27/06	290.86	181.67	121.25	187.29
PS-9/06	222.07	137.94	51.61	142.20
PS-3/05	188.04	117.02	32.76	113.39

Table III
The amount of asparagine, aspartic acid, glutamine and glutamic acid in the samples of oat grains

Oat grains	Asn [mg kg^{-1}]	Asp [mg kg^{-1}]	Gln [mg kg^{-1}]	Glu [mg kg^{-1}]
Detvan	792.24	268.85	396.91	468.79
Vendelin	567.55	144.62	211.15	261.89
Valentin	546.22	156.83	240.65	269.36
Zvolen	720.76	160.05	312.94	285.28
Atego	522.36	201.01	199.48	305.56

It is evident that in the wheat flour there is the aspartic acid which has the highest portion among determined amino acids. The amount of free L-asparagine varies between 78.51 mg kg^{-1} and 115.22 mg kg^{-1}. On the other hand, in the whole grains of wheat and oat the free L-asparagine occupies the highest ratio of free amino acids. Moreover, the oat grains contain approx. two or three fold higher amount of L-asparagine comparing to wheat grains. It is important from the point of view of the next usage of these grains at their heat treatment in the process of production of bakery ware or breakfast cereals. Furthermore, there were observed some differences between the amino acid profile in the similar kinds of wheat flour. These differences should be considered at the choice of flour which should be used for production of high heat treated products.

Conclusions

Human exposure of acrylamide from cereal products represents approx. one half of daily exposure of this contaminant. The determination of amino acids in flours as well as in the whole grains which are responsible for acrylamide formation after heat treatment could be helpful at the choice of suitable flour and cereal variety with the lowest asparagine amount and could decrease of human exposure of acrylamide from cereal products.

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P19 CHARACTERIZATION OF BETA-CAROTENE ENRICHED BIOMASS PRODUCTION BY RED YEASTS

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Introduction

During the last decades fast progress has been made within the field of biochemistry of carotenoid biosynthesis in bacteria, fungi, and plants. Although more than 600 different carotenoids have been identified in nature, only a few are used industrially. There are many yeast strains able to produce different carotenoids. However, the heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous*, are the only microbial systems with commercial potentials for the production of astaxanthin. Several genes involved in the astaxanthin biosynthetic pathway of *X. dendrorhous* have been cloned and characterized recently. Analysis of DNA in other red yeast is very complicated and only several genes were described yet.

There are many yeast strains able to produce different carotenoids, mainly as a part of stress response. In this work three different red yeast strains (*Sporobolomyces roseus, Rhodotorula glutinis, Rhodotorula rubra*) were enrolled into a comparative study. To increase the yield of carotenoid pigments at improved biomass production, several types of exogenous as well as nutrition stress were tested. Each strain was cultivated at optimal growth conditions and in medium with modified carbon and nitrogen sources. Synthetic media with addition of complex substrates (e.g. yeast extract) and vitamin mixtures as well as some waste materials (whey, potato extract) were used as nutrient source. Some types of exogenous stress – peroxide, salt were applied too. The production of carotene-enriched biomass was carried out in flasks as well as in laboratory fermentor. Changes in yeast cells on metabolome level were studied using LC/MS techniques to carotenoid analysis.

Materials and Methods

Strains

In the study following red yeast strains were tested: *Rhodotorula glutinis* CCY 20-2-26, *Sporobolomyces roseus* CCY 19-4-8; *Rhodotorula rubra* CCY 20-7-31; *Phaffia rhodozyma* CCY 77-1-1, *Sporobolomyces salmonicolor* CCY 19-4-10.

Cultivation

Red yeasts were cultivated in a simple glucose medium aerobically at 28 °C. Physilogical stress was induced by nutrition components (C and N source) and by addition of 5 mM peroxide and 2% and/or 5 % NaCl. Stress cultivations with *S. salmonicolor*, *R. glutinis* and *P. rhodozyma* were realized in flasks as well as in 2 L-laboratory fermentor (B.Braun Biotech).

Three series of cultivations were realized with each strain. Two-step inoculation was done. All strains were firstly inoculated into a medium containing yeast autolysate (7 g), $(NH_4)_2SO_4$ (5 g), glucose (40 g), KH_2PO_4 (5 g), $MgSO_4$ (0.34 g) per liter (INO I) and cultivated at 28 °C for 24 hours at permanent shaking and lighting. Second inoculum (INO II) was prepared similarly, in 1st series was used the same medium as INO I, in 2nd series lyophilized whey was added (7 g dm^{-3}) and in 3rd series potato extract (7 g dm^{-3}) was added into INO II. Cultivation in INO II undergo at 28 °C for 24 hours at permanent shaking and lighting. Production media contained $(NH_4)_2SO_4$ (5 g), glucose (40 g), KH_2PO_4 (5 g), $MgSO_4$ (0.34 g) per liter. Several waste substrates were added and cultivation was done for 80 hours at 28 °C under permanent lighting and shaking. Production media were prepared according to following scheme:

- 1st series: INO I --- INO II --- production: 1 – control, 2 – 5 mM peroxide, 3 – 2% NaCl, 4 – 5% NaCl, 5 – lyophillized whey non-processed (7 g dm^{-3}), 6 – lyophillized whey processed by deproteination agent (7 g dm^{-3}), 7 – liquid whey (250 ml dm^{-3}), 8 – potato extract (Hi Media; 7 g dm^{-3})
- 2nd series: INO I --- INO II (whey, 7 g dm^{-3}) --- production: 1 – control, 2 – 5 mM peroxide, 3 – 2% NaCl, 4 – 5% NaCl, 5 – lyophillized whey non-processed (7 g dm^{-3}), 6 – lyophillized whey processed by deproteination agent (7 g dm^{-3}), 7 – liquid whey (250 ml dm^{-3})
- 3rd series: INO I --- INO II (potato extract 7 g dm^{-3}) --- production: 1 – control, 2 – 5 mM peroxide, 3 – 2% NaCl, 4 – 5% NaCl, 5 – potato extract (7 g dm^{-3}).

Analyzed Parameters

In all samples biomass and carotenoid content were evaluated. Biomass was determined gravimetrically. Levels of carotenoids – lycopene and beta-carotene were analyzed using HPLC/VIS (450 nm) and verified by HPLC/MS. Ergosterol and phytoene were analyzed by RP-HPLC (280 nm).

Results

In this work the growth of some red yeasts on waste substrates and subsequent effect of these substrates on beta-carotene production was studied. It was observed that addition of non-processed or deproteined whey or potato extract to media can increase beta-carotene production.

In *Rhodotorula glutinis* addition of deproteined whey into production medium led to 3.5× increased production of beta-carotene without changes in biomass. Non-processed whey or potato extract added to production media led to about 3× increase of beta-carotene production but it was accompanied by lost in biomass. The highest yield was reached after addition of lyophillized whey to INO II as well as

Table I
Production of biomass and beta-carotene by stressed red yeasts in laboratory flasks

	R. glutinis biomass	*R. glutinis* Beta-carotene	*S. roseus* biomass	*S. roseus* Beta-carotene
control	11.1	0.18	11.5	0.16
2 mM peroxide/INO	10.6	0.17	10.6	0.26
2% salt/ INO	11.8	0.38	11.6	0.05
2% salt/ INO/ 5 mM peroxide/prod.	10.1	0.56	11.7	0.17
2 mM peroxide/INO/ 5 mM peroxide/prod.	10.0	0.29	10.0	0.29

to production media. Liquid whey exhibited negative effect. Also potato extract added into INO II led to increased beta-carotene production, while biomass yield was lower.

Rhodotorula rubra is poor producer of carotenoids when compared with other strains. However, cultivation in presence of potato extract in INO II combined with salt stress in production medium led to the highest biomass as well as beta-carotene production observed yet.

Sporobolomyces roseus exhibited substantial changes in biomass : carotene ratio dependent on whey addition. Substantial biomass decrease in presence of lyophillized whey in INO II (under 5 g dm^{-3}) was accompanied by very high beta-carotene yield.

In flasks combined stress led to induction of carotenoid production in all studied strains; 3 × increase of beta-carotene (*R.glutinis*) was obtained. Addition of stress factor into inoculation medium induced slight increase of biomass production (salt - *R.glutinis*) and beta-carotene production (*R. glutinis*).

Conclusions

Changes in medium composition can lead to substantial changes in biomass as well as carotenoid production. Waste substrates can be used as medium component, which can in particular strains and conditions induce carotenoid as well as biomass production. Thus, waste substrates could be used industrially for carotenoid-rich biomass production.

Predominantly strain *Rhodotorula glutinis* CCY 20-2-26 can be used for industrial production of carotenoid-rich biomass using processed waste substrates and/or mild physiological stress.

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P21 BIOGENIC AMINES IN CHEESES AND HUMAN HEALTH

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Introduction

Food safety is an increasingly important public health issue. Governments all over the world are intensifying their efforts to improve food safety. These efforts are in response to an increasing number of food safety problems and rising consumer concerns[1]. Like of other processed foods, also cheeses result from a manufacturing process involving the selection of raw material (e.g. milk, lactic acid bacteria, rennet), preparatory treatments such as milk pasteurization, renneting, drainage, salting, the ripening operation proper, preservation (if needed), packaging and storage. Last but not least, treatment of the food by the consumer influences its condition. Thus, an integral approach should be taken when assign the implications of individual risk factors for the safety of the consumer[2].

Cheeses are among those high-protein-containing foodstuffs in which enzymatic and microbial activities cause the formation of biogenic amines from amino acid decarboxylation (Innocente et al., 2007). Codex Alimentarius of the Slovak Republic stated the maximum limit only for the tyramine in hard cheeses, in which the concentration of tyramine must not exceed value of 200 mg kg^{-1}.

The aim of this study was to determine cadaverine (CAD), histamine (HIS), tyramine (TYR) and putrescine (PUT) in some commercial cheeses commonly consumed in Slovakia using ion-exchange chromatography. Chemical analyses were carried out in parallel with determination of sensory assessment of cheeses and their microbiological quality with the respect of *Enterobacteriaceae*.

Experimental

In total 31 samples of five cheese typologies were taken from supermarkets and retail shops: 5 high cooked cheeses, like Emmental, 8 low cooked cheeses, like Edam, 9 mozzarella like cheeses, 5 mould cheeses, and 4 sour natural ripened cheeses containing yeasts and *Brevibacterium linens.* All products were sampled during the correct their shelf life. Samples were stored at 4 °C until to analysis.

Biogenic Amines Analysis

The five biogenic amines (BA) studied were: cadaverine (CAD), histamine (HIS), tyramine (TYR) and putrescine (PUT). Ion exchange chromatography with amino acid analyzer (AAA 339 – T Microtechna, Czech Republic) was used for both qualitative and quantitative studies of biogenic amines in cheeses[3].

Each sample (100–200 g) was homogenised and mixed thoroughly with a Moulinex blender on a day of collection or purchase. A 10 g sample was extracted with 5% trichloracetic acid, centrifuged and filtrated through a membrane filter with a pore size of 0.45 μm. All the samples were prepared and analysed in duplicates.

Microbiological Analysis

All cheese samples were subjected to microbiological routine analysis: Enterobacteriaceae, E. coli, Staphylococcus aureus, yeasts and moulds. The cultured selective agar plates (blood agar, VRBG Agar, Endo Agar, Baird-Parker Agar; Oxoid, UK) were used. Petri dishes were inoculated with 0.1 ml of diluting sample and cultivated in aerobic conditions 24–48 h at 37 °C and 25 °C for five days, respectively. Pure cultures were identified at species by routine microscopical, cultivation and biochemical methods. Micromycetes were identified macroscopically and microscopically, using the method according to St-Germain and Summerbell[4].

Sensory Evaluation

Six volunteers (all women) were evaluated attributes related to the flavour and mount feel of all type of cheeses used in the experiment. The descriptor chosen were: colour, consistency, mouth couting, cheese flavour intensity, sourness, bitterness, off-flavour, and overall acceptability.

Results

The mean value of biogenic amine sum (CAD + HIS + TYR + PUT) concentration in mg kg^{-1} varied from 9.82 in cheese with moulds to 1,092.33 in sour natural ripened cheeses. In both groups of hard cheese the mean value of the sum biogenic amine concentration was very similar (175.05 mg kg^{-1} in high cooked cheeses *versus* 198.32 mg kg^{-1} in low cooked cheeses). The prevailing amine in all cheeses included into the experiment was tyramine, following by histamine, although HIS was not detected in any case of mozzarella-like cheeses evaluated.

Enterobacteriaceae count ranged from 1.12×10^3 CFU g^{-1} (high cooked cheese samples) to 21×10^3 CFU g^{-1} in cheese with mould. Totally, *Enterobacteriaceae* were isolated in 19 (61.29 %) of 31 evaluated cheese samples in this study.

According to the sensory assessment, 87.01 % of evaluated cheese had characteristic organoleptic properties depending on the kind of cheese. In four of 31 evaluated cheeses, the cheese samples did not show excellent sensory qua-

Table I
Concentration of BA in low cooked cheeses and *Enterobacteriaceae* count (Ent) (CFU × 10^3 g^{-1})

Col. stats	HIS	TYR	PUT	CAD	Sum	Ent
		[mg kg^{-1}]				
Mean	8.5	134.8	13.2	18.0	173.5	8.2
sd	10.6	128.6	16.5	25.0	138.9	12.1
Minimum	1.0	1.30	0.0	0.0	8.00	0.0
Maximum	33.2	305.1	39.9	64.8	324.0	32.5
Median	4.5	111.7	3.9	8.2	173.5	1.0

lity mainly in the flavour. In one of did not accepted cheeses from sensory viewpoint, the cheese sample from high cooked smoked and grate cheese, the original packing of cheese from the hypermarket we detected large defects in sensory cheese characteristic.

Table II
Concentration of BA in high cooked cheeses and *Enterobacteriaceae* count (Ent) (CFU × 10^3 g^{-1})

Col. stats	HIS	TYR	PUT	CAD	Sum	Ent
		[mg kg^{-1}]				
Mean	24.7	150.7	13.5	9.3	196.6	1.1
sd	28.9	180.6	18.4	12.1	168.5	1.5
Minimum	1.0	1.30	0.0	0.0	62.5	0.0
Maximum	67.7	432.6	43.2	29.7	464.0	3.1
Median	12.8	102.6	4.9	4.9	174.0	0.0

Table III
Concentration of BA in mould cheeses and *Enterobacteriaceae* count (Ent) (CFU × 10^3 g^{-1})

Col. stats	HIS	TYR	PUT	CAD	Sum	Ent
		[mg kg^{-1}]				
Mean	2.4	1.6	0.0	0.35	3.7	21.1
sd	1.7	2.5	0.0	0.7	3.5	46.3
Minimum	1.0	0.0	0.0	0.0	1.0	0.0
Maximum	4.9	5.3	0.0	1.4	9.0	104.0
Median	1.9	0.6	0.0	0.0	2.5	0.6

Table IV
Concentration of BA in mozzarella like cheeses and *Enterobacteriaceae* count (Ent) (CFU × 10^3 g^{-1}) (*$p < 0.05$)

Col. stats	HIS	TYR	PUT	CAD	Sum	Ent
		[mg kg^{-1}]				
Mean	0.0	1.2	3.7	2.0	6.7*	1.2*
sd	0.0	2.6	5.8	4.3	11.5	1.5
Minimum	0.0	0.0	0.0	0.0	0.0	0.0
Maximum	0.0	8.0	14.1	12.6	34.0	4.0
Median	0.0	0.0	0.0	0.0	0.0	0.5

Table V
Concentration of BA in sour natural ripened cheeses and *Enterobacteriaceae* count (Ent) (CFU × 10^3 g^{-1}) (*$p < 0.05$)

Col. stats	HIS	TYR	PUT	CAD	Sum	Ent
		[mg kg^{-1}]				
Mean	66.2	249.2	207.4	673.2	1,194.0*	2.0*
sd	51.2	169.2	194.3	462.8	783.4	4.1
Minimum	14.2	83.8	18.2	190.9	443.0	0.0
Maximum	152.1	454.2	526.5	1,408.0	2446.0	8.2
Median	168.1	151.4	653.9	907.0	2446.0	0.0

Cheese consisted of the gross crust of dark brown colour with a rare presence of cheese curd of golden – yellow colour. The consistency of cheese was dry and hard, odour expressive smoked and taste was biter and tarry (sum of BA = 466.3 mg kg^{-1}, *Enterobacteriaceae* were not isolated).

According to the sensory assessment, 87.01 % of evaluated cheese had characteristic organoleptic properties depending on the kind of cheese. In four of 31 evaluated cheeses, the cheese samples did not show excellent sensory quality mainly in the flavour. In one of did not accepted cheeses from sensory viewpoint, the cheese sample from high cooked smoked and grate cheese, the original packing of cheese from the hypermarket we detected large defects in sensory cheese characteristic. Cheese consisted of the gross crust of dark brown colour with a rare presence of cheese curd of golden – yellow colour. The consistency of cheese was dry and hard, odour expressive smoked and taste was biter and tarry (sum of BA = 466.3 mg kg^{-1}, *Enterobacteriaceae* were not isolated).

Conclusions

From a "good manufacturing practice" point of view, total of 100–200 mg kg^{-1} of biogenic amines in fermented foods are regarded as acceptable[2]. The major BA producers in foods are except some lactic acid bacteria which do not produce significant levels of BA, the undesirable bacteria mostly *Enterobacteriaceae* and *Enterococcus* sp.5 According to our results, the count of Enterobacteriaceae and sum of biogenic amines was statistically significant only in mozzarella like cheeses and in sour natural ripened cheeses ($p < 0.05$).

From a health point of view the sum BA as well as the concentration of individual biogenic amines should not to increase some certain levels. According to Spanier et al. (1991) the sum of BA (HIS+TYR+PUT+CAD) 900 mg g^{-1} in cheese is recommended as the highest acceptable concentration. This limit was exceeded in sour natural ripened cheeses in which the sum of BA was determined in a value of 1,194 ± 783.4 mg kg^{-1} of cheese sample.

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P22 PHYTOSTEROL AND FATTY ACID PROFILE OF FOUR EDIBLE OILS PROCESSED IN ROMANIA

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Introduction

Triglycerides represent the principal component (95–98 %) of most edible oils obtained from seeds and fruits. Small amounts of sterols, free fatty acids, hydrocarbons, tocopherols, phospholipids and triterpenic acids are also present.

Fatty acids occur predominantly as esters of glycerol, i.e., triacylglycerol, in natural fats of animal and plant origin. Two distinct families of essential fatty acids exist in the human body: the ω-3 family (derived from α-linolenic acid) and ω-6 family (derived from linoleic acid)[1].

Phytosterols are a group of natural compounds found in all plants and in food products of plant origin. In vegetable oils, sterols are found primarily as free and esterified forms[2].

Four edible oils: walnuts, sesame, peanut and poppy seed oil, processed by minimal technologies (cold pressing) in Romania were investigated. The goal of the present study was the identification and quantification of fatty acids as FAMEs (fatty acid methyl esters), in the total lipid extracts (TLE) and in the esterified sterol fractions (ESF).Total, free and esterified sterols were also quantified as trimethylsilyl ether derivatives (TMS), using gas-chromatography (GC) with flame ionization detector (FID).

Experimental

Sample Preparation

The FAMEs were prepared by transesterification of the oil samples and the esterified sterol fractions by sodium methoxide catalysis[1].

Saponification, extraction, purification and preparation of TMS derivatives of the total, free and esterified sterols were made according to Phillips et al.[3].

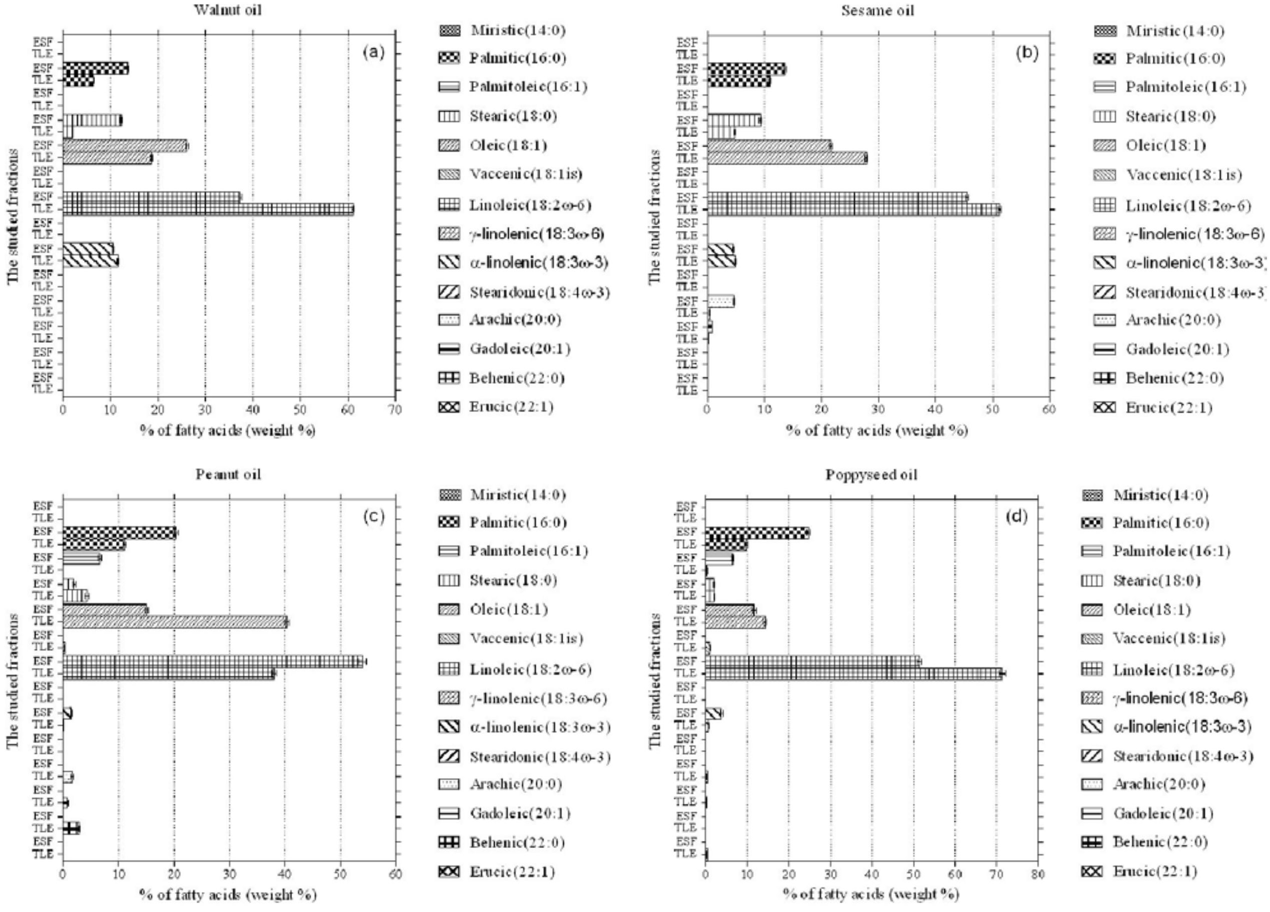

Fig. 1. **The fatty acid compositions [weight %] in total lipid extracts (TLE) and esterified sterol fractions (ESF) of: (a) walnut, (b) sesame, (c) peanut and (d) poppysed oils**

GC Analysis

GC conditions (for FAMEs): gas chromatograph SHIMADZU GC-17-A equipped with FID detector and capillary column Alltech ATWAX (30 m × 0.25 mm × 0.25 μm) with temperature programme 150 °C held for 5 min, ramp 4 °C min^{-1} up to 235 °C, held for 5 min. The flow rate of the carrier gas helium was 1.8 ml min^{-1} (split ratio 1 : 20). The injector and the FID temperature was 260 °C. The identification of FAMEs was based on retention times and comparison with those of standard FAMEs.

GC conditions (for derivatized sterols): GC-FID apparatus was the same. An RTX-5™ fused silica capillary column (30 m × 0.25 mm × 0.25 μm) was used. The temperature programme was: 200 °C held for 5 min, ramp 10 °C min^{-1} up to 300 °C (held 20 min); injector temperature 280 °C; detector temperature 300 °C. The flow rate of the carrier gas helium was 0.58 ml min^{-1} (split ratio 1 : 17). Identification of sterols was based on comparison of their relative retention times (RR_t to β-sitosterol) with data from the literature[4, 5]. A mixture of sterol standards was studied in the same conditions and the retention times (R_t) were used to assist the peak identification. The sterol concentrations were calculated using the area of the internal standard peak.

All extractions and GC-FID runs were performed in triplicate. The mean values and standard deviations were calculated.

Results

The fatty acid and sterol compositions reported in this study are in good agreement with those reported in the literature.[3,4,6,7]

Table I
Total sterol concentrations after direct saponification of the oils (mg $100g^{-1}$)

Sterols	Walnut	Sesame	Peanut	Poppyseed	RR_t
1.	1.01 ± 0.12	n.d.	n.d.	n.d.	0.80
2.	n.d.	n.d.	n.d.	n.d.	0.83
3.	13.10 ± 0.74	71.00 ± 0.32	23.90 ± 0.34	41.18 ± 0.25	0.90
4.	0.60 ± 0.07	1.11 ± 0.11	1.53 ± 0.06	4.09 ± 0.11	0.91
5.	0.42 ± 0.05	34.10 ± 0.63	14.60 ± 0.32	11.44 ± 0.57	0.94
6.	147.00 ± 1.03	345.00 ± 0.66	114.00 ± 0.86	187.40 ± 0.56	1.00
7.	7.15 ± 0.23	2.71 ± 0.15	2.46 ± 0.10	2.33 ± 0.08	1.02
8.	28.00 ± 0.94	50.70 ± 0.50	15.30 ± 0.49	23.29 ± 0.70	1.03
Total	197.28 ± 3.18	504.62 ± 2.37	171.79 ± 2.17	269.73 ± 2.27	

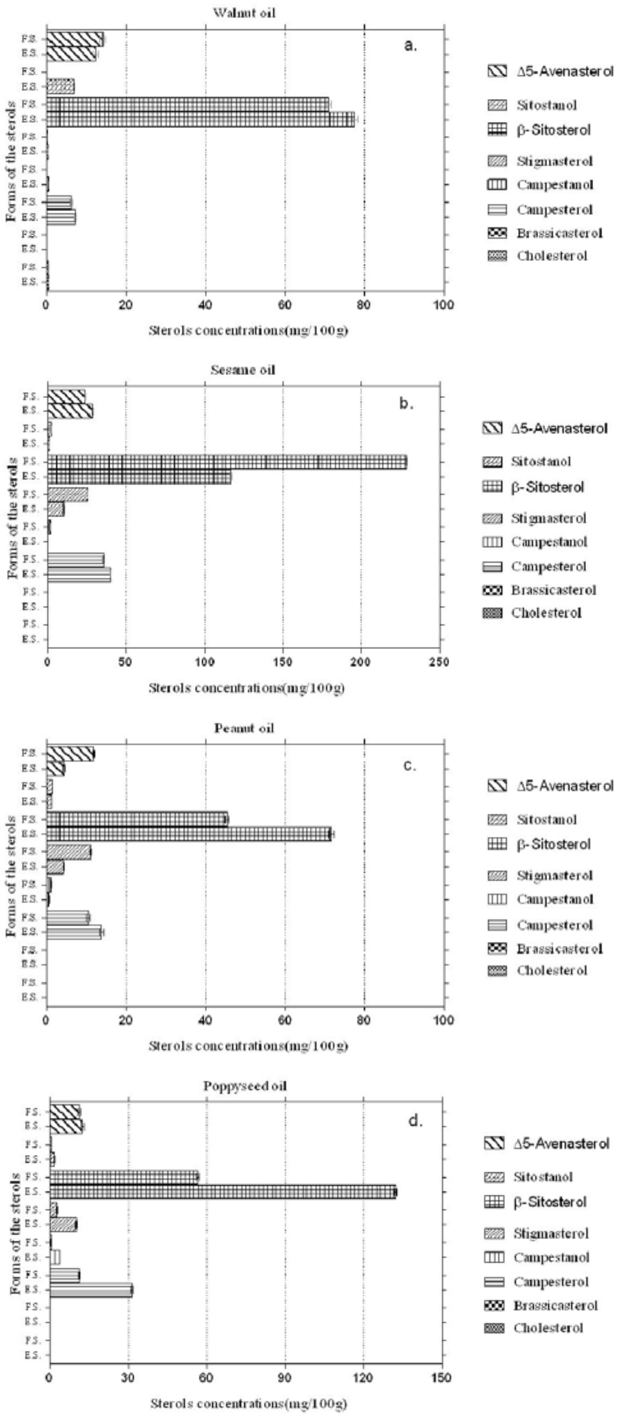

Fig. 2. **Free and esterified sterol concentrations in four edible oils: (a) walnut, (b) sesame, (c) peanut and (d) poppysed oil**

Fatty Acid Composition

Fig. 1. illustrates the fatty acids compositions of edible oils used in this study. Three fatty acids were predominant in the analyzed oils (TLE) and theirs subfractions (ESF): linoleic acid (37.27–71.41 %), oleic (11.58–40.36 %) and palmitic acid (6.54–24.62 %).The unsaturated fatty acids/saturated

fatty acids ratios varied from 3.96 to 10.62 for the total lipid extracts. For the studied ESF's, these ratios varied from 2.64 to 3.44.

The polyunsaturated fatty acids were the most abundant out of the unsaturated fatty acids of TLE's, in all studied oils, except for peanut oil. The polyunsaturated/monounsaturated fatty acids ratios ranged from 0.92 to 4.49 for the analyzed TLE's and from 1.83 to 3.06 for ESF's, respectively.

Sterol Composition

The predominant phytosterols in all four samples were sitosterol, campesterol and Δ5-avenasterol (see Table I).The proportion of free and esterified sterols varied widely among samples (see Fig. 2.). In all oils except sesame oil, most of the β-sitosterol was esterified.

Conclusions

The determination of fatty acids and sterols' composition is essential for the analytical assessment of the quality, origin, extraction method, refining procedure and possible adulteration of the vegetable oils.

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P23 ANTIOXIDANT ACTIVITY OF FLAVANOLS FROM GRAPE SEED EXTRACTS

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Introduction

Natural antioxidants, particularly from fruits and vegetables, have gained increased interest among consumers and scientific community, due to their lower risk for cardiovascular diseases and cancer demonstrated by many studies[6].

Grape seeds are a rich source of flavanols, having monomers such as catechin, epicatechin, epicatechin-3-*o* gallate and dimers, trimers, teramers. Extracts from four varieties of Romanian grapes: Merlot, Mustoasa, Feteasca, and Chasla were first analyzed by HPLC (high performarce liquid chromatography) regarding their flavonol content.

The aim of this research was to evaluate the efficiency of ORAC, DPPH and ABTS radicals and to estimate the antioxidative capacity of grape seed extracts. The antioxidative activity of the extracts was determined through:

- Oxygen radical absorbance capacity assay (ORAC)
- 2,2'-diphenyl-1-pichrylhydrazyl assay (DPPH)
- Trolox equivalent antioxidant capacity assay (TEAC)

Total phenolic compounds content was determinated colorimetrically using Folin-Ciocalteu reagent.

Material and Methods

Preparation of Grape Seed Extracts

Seeds of the Vitis vinifera grape were obtain from Recas area (Bihor, RO). Grape seed were first grinded until a powder was obtained, which was afterwards deoiled with hexan (1 part of powder to 10 parts of hexane w/v). The solid residue was kept under the hood in the dark to evaporate the hexane. Seed extracts were prepared for analysis by mixing the powder with metanol/water/acetic acid (70 : 29.05 : 0.5, v/v/v) for a ratio of 1 part powder to 10 parts solvent (w/v). The mixture was sonicated for 15 min and shaken for 30 min at 4,000 rpm. The extract was concentrated in vacuum rotary evaporator at 40 °C. Volume of the concentrate was then adjusted to obtain a concentration of 1 g solids ml^{-1} by adding a predetermined volume of methanol[7].

ORAC Assay

ORAC assay measures antioxidant inhibition of peroxyl radical induced oxidation, reflecting classical radical chain breakage of antioxidant activity, by hydrogen atom transfer. The ORAC assay was performed as described by Ou et. al.[4].

DPPH Assay

DPPH assay is based on the measurement of the reduction ability of antioxidants toward DPPH$^{\bullet+}$. The kinetics of 400 µl grape seed extract in 2.8 ml of DPPH (80 µM in etanol) were registered in 30 min by monitoring DPPH disappearance at 515 nm.

TEAC Assay

TEAC assay asseses the capacity of a compound to scavenge ABTS radical (ABTS$^{\bullet+}$). Intensely colored radical cation ABTS$^{\bullet+}$ is formed by peroxyl radical oxidation of ABTS. The antioxidant ability is measured as the ability of test compounds to decrease the color formation. Using the method of Arnao et. al. (2002)[1] the interaction between the antioxidants and the ABTS$^{\cdot+}$ was monitored spectrophotometricaly at 734 nm.

Folin-Ciocalteu Assay

In Folin-Ciocalteu assay 250 µl extract were mixed with 1.25 ml of Folin-Ciocalteu reagent and 1.9 ml of sodium carbonate respectively and allowed to react for 2 hours. The absorption was measured with a Biotek Synergy HT spectrophotometer. The total phenolic compounds content was expressed as gallic acid equivalents (GAE mg g^{-1} dry weight DW).

Results and Discussion

The four grape seed extracts were analyzed by HPLC-UV, Merlot variety having 80 % of flavanols content, Feteasca 50 %, Mustoasa 40 %, and the poorest flavanol content was for Chasla, only 20 % (data not shown). Table I and Fig. 1. shows the value obtained for the antioxidant capacity assays. ORAC, TEAC and DPPH values are expressed in µM Trolox g^{-1} DW. The antioxidant activity using TEAC and ORAC ranged from 86 to 280, and respectively from 100–183 µM Trolox g^{-1} DW. From the four grape seed extracts, Merlot and

Table I
Antioxidant activity of grape seed extracts as determined by ORAC, DPPH, TEAC and Folin-Ciocalteu assay

Sample name		µM Trolox g^{-1} DW		mg GAE 100 mg^{-1} DW
	ORAC	DPPH	TEAC	Folin-Ciocalteu
Merlot	183.73 ± 2.4	1,140 ± 0.08	280.3 ± 1.8	651.2 ± 0.02
Mustoasa	123.1 ± 0.2	424 ± 0.09	129.6 ± 0.6	467.5 ± 0.04
Feteasca	155.3 ± 2.1	656.1 ± 1.3	202.1 ± 0.6	645.2 ± 0.01
Chasla	100.7 ± 1.8	67.2 ± 1.1	86.18 ± 3.1	423.2 ± 0.01

Chasla varieties have the highest (280.3 ± 1.8), and respectively the lowest (86.18 ± 3.1) TEAC values. Chasla have also the lowest ORAC value (100.7 ± 1.8).

The antioxidant activities determined by TEAC and ORAC showed a very good correlation (r = 0.99). A good correlation was obtained also for ORAC and DPPH assay (r = 0.97). Both assays are based on Trolox (a water soluble derivative of vitamin E) equivalents, although the ORAC assay represents a hydrogen atom transfer reaction mechanism and the TEAC assay represents a single electron transfer-based method[5]. Ou et al. (2002) reported no correlation between the FRAP and ORAC techniques among most of 927 freeze and dried vegetable samples, whereas these methods reveald a hight correlation in blueberry fruit (Connor et al, 2002)[3,4]. Similarly, Awika et al, 2003 observed high correlation between ABTS, DPPH, ORAC among sorghum and its products[2]. DPPH assay values shows significant differences between the flavanol richest Merlot sample (1,140 ± 0.08) and the flavanol poorest Chasla sample (67.2 ± 1.1) respectively. The amounts of total phenolic compounds were of the same order of magnitude among the studied grape seed varieties from 423 mg GAE 100 g^{-1} DW to 651 mg GAE 100 g^{-1} DW material (Table I). This is most probably due to the presence of the same type of phenolic compounds in the samples[5].

Conclusions

The flavanols contribute to the antioxidant activity of grape seed extracts. Ranking of antioxidant capacity was higly consistent across the different methods used, obtaining the same following ranking from the varities of the grapes seed extracts tested:

Merlot> Feteasca> Mustoasa > Chasla

This study confirms also the correlation between ORAC, DPPH and TEAC assays, their values being proportional with phenolic compounds contents.

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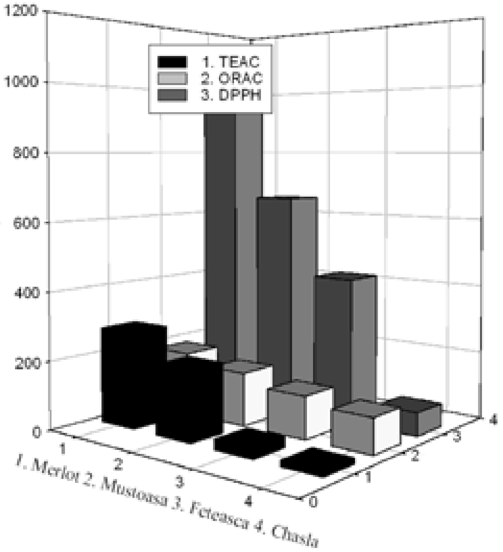

Fig. 1. **The antioxidant activity of grape seed extracts determined by TEAC, ORAC, DPPH assays**

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P24 INFLUENCE OF LONG-TERM STORAGE CONDITIONS ON ANTIOXIDANT AND OTHER ACTIVE COMPONENT CONTENT IN SEVERAL SORTS OF APPLES

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Introduction

Apples are one of the most common sources of natural antioxidants in Czech population. This work was focused on study of changes of antioxidant content, enzyme activities, and protein composition in several local sorts of apples stored for a long time under controlled atmosphere with reduced oxygen content.

To qualitative as well as quantitative analysis of individual low-molecular weight antioxidants RP-HPLC/UV-VIS and LC/MS were used. Activites of superoxide dismutase, peroxidase and polyphenoloxidase were measured spectrophotometrically, antioxidant activity was measured by Randox kit. Proteins were analyzed by 1D microfluidic system Experion, saccharides by HPLC/RI. Except long-term storage conditions also influence of some other commonly used technological processes were tested (freezing, drying).

Material and Methods

Plant Material

Apples of three cultivars (Idared, Golden Delicious and Jonagored) were harvested and stored under Regular Atmosphere (RA) and controlled atmosphere-Fluctuated ANaerobiosis (FAN) for 158 days at 1 °C.

Total Phenolic, Flavonoid and Antioxidant Capacity Assays

Total solube phenolics were analyzed colometrically with Folin Ciocalteu reagent using photometric detection (750 nm) and results were expressed as mg gallic acid per 1 g of apple.

Total flavonoid content was analyzed colometrically with $NaNO_2 + AlCl_3$ using photometric detection (510 nm). Results were expressed as mg catechin per gram of apple tissue.

Total antioxidant capacity was measured by Randox kit. This colorimetric method use radical $ABTS^{\cdot+}$ (2,2'-Azino-bis(3-ethyl-2,3-dihydroBenzoThiazol-6-Sulphonate))and photometric detection (600 nm). Results were expressed as mmol Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) per liter of raw apple juice.

Individual Antioxidants Assays

Individual flavonoids were analyzed using RP- HPLC method with the extrenal standarts ((–)catechin, catechin gallate, chlorogenic acid, epicatechin, morin, phlorizin, quercetin, rutin). Spectrophotometric detection (UV-VIS) after their extraction was used. In these assays two types of extraction were used:

- water extraction
- extraction by mixture of 1% HCl + ethylacetate

Samples (20 µl) were injected into the RP-18 column (Biospher PSI 200 C18, 7 µm, 150 mm × 4.6 mm). Mobile phases were methanol/water (55 : 45) for water extraction and methanol/acetonitrile/water with 1% phosporic acid (20 : 30 : 50) for organic extration. The flow rate was maintained at 0.75 ml min^{-1}, analysis was performed at 30 °C.

Carotenoids (beta-carotene, lycopene, luteine) were analyzed by RP-HPLC with spectrophotometric detection, organic extraction (acetone + diethylether) was used for pigment isolation. Samples (20 µl) were injected into the RP-18 column (Hypersil C18, 5 µm, 250 mm × 4.6 mm). As mobile phase for isocratic elution methanol was used. The flow rate was 1.1 ml min^{-1}, analysis was done at 45 °C.

Ascorbic Acid Assay

Ascorbic acid was determined using RP-HPLC on Hypersil APS-2, NH_2, 5 µm, 150 mm × 4.6 mm column. Samples were stabilized by 2% HPO_3, 20 µl was injected. Mobile phase was natrium acetate/acetonitrile (95/5). Analysis was performed at flow rate 0.6 ml min^{-1} and 30 °C.

Microtitration method with 2,6-dichlorindofenol was used as comparative method too. The end point of titration was determined by pink colour.

Results were expressed as mg of ascorbic acid per kg of apples.

Surface Microflora Assay

Natural microflora was determined using Evirocheck® kit (Merck). Two types of kit were used:

- Contact TVC – Total Viable Counts
- Contact YM(R) – Yeasts and Mould

These tests can be used for analysis of liquid material as well as for surface testing. Results were expressed as KBE cm^{-2} or cfu cm^{-2}. Artificial injection of *Gloeosporium* and *Penicillium* moulds was done for comparison of effect of surface infection on apple quality. Infected apples were stored in darkness and cold (6 °C). After 4–8 weeks surface changes were observed and surface microscopy of apples was done too.

Sensory Analysis

A group of 21 respondents were enrolled into orientation sensory study. They tested several apple varieties and evaluated basic sensory parameters. Apple variety preferences and apple intake was studied too. The group of respondents was divided into two age-different groups:

- seniors: total 13, age 68.5 ± 7.16; 10 F/3 M,
- juniors: total 8, age 27.13 ± 3.63; 6 F/2 M.

Results

Antioxidant levels in apples are relatively low. Differences were observed according to fruit colour (variety) – high content was found mainly in yellow sorts (Golden Delicious). Freezing led to very low changes of antioxidant levels. Controlled drying caused concentration of all antioxidants including ascorbate.

Apple surface natural microflora contained mostly moulds and yeasts. Artificial infection of long-term stored apples (158 days) was more intensive in apples stored under normal atmosphere than under controlled storage conditions (fluctuated anaerobiosis).

Total phenolic and flavonoid levels differed according to apple sort and colour. A part of phenolics formed by flavonoids was in range of 30–60 %. Ascorbate levels detected in apples were in range 12–51 mg kg^{-1}. In Idared apples about 4× higer content of ascorbate was found when compared with Jonagored apple. Concentrations of individual flavonoids (chlorogenic acid, morin, quercetin, rutin) carotenoids (lycopene, beta-carotene) and catechins ((–)catechin, catechin gallate, epicatechin) differed slighty according to apple sort. These substances exhibited significant contribution to final colour of apple fruits. Their content in apples was relatively low. TAS levels were related to the content of phenolics and flavonoids, no direct correlation was found. Antimutagenicity corresponded to TAS.

Freezing is very suitable procedure for long-term conservation without significant changes of active substance content. After drying under regulated conditions (12 hours, 50 °C) the highest decrease was observed in ascorbate level (62 %), TAS level decreased by 42 %, flavonoids and phenolics content decreased by 40–50 %. Regulated drying has probably no significant negative effect on biological activity of apples with regard to amount of dried fruit intake.

Long-term storage (158 days) led to some increase in levels of total as well as individual antioxidants in all studied sorts. The highest level of all antioxidants was found in Idared variety, which is characteristic by the lowest long-term stability. Levels of most of antioxidants in apples stored under modified atmosphere were also lower than under RA. It seems that great group low molecular weight as well as high molecular weight antioxidants is mobilized in overriped or damaged fruits, the higest response was observed in Idared apples. Simultaneously, this variety was very sensitive to artificial mould infection, fruit damage after artificial infection was in this sort the highest from all studied apple varieties. Thus, some unbalance in apple antioxidant status could be accompanied with increased sensitivity to mould infection.

In stored apples some antioxidant enzymes were analyzed too. The highest activity exhibited catalase (CAT) followed by superoxide dismutase (SOD). Level of polyphenoloxidase (PPO) was very low. Activities of CAT and PPO were lower in apples stored under regular than under modified atmosphere, while SOD activity was higher in apples stored under FAN. In red varieties the antioxidant enzymes exhibited higher values than in the green ones. Mainly CAT and PPO are mobilized on overriped and/or slightly damaged apples.

Sensory analysis results obtained in two gorups of age-different subjects showed that in both groups no significant differences in evaluation taste, aroma, colour and texture in 4 sorts of apples were found. The lowest degree of consumer acceptance was found in Idared apple, while Jonagold was the most prefferd sort from analyzed apples. In general, red apple sorts were better accepted than green sorts. Both group preffered fresh apples than dried fruits. Apple intake in senior group (1 apple daily, on average) was about 2× higher than in junior group.

Conclusions

In general, apples contain a large number of different low-molecular weight as well as enzyme antioxidants, but levels of individual derivatives are relatively low. However, high intake of apples confirmed by questionnaire analysis can lead to cumulative effect. Thus, apples are one of the most important sources of natural antioxidants in Czech population (average intake in population is about 600 g per week).

At present, more than 90 % of produced apples are being stored and the losses range from 5 % to 25 %. Study of modern storage technologies and their influence of fuit quality and consumer acceptability is very important problem.

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P25 CONTENT OF POLYPHENOLS AND ANTIRADICAL ACTIVITY OF BEE POLLEN

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Introduction

Recently, many investigations have been concerned with antioxidant properties of different nutritional products. Bee gathered pollen is regarded as valuable special food and is used also in apitherapy[1]. This beehive product also has several useful pharmacological properties, such as antibiotic, antineoplastic, antidiarrhoeatic and as an antioxidant agent[2]. The antioxidant activity of honeybee-collected pollen has been recognized as a free radical scavenger and as a lipid peroxidation inhibitor[2,3]. This activity has been associated with the phenolic pollen content[2].

The aim of the study was to measure content of polyphenols and the antiradical activity of dried bee pollen.

Materials and Methods

The pollen loads were collected by 20 honey bee colonies (*Apis mellifera*) settled in hives with bottom-fitted pollen traps, from different areas of Slovakia, during the season 2007.

The fresh bee pollen was stored at –18 °C for approximately half of year, with moisture 20 %, until analysed. The dried pollen samples were dried (moisture 9–11 %). The moisture was tested by thermo-gravimetric analyzer. The pollen loads for analysis were taken from the following plant species: *Papaver somniferum* L.*, Brassica napus* subsp. *napus* L., *Helianthus annuus* L.

The pollen samples (10 g) were milled, homogenized and diluted in 100 ml 90% ethanol. The ethanol extracts of pollen were stored at 5 ºC for further analysis.

The modified method by Brand-Williams was used[4,5]. Antiradical activity of various bee pollen samples was determined using the free DPPH$^\bullet$ radical. Absorbance at 515.6 nm was measured at different time intervals using Shimadzu 1601 UV/VIS spectrophotometer (UV-1601, Shimadzu, Tokyo, Japan) until the reaction reached a plateau. The absorbance of the 2,2-diphenyl-1-pikrylhydrazyle radical (DPPH$^\bullet$) without an antioxidant (i.e. the control), was measured first. The percent of inhibition of the DPPH$^\bullet$ radical by the sample was then calculated according to the formula:

$$\% \text{ inhib} = [(A_{C0} - A_{At})/A_{C0}] \cdot 100, \qquad (1)$$

where A_{C0} is the absorbance of the control at t = 0 minute, A_{At} is the absorbance of the antioxidant at time t minutes, % inhib equals percentage of free DPPH$^\bullet$ radicals.

Total polyphenols content was quantified according to the Folin-Ciocalteau spectrophotometric method using tanin as reference standard[6]. Results were expressed as milligrams of tanins equivalent per kilogram of pollen and were presented as the mean of triplicate analyses. All values of antioxidant and antiradical activity are expressed as mean ± standard deviation.

Results and Discussion

The antiradical activity was in the particular samples in range from 48.83 to 86.12 % (average 71.39 ± 16.45 %). Antiradical activity as determined by the DPPH radical scavenging method decreased in the order: *Brassica napus* > *Papaver somniferum* > *Helianthus annuus*. In samples of bee pollen was the polyphenol content in the range from 763.67 to 1377.67 mg kg^{-1} (average 1026.67 ± 258.31 mg kg^{-1}). Antiradical activity increased in the same order than content of polyphenols (Table I). In present investigations, great variability regarding content of polyphenols as well as antiradical activity in 3 pollens was found.

Table I
Antiradical activity and content of polyphenols in pollen

Pollen	DPPH [% of inhibition]	Polyphenols [mg kg^{-1}]
H. annuus	48.43 ± 0.29	763.67 ± 5.56
P. somniferum	79.61 ± 0.45	938.67 ± 3.09
B. napus	86.12 ± 0.48	1377.67 ± 3.68

Almaraz-Abarca et al.[7] reported that antioxidant activities were different for each species and were not clearly associated to the flavonol content in pollen. Pollen from different botanical origin had different antioxidant capacity. The flavonol and phenolic acid composition, rather than the concentration, could be the determinant factor.

Great variability of phenolic contents was observed in the pollen of 12 plant species. Great differences in the radical-scavenging activity (RSA) were observed and were not correlated with the content of phenolic compounds. The pollen species can be divided into three groups: those of high (61–91.3 %), medium (23.5–29.6 %), and low RSA (8.6–16 %). In some of them a very high RSA corresponded to high levels of total phenols, phenylpropanoids and flavonols.[8]

In the present investigations very high and high antiradical activity in the case of *Brassica napus* and *Papaver somniferum*, respectively, were manifested by very high and high content of polyphenols. The above results are partially in agreement with the Leja et al.[8] reports. *Helianthus annuus* pollen integrated to second group with medium RSA reported by Leja et al. (2007). None of investigated pollens from our research is being possible to integrate to third category with the low RSA. High levels of phenolic constituents are often accompanied by high antioxidative capacity of pollen; however, according to reports of Campos et al.[9,10], no direct correlation between flavonoids and radical-scavenging activity was found.

Conclusions

Further studies of the antioxidant properties (include antiradical activity) and the antioxidant components of bee pollen from different botanical origins are required, especially identification and quantification of individual antioxidants contained in pollen.

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P26 REDUCTION POWER, POLYPHENOLS CONTENT AND ANTIMICROBIAL ACTIVITY OF HONEY

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Introduction

Recent views propose honey not only as a health-promoting dietary supplement, but shed light on its antioxidant properties. Honey has been reported to have antifungal activity, but not many species of fungi have been tested[1]. Superficial fungal infections are amongst the most difficult diseases to successfully treat, antibiotics which successfully combat bacterial diseases being largely ineffective against fungi. So a treatment which has both antifungal and antibacterial activities would be most beneficial. Therefore the effectiveness of honey against the dermatophyte species which most frequently cause superficial mycoses (tineas such as ringworm and athletes foot) was investigated[2]. The aim of the study was to measure the reduction power, content of polyphenols and antimicrobial activity of honey.

Materials and Methods

Two honey samples were obtained directly from beekeepers during the 2007 harvest, from different locations across Slovak Republic. The floral origin of the samples was specified by microscopic analyses of pollen grains at Institute of beekeeping in Liptovsky Hradok. Honeys were derived from different plant species namely *Castanea sativa* Mill. and *Brassica napus* subsp. *napus* L. Honey samples were stored at 4 °C in the dark until analysed.

Reduction power was evaluated by the method of Prietto et al.[3]. This method is established on reduction of Mo (VI) to Mo (V) with an effect of reduction parts in the presence of phosphor under formation of green phosphomolybdenum complex. Solution absorbance of reducing sample was measured at 705 nm (UV-1601, Shimadzu, Tokyo, Japan) toward black experiment (distilled water). Reduction power of compounds (RP_{AA}) expressed as quantity of ascorbic acid necessary to achieve the same effect in $\mu g\,ml^{-1}$ was calculated using the equation (1).

Total polyphenols content was quantified according to the Folin-Ciocalteau spectrophotometric method using tanin as reference standard[4].

$$RP_{AA} = (A_{705\,nm} - 0.0011)/0.00236 \qquad (1)$$

The potential antimicrobial activity of selected honey samples against *Alternaria infectoria*, *Scopulariopsis brevicaulis*, *Trichophyton ajelloi* and *Saccharomyces cerevisiae* was studied using the agar well diffusion method. The strains of fungi were maintained on Czapek-Dox agar (CDA, HiMedia). Honey solutions were prepared in three fractions: 50, 25 and 10 % (by mass per volume). Experimental results were expressed as means ± standard deviation. All tests were performed in triplicate.

Results and Discussion

Reduction power of chestnut honey compounds was higher ($4{,}083.67 \pm 4.50\ \mu g\,ml^{-1}$) than of rape honey ($3{,}618.33 \pm 3.30\ \mu g\,ml^{-1}$). Comparison of polyphenols content also refers on higher values in chestnut honey than in rape honey ($65.33 \pm 3.86\ mg\,kg^{-1}$ versus $41.00 \pm 2.16\ mg\,kg^{-1}$). The value of polyphenols for chestnut honey was approximately 1.6-fold higher than that for rape honey.

Bertoncelj et al.[5] reported that total phenolic content (determined by the modified Folin-Ciocalteu method), antioxidant activity and colour parameters differ widely among 7 different Slovenian honey types. In the case of chestnut honey the phenol content was $199.9 \pm 34.1\ mg\,kg^{-1}$, i.e. 3-fold more than in our present study. The results obtained from the study by Beretta et al.[6] showed that the total phenol content in chestnut honey was $211.2\ mg\,kg^{-1}$, i.e. 3.2-fold more than in our present study.

Vela et al.[7] observed that the phenolic compounds are partly responsible for the antioxidant effects of honey, but obviously there are other factors involved.

Table I
Antimicrobial effect of honey samples

	Honey concentration [%]		
	10	25	50
	Castanea sativa Mill.		
A. infectoria	11.46 ± 0.45	15.83 ± 0.99	18.21 ± 0.40
S. brevicaulis	11.70 ± 0.67	14.40 ± 0.99	18.75 ± 0.25
T. ajelloi	14.55 ± 1.12	18.11 ± 0.39	21.75 ± 0.35
S. cerevisiae	8.15 ± 0.18	10.32 ± 0.30	12.84 ± 0.93
	Brassica napus subsp. *napus* L.		
A. infectoria	10.94 ± 1.59	15.77 ± 0.12	17.22 ± 0.35
S. brevicaulis	9.93 ± 0.09	12.36 ± 0.44	17.37 ± 0.43
T. ajelloi	13.77s628 ± 1.01	17.38 ± 0.83	20.99 ± 0.52
S. cerevisiae	7.51 ± 0.47	11.83 ± 0.89	14.48 ± 0.86

Antifungal activities of the two honey samples with different concentration against fungi *Alternaria infectoria*, *Scopulariopsis brevicaulis*, *Trichophyton ajelloi* and *Saccharomyces cerevisiae* strains are presented in Table I. The obtained results characterize honey samples as a product with a broad antimicrobial effect.

A comparative method of adding honey to culture media was used to evaluate the action of starch on the antifungal activity of honey[8]. Brady et al.[9] studied antifungal activity of honey to dermatophytes. The results of this investigation show that the common dermatophytes are sensitive to the antimicrobial activity of honey, indicating that clinical evaluation of honey in the treatment of tineas is warranted.

Conclusions

Phenolic compounds appear to be responsible for the antioxidant activity of honey. Further studies are necessary to clarify the antioxidant effect of honey.

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P27 COMPARISON OF PECTATE HYDROLASES FROM PARSLEY ROOT CELLS

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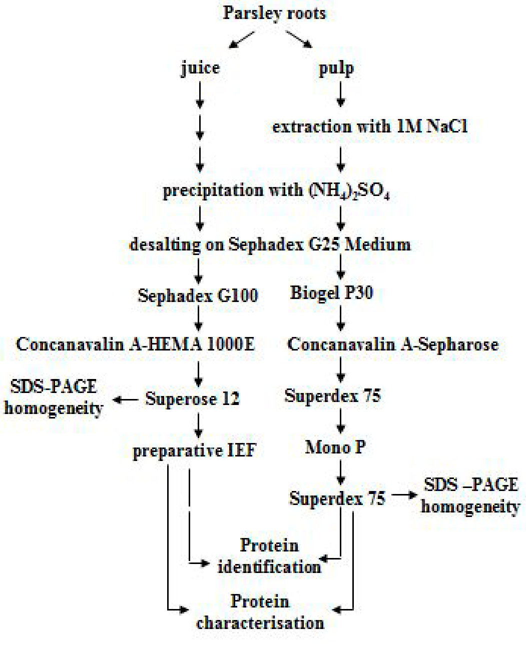
Introduction

Plant pectate hydrolases are in general supposed to be bound on primary cell wall where they cause the homogalacturonan degradation. Polygalacturonases can be classified into two groups depending on action pattern; enzymes randomly cleaving substrate (polygalacturonases, EC 3.2.1.15) and enzymes terminally cleaving substrate (exopolygalacturonases, EC 3.2.1.67)[1]. The biological function, structure as well as gene-expression of polygalacturonases have been studied in detail, while the research of exopolygalacturonases (exoPGs) is still on its beginning. ExoPGs have not yet been fully characterized in terms of developmental roles but could clearly have significant involvement in cell expansion processes. These enzymes are supposed to play a key role in the turnover of biologically active oligogalacturonates as signalling molecules affecting plant growth and development.

Plant exoPGs were supposed to prefer only polymeric substrate and the ability for cleaving substrate with lower degree of polymerization (DP) was strictly attributed to enzymes produced by microorganisms. First description of plant enzyme preferring oligogalacturonates (oligogalacturonate hydrolase, OGH) appeared in 2005 when an enzyme from carrot roots was described[2].



Scheme 1

Experimental

Purification of Exopectate Hydrolases

In this work, exopectate hydrolases were isolated from parsley root juice and pulp extracts. The pulp protein mixture presents a very heterogeneous material what requires more complicated purification process. Accordingly, two different purification pathways were developed using different separation methods involving gel-permeation, affinity chromatography, chromatofocusing as well as preparative IEF (Scheme 1.).

Characterization of Exopectate Hydrolases

Activities of pectate hydrolases, utilizing substrates with various DP and different pHs, were determined by Somogyi assay[3]. The zymogram technique after IEF[4] with a colourless D-galacturonan DP 10 followed by staining of non cleaved substrate with ruthenim red was used for determination of pI. Molecular mass analysis of native and deglycosylated enzymes was done using SDS-PAGE with silver or Commasie Blue staining method for band visualization. Presence of

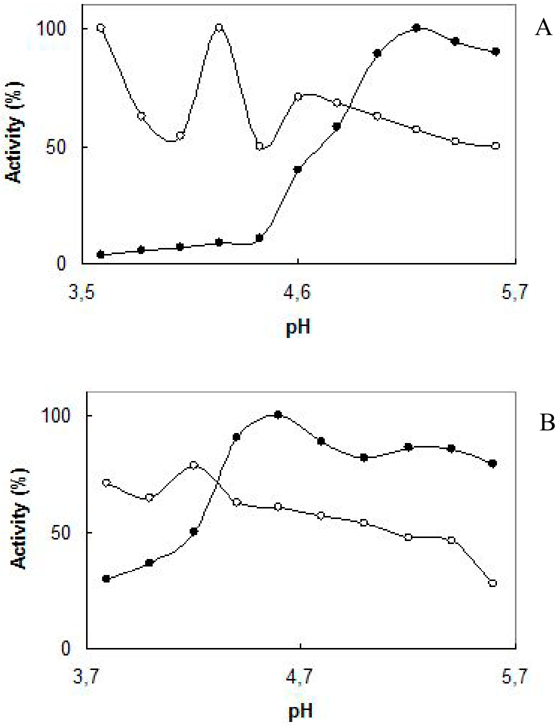


Fig. 1. **The pH optima of pectate hydrolases in parsley roots; enzyme activity on: • – 0,5% sodium pectate, ○ – 1 μmol ml^{-1} pentagalacturonate, A – enzymes from parsley juice, B – enzymes from parsley pulp**

these enzyme forms in individual cell structures was determined using the differential centrifugation method[5].

Results

Only pectate hydrolases with terminal action pattern on substrate were found in parsley roots cells[6,7]. ExoPG and two types of OGH were isolated, partially purified, further characterized and in-between compared. The comparison of their molecular masses, isoelectric points (Table I), temperature optima, thermal stability and also action pattern showed very similar results. On the other hand there were observed differences in their pH optima (Fig. 1., Table I) and substrate specificity in respect to DP of substrate (Fig. 2., Table I). In addition, the individual enzyme forms occurred in different cell structures as is partially shown (Fig. 3.).

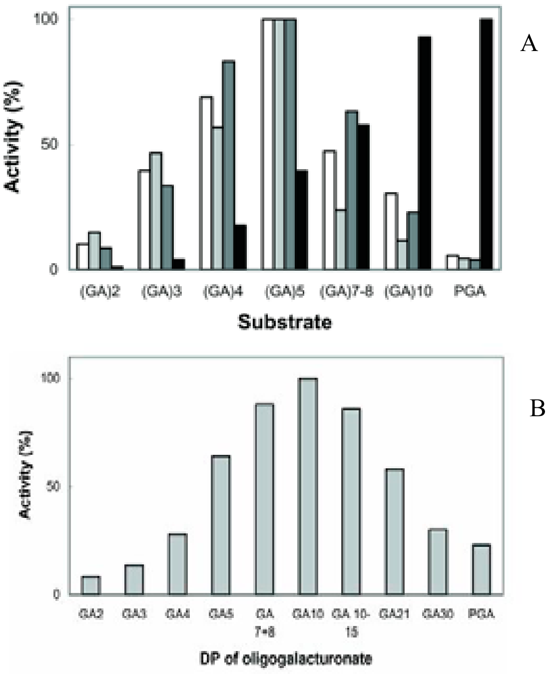


Fig. 2. **The initial rates of pectate hydrolases from parsley roots on substrates with various DP: A – enzymes isolated from parsley juice with pH optimum 3.6 – □, 4.2 – ▒, 4.6 – ▓ and 5.2 – ■, B – enzyme found in parsley pulp with pH optimum 4.7**

Table I
Characterization of pectate hydrolases from parsley root cells

Enzyme	pH optimum	DP of preferred substrate	M_r	pI	Occurrence
OGH6a	3.6	6	55.3	5.45	organelles
OGH6b	4.2	6	55.3	5.35	plasts, walls
OGH6c	4.6	6	55.3	5.60	intracel. comp.
OGH10	4.7	10	53.5	5.30	wall
exoPG	5.2	PGA	55.3	5.55	wall, ER

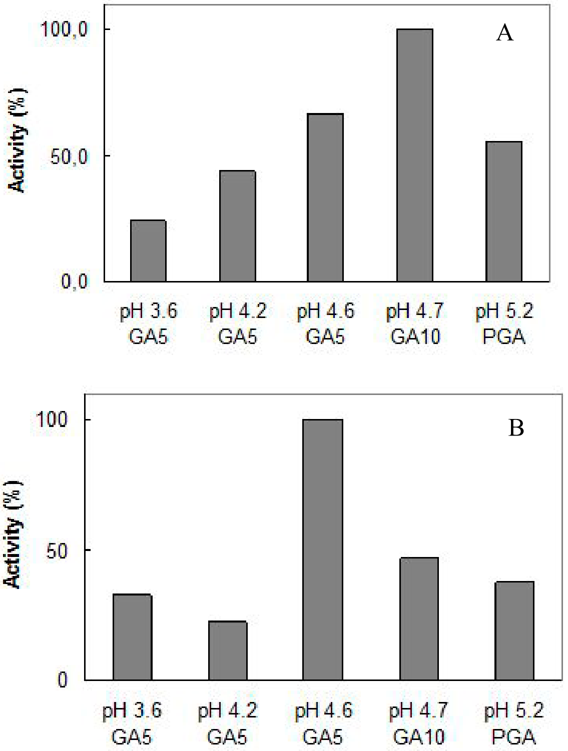


Fig. 3. **Abundance of individual exopectate hydrolase activities in different cell component: A – cell wall, B – cytosol**

ExoPG preferring the polymeric substrate has its pH optimum at 5.2. In contrast, the other types of pectate hydrolases, OGHs, favour oligomeric substrates, hexagalacturonate (OGH6) and decagalacturonate (OGH10) respectively. OGH10 has its pH optimum at 4.7.

OGH6 includes three isoforms with pH optima at 3.6, 4.2 and 4.6 (Table I). All forms showed temperature optima between 60–70 °C and were 100% stable at 55 °C (all unbound forms) and 50 °C (OGH10) respectively.

Conclusions

Five forms of pectate hydrolases purified from parsley root cells were described[6,7]. The main difference between these enzymes is mainly the substrate preference in relation to the chain length. It is accompanied by the sharp decrease of pH optima with decrease in DP of preferred substrate. The relationship between pH decrease in primary cell wall during auxin activation of proton pump bound on plasmatic membrane[8] and DP decrease in linear parts of pectin molecule side chains[9] regulated by pectate hydrolases with terminal action pattern can be indicated.

One enzyme only (OGH10), with preference to decameric substrate, was strictly bound to the cell wall. Next three forms, with preference to hexagalacturonate, were found in other cell structures. Only one typical form of exoPG with preference to polymeric substrate was identified; partially bound to the cell wall and partially free in the cytosol.

The OGH10 enzyme with unique substrate preference to decamer could also be important in determining the half-live of oligogalacturonic acids in the infected plant tissue. It could be the endogenous signals regulating aspect of programmed developmental events or responses to "invanders". Therefore, this enzyme and other pectate hydrolases may be of importance to the plant from several perspectives.

This research was supported by grants 2/6133/26, II/2/2005 and MSM 021630501

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P28 REACTION MECHANISM OF XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET) FROM *PETROSELINUM CRISPUM* ROOTS

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Introduction

Plant xyloglucan endotransglycosylases (XETs, EC 2.4.1.207) catalyze the random cleavage of β-1,4-polyglucose backbone of the donor molecule (xyloglucan, XG) and the transfer of part of XG carrying the newly created reducing end to hydroxyl group at C-4 on the non-reducing end of another XG molecule or XG-derived oligosaccharide (acceptors). The process of transglycosylation can be described by Bi Bi reaction mechanism. Generally, there are two possible models of Bi Bi reaction mechanism: Pin-Pong and Sequential. In the Ping-Pong mechanism, the cleavage and transfer of part of polyglycan chain to the acceptor run in two steps. The first product (represented by the part of polyglycan chain carrying the original reducing end) is released from the stable enzyme-substrate complex before the acceptor substrate can bind. In contrast to the Ping-Pong mechanism, the sequential mechanism is observed like one step reaction, where a ternary complex of enzyme with the both substrates is formed. The products are released at the same time.

The main XET form of parsley roots with the isoelectric point 4.6 has a broad pH optimum in the region of its stability (pH 4.5–9.0) with one maximum in acidic (pH 5.8) and the second one in alkalic (pH 8.8) region[1]. The kinetic analysis at these two pH optima was performed using radioactive alditols of XG octasaccharide (XLXGol + XXLGol) as an acceptor substrate as well as in dependence on the degree of polymerization (DP) of reducing xyloglucan oligosaccharides (XGOs). XGOs with DP 7, 8 and 9 were used.

The mechanism of BiBi reaction was suggested from the nonlinear regression of kinetic data.

Experimental

Extraction of XTH from Parsley Roots

XET from parsley roots (*Petroselinum crispum* cv. Olomoucká dlouhá) was isolated and partially purified as described previously[1].

Substrates

Tamarind seed xyloglucan used in this study was from Dainippon Pharmaceutical Co., Ltd, Osaka, Japan.

XGOs with DP 7–9 were prepared by digestion of tamarind xyloglucan with *Trichoderma* cellulase[2]. They were purified on Biogel P2 column and further fractionated by preparative HPLC on TSK Gel Amide column (Tosoh) as described[3]. XG octasaccharide was converted to the corresponding 1-deoxy-1-aminoalditols (glycamines) by reductive amination[4]. Radioactive alditol of XG octasaccharide or reducing XGOs with DP 7, 8 and 9 were prepared by their reduction with Na-borotritide [^{3}H]NaBH$_4$ (ICN Radiochemicals, San Diego, CA) as described earlier[5].

Kinetic Parameters

Standard XET assays were performed at 29 °C utilizing the radiometric method according to Fry et al. The initial rates were determined at pH 5.8 and 8.8 using five different concentrations of xyloglucan (0.5–5 g dm^{-3} at pH 5.8 and 0.5–1.5 g dm^{-3} at pH 8.8, respectively) and of radioactive alditols of XG octasaccharide (0.6–3.1 μM). Further experiments were all performed at pH 5.8 using the same XG concentration range as described previously for this pH. Reducing XGOs with DP 7, 8 and 9 were used in the range of 5–200 μM. The incorporation of each [1–^{3}H]-labelled XGOs was measured by scintillation counting (Liquid Scintillation Analyzer Tri-Carb 2800TR, PerkinElmer, Illinois, USA) as described previously (Sulová et al., 1995). The K$_M$ values were calculated from nonlinear regression using Origin 6.0.

Results

XET as a member of glycoside hydrolase family GH16 utilize a double displacement/retaining mechanism of transfer. This mechanism involves the formation of a covalent enzyme-substrate intermediate[6], what is characteristic for

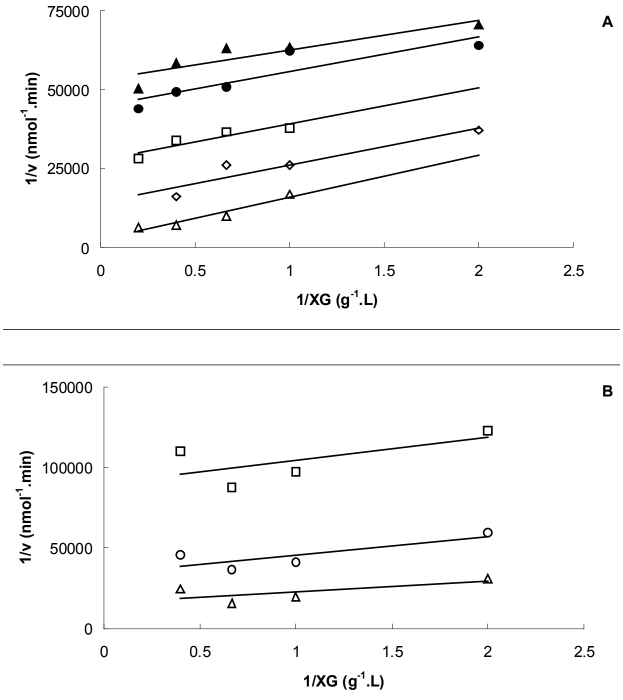

Fig. 1. **Plot of 1/v = f(1/XG) at different concentrations of mixture of XLXGol + XXLGol: 0.86 μM (▲), 1 μM (●), 1.24 μM (□), 1.54 μM (◊), 2.1 μM (○) and 3.1 μM (Δ). Michaelis parameters were determined at pH 5.8 (A) and 8.8 (B), respectively**

retaining glycanase operating by Ping-Pong mechanism. This is supported by the most of works published to this date[3,7,8]. On the other hand, the kinetic data of XET isolated from suspension-culture poplar cells are consistent with the sequential mechanism[9].

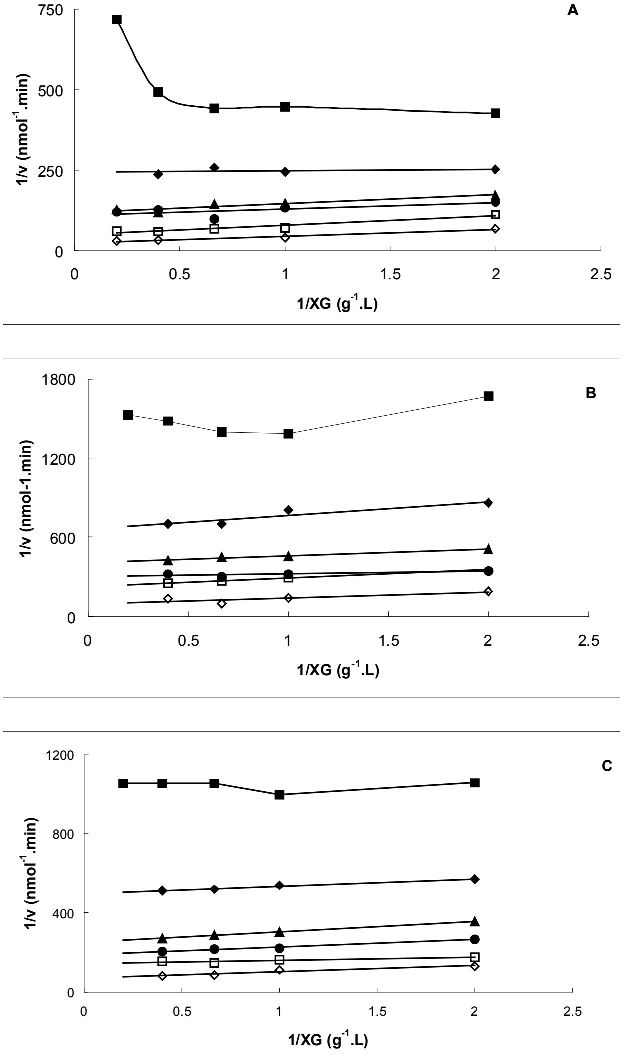

Fig. 2. **Plot of 1/v = f(1/XG) at different concentrations of nonasaccharide (A), octasaccharide (B) and heptasaccharide (C): 5 μM (■), 10 μM (♦), 20 μM (▲), 30 μM (●), 50 μM (□) and 200 μM (◊)**

Kinetic analysis was performed at both pH optima of XET (Figure 1 A, B) using radioactive alditols of XG octasaccharide (XLXGol + XXLGol).

While K_M values for mixture of XLXGol + XXLGol were very similar at both pH optima (123 μM at pH 5.8 and 137 μM at pH 8.8, respectively) for XG they differed (0.565 g dm^{-3} at pH 5.8 and 2.42 g dm^{-3} at pH 8.8, respectively). A strong inhibition with higher concentrations of XG was observed especially at pH 8.8 where the linearity of reaction in dependence on XG concentration was limited. From this reason the further study of kinetic parameters was carried out only at acidic pH using reducing XGOs.

Table I
Kinetic parameters calculated from nonlinear regression

Acceptor substrate	Parameter values ± S.D. K_{MXG} [g dm^{-3}]	K_{MXGO} [μmol dm^{-3}]
nonasaccharide	0.854 ± 0.21	42.199 ± 6.546
octasaccharide	0.589 ± 0.22	92.396 ± 10.120
heptasaccharide	0.755 ± 0.08	117.612 ± 5.588

The Lineweaver-Burk plots for XET showed parallel lines at higher concentrations of reducing XGOs with DP 7, 8 and 9 as acceptor substrates (Fig. 2. A, B, C).

The kinetic parameters K_M were determined by measuring initial transfer rates of reducing XGOs with DP 7, 8 and 9 into XG (Table I). The data concluded in Table I show that the K_M values for acceptor substrates increase with their decreasing DP. On the other hand the lowest K_M value for donor substrate was calculated for octasaccharide as an acceptor.

Conclusions

The kinetic study analyzed at both pH optima of XET (pH 5.8 and 8.8, respectively) indicate that the affinity of enzyme to acceptor substrate (mixture of XLXGol + XXLGol) is independent on pH unlike the K_M values for XG, where significant differences can be seen.

The relationship between the K_M values and DP of acceptor substrates shows the decrease of enzyme affinity to reducing XGOs with their decreasing DP. As a consequence, the best acceptor substrate seems to be the nonasaccharide.

The comparison of K_M values for octasaccharides indicates that parsley XET has a higher affinity for reducing oligosaccharide than to its alditol.

In all cases excepting the kinetic analysis using low concentrations of XGOs, the Lineweaver-Burk plots for XET showed parallel lines. These results indicated that the enzyme catalyzed the reaction utilizing a Ping-Pong (Bi Bi) mechanism rather than a sequential one. Hence, the non-parallel lines at lower concentrations of XGOs can be explained by the stronger influence of side reactions like interpolymeric transglycosylation and inhibitions at such concentrations of acceptor substrate.

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P29 MULTIELEMENTAL PROFILING AS FINGERPRINT OF WINES BY SIZE-EXCLUSION COUPLED TO UV AND ICP-MS

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Introduction

A number of studies describes the levels of metals in wines[1,2] and organic compounds with high complexation capacity with them like anthocyanins[3], phenolic compounds[4,5], and so on. Correlation of metals and organic compound has been pointed out but it can not be probed since the measurements were performed off-line. Only in the case of Pb, the size exclusion chromatography coupled to an inductively coupled plasma mass spectrometry (SEC-ICP-MS) was used to determine biomolecular complexes[6]. However, the multielemental profiling of wines by SEC-ICP-MS has not been performed until now. This approach has been successfully applied to the fractionation of several elements in nuts[7,8], soybean flour[9], and organs from *Mus musculus*[10]. Multielemental fractionation studies have also been performed by SEC-ICP-MS in premature human[11,12] and whey milk[13]. The aim of this work is to determine the metal-binding molecules profiles in red and white wines from different grape varieties as a preliminary step to find key compounds that can be used as fingerprint as well as to know all the metallospecies present in wines. The analytical methodology to obtain the distribution patterns of these elements was based on SEC on-line coupled to UV and ICP-MS.

Experimental

Samples

Samples of red and white wines were purchased in a local supermarket. All the samples were stored al 4 °C until analysis. Table I shows the list of wines analyzed and the assigned codes.

Procedures

The SEC was carried out in a 26/70 XK column packed with Sephadex LH-20 (hydroxypropylated dextran beads crosslinked to yield a polysaccharide network) all from Amersham Biosciences (Uppsala, Sweden). An AKTA-Prime system (pump and UV detector at 254 nm) (Amersham) was used as the eluent delivery system, equipped with a 200 µl sample loop. ICP-MS Agilent, Model 7500 ce (Agilent Technologies, Tokyo, Japan). The instrumental operating conditions are given in Table II. Samples were two-fold diluted with mobile phase and 200 µl was injected in the SEC-UV-ICP-MS system.The mobile phase for SEC was daily prepared with a pH 4 methanol-buffer (50 : 50, v/v) solution.

Table I
Analysed samples

Sample	Origin	Grape Variety	Comments
1	NAVARRA	Tempranillo, Garnacha	Red
2	NAVARRA	Not determined	Red
3	JUMILLA	Monastrell	Red
4	NAVARRA	Garnacha	Red
5	RIOJA	Tempranillo, Garnacha, Graciano	Red
6	JUMILLA	Monastrell, Tempranillo	Red
7	PENEDÉS	Tempranillo, Garnacha, Carieña	Red
8	RIOJA	Tempranillo, Garnacha	Red
9	VALDEPEÑAS	Tempranillo	Rosé
10	CAMPO DE BORJA	Tempranillo, Garnacha	Red
11	PENEDÉS	Garnacha, Cariñena	Red
12	LA MANCHA	Tempranillo	Red
13	RIBERA DEL DUERO	Not determined	Red
14	JUMILLA	Monastrell	Red
15	RIOJA	Tempranillo, Garnacha	Red
16	CATALUÑA	Macabeo	White
17	CONDADO DE HUELVA	Zalema	White
18	CONDADO DE HUELVA	Zalema, Palomino	White
19	PENEDÉS	Chardonnay, Parellada	White

Table II
Instrumental operating conditions for SEC-UV-ICP-MS

SEC-UV	
Column	Sephadex LH-20
Exclusión limit	5,000 Da
Mobile phase	Methanol-water (pH 4.0)
Flow rate	2 ml min^{-1}
Injection volumen	200 µl
UV-visible wavelength	254 nm
ICP-MS	
Forward power	1,500 W
Carrier gas flow rate	0.8 dm^3 min^{-1}
Make up gas	0.1 dm^3 min^{-1}
S/C temperature	–2 °C
Sampling depth	8 mm
Sampling and skimmer cones	Pt
Dwell Time	0.1 s per isotope
Isotopes monitored	^{55}Mn, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{208}Pb, ^{53}Cr, ^{57}Fe, ^{59}Co, ^{82}Se, ^{111}Cd

Results

The fractionation profiles of the elements in wines are shown in Figs. 1.–4. Only the most interesting profiles are discussed in this paper.

Manganese Fractionation Profiles

Fig. 1 shows the chromatographic profiles of ^{55}Mn in the studied samples. The typical molecular mass distribution pattern of this element is the presence of only one highly abundant fraction between 2,126–7,000 Da. This fact indicates that manganese in wines is mainly associated with polysaccharides, peptides, proanthocyanidins or low molecular mass proteins. Condensed tannins and anthocyanins are the most important metal ligands, since these species have numerous coordination sites capable of binding metal cations. In addition, the molecular mass indicates that the organic compound is not an anthocyanin. It has been reported that cyanidin-3-O-glucoside anthocyanin with two –OH groups in the ortho position can complex Mn at the ratio 2 : 1 ref.[4], but the chromatograms shows the absence of Mn in the retention time of this anthocyanin or the abundance is too low.

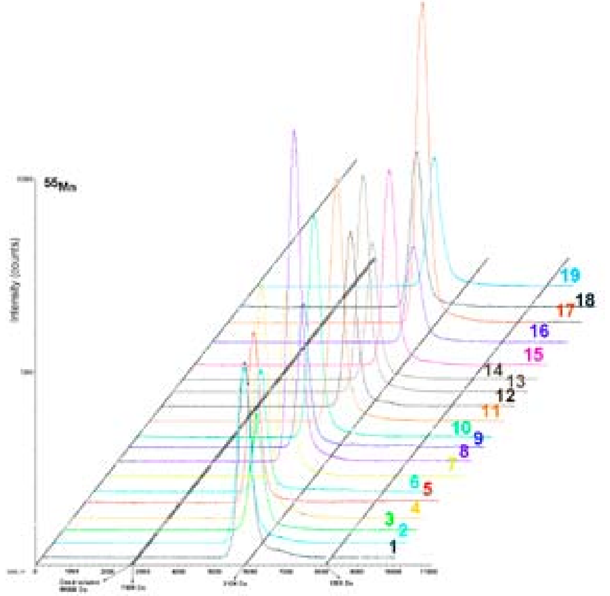

Fig. 1. **Molecular mass distribution of manganese species in wines**

Lead Fractionation Profiles

Fig. 2. shows the superimposed chromatographic profiles of ^{208}Pb in wines. Lead is mainly bound with high molecular mass compounds as can be concluded from the co-elution with Metallothionein I (7,000 Da). All the samples present around the same abundance of this Pb-containg fraction. It is remarkable the presence of two peaks in this molecular mass region in sample 5. Sample 10 is the only one that presents a Pb-containing fraction of about 2,126 Da. In several samples[1,4,11,17,18], a low abundant fraction with a molecular mass from 1,325 to 2,126 Da, have also been detected. Experiments with untreated wine passed through a minicolumn packed with polyurethane foam modified by 2-(2-benzothiazo-lylazo)-p-cresol indicated that Pb(II) is strongly associated to other constituents, possibly bound with pectic polysaccharides and/or other related high-molecular-weight natural organic species[14]. As previously reported[6], lead in wine can be bound with a structurally complex pectic polysaccharide (rhamnogalacturonan II-RG II) that present the ability to form dimmers cross-linked by 1 : 2 borate diol esters (dRG II). RG-II is a major polysaccharide of wine and is mainly present as a dimmer, although the monomeric form can also be detected. The molecular mass of dRG II is about 10 kDa, so the presence of B and Pb in this region is a marker of the presence of the complex. For this reason 11B was monitored together with ^{208}Pb and they were detected in all the samples in the fraction of about 7,000 Da. Fig. 3 shows the presence of both elements in the most abundant fraction of lead in wines (about 7,000 Da) in sample 1.

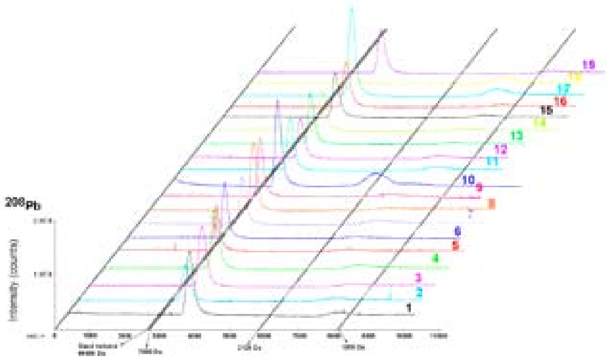

Fig. 2. **Molecular mass distribution of lead species in wines**

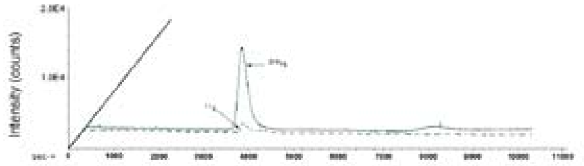

Fig. 3. **Coelution of Pb and B in sample 1**

Arsenic Fractionation Profiles

The molecular mass fractionation profiles of arsenic (Fig. 4.) are very different to those previously considered for other elements. Although the abundance of As-containing peaks is low and not all the samples present this element above the detection limits, we can find that arsenic is present in two fractions, one of low molecular mass (526–1,355 Da) and other of about 2,126 Da. It has been reported (15) that the inorganic arsenic, As (III) (arsenite) is the major arsenic specie in wines but the organic species such as dimethylarsinic acid (DMAA) and monomethylarsonic acid (MMAA), are under the detection limits of hidride generation-atomic spectroscopy fluorescence (HG-AFS). Other papers report that, in most of wines, DMAA is the most abundant specie, but the total inorganic aresenic fraction is considerable[16]. Some arsenosugars have also been determined in wines with a molecular mass from 326 to 478 Da[17]. However, the association of As in wines with an organic compound of high molecular mass (2,126 Da) have not been reported until now.

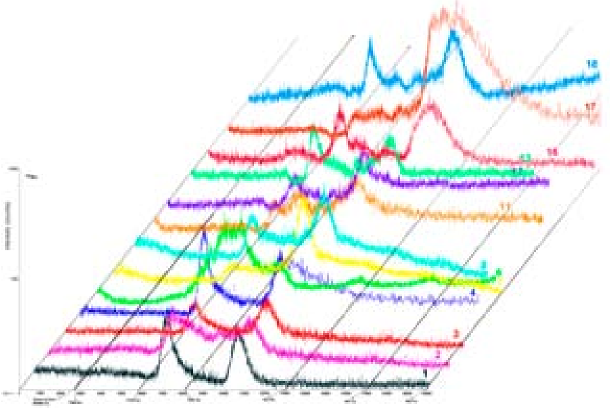

Fig. 4. **Molecular mass distribution of arsenic species in wines**

Conclusions

The identification of chemical forms of elements in wine enables their toxicity and bioavailability to be estimated. The use of ICP-MS allows the multielement speciation in wines in only one chromatographic run, which constitutes a reliable technique with high throughput. The possibility to screen many elements in a given chromatographic peak from a sample is critical since there are no errors associated with different measurements. On the other hand, food samples are complex and the selectivity of the ICP-MS avoids the tedious sample preparation. Therefore, ICP-MS is an excellent guide for further studies of the fraction of interest like reversed phase coupled to ICP-MS, refractive index and UV detectors. The simultaneous determination of elements, carbohydrates, polysaccharides and polyphenols will be allowed. Finally, complexes will be identified by organic mass spectrometry.

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P30 SOLATION OF Cu-METALLOPROTEINS IN *MUS MUSCULUS* BRAIN EXTRACTS BY REVERSE PHASE-HPLC COUPLED TO ICP-MS

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Introduction

Orthogonal chromatography systems coupled to ICP-MS and organic mass spectrometry has been widely used for metal-biomolecules characterization in biological samples connected with in environmental issues. The use of these analytical approaches provides very interesting information to deep insight on toxicological concern of metals in the environment. The use of heteroatoms can be used as markers that simplify the traditional proteomic approaches[1,2].

In a previous study, several organs of *Mus musculus* mice were extracted and analyzed by size exclusion chromatography coupled to UV and inductively coupled plasma mass spectrometry (SEC-UV-ICP-MS)[3]. Some differences were found in the molecular mass distribution patterns of elements in the studied organs as in the case of a Cu-containing fraction that was only present in the brain. This fraction was collected, lyophilized and separated by reversed-phase (RP) high performance liquid chromatography (HPLC) following the Cu with the ICP-MS.

2D-PAGE study of the extracts was performed in parallel, for the identification of overall proteins, after tryptic digestion of the spots. Comparison of metallomics and proteomics results was performed and correlated with *Mus musculus* genome information from data base.

Experimental

Standard Solutions and Reagents

Methanol (Teknokroma, Barcelona, Spain) was of LC gradient grade. Double de-ionized water (18.2 MΩ CM) obtained from a Milli-Q water system (Millipore, Bedford, MA, USA) was used throughout. All the reagents used were of the highest available purity.

The buffer solution was prepared by dissolving 100 mM of ammonium acetate (Merck, Darmstadt, Germany) in water containing 1 % (v/v) methanol (buffer A) and in methanol (buffer B), adjusting the pH to 7.4. Suprapur acetic acid (100% m/m) and ammonia (25% m/m) used for pH adjustment of the mobile phases were purcharsed from Merck[4].

Instrumentation

HPLC was performed with an Agilent 1100 Series (Waldbronn, Germany). Reversed-phase HPLC was performed with a Spherisorb ODS 2 column (250 mm × 4.6 mm, 5 µm particle size: type PEEK) (Waters, Massachusetts, USA). The reversed-phase HPLC column was directly connected to the nebulizer of the ICP-MS instrument via PEEK tubing.

Elemental detection was performed using a model Agilent 7500ce ICP-MS instrument (Waldbronn, Germany)

Table I
Instrumental conditions for (RP-HPLC) and (ICP-MS)
Instrumental operating conditions

Reverse phase-HPLC conditions	
Columns Spherisorb ODS 2 (250 mm × 4.6 mm, 5 µm)	
Mobile phase Concentration gradient methanol (pH 7.4)	
A: 100 mM NH_4Ac in 1% methanol	
B: 100 mM NH_4Ac in methanol	
Time [min]	Buffer [%]
0	0
5	30
15	30
25	0
Flow rate	0.4 ml min^{-1}
Injection volume	50 µl
ICP-MS conditions	
Forward power	1,500 W
Plasma gas flow rate	15.0 dm^{-3} min^{-1}
Auxiliary gas flow rate	1.00 dm^{-3} min^{-1}
Carrier gas flow rate	0.86 dm^{-3} min^{-1}
Sampling depth	6.5 mm
Sampling and skimmer cones	Platinum
Dwell time	0.3 s per isotope
Isotopes monitored	^{63}Cu

Procedures

Animals and sample preparation

Mus musculus (inbred BALB/c strain) mice were from Charles River Laboratory (Spain). Mice of 7 weeks of age were fed ad libitum with feed conventional pellets. This feed contained 11.9 % moisture, 16.1 % crude protein, 3.1 % crude oil, 60 % N-free extract (including starch, sugars, crude fibre, etc.) and 5.1 % total minerals. Cu concentrations included in the feed were as follows (the limits recommended for this element is indicated in parentheses): 17 mg kg^{-1} Cu (10–35 mg kg^{-1}).

Mice were individually killed by cervical dislocation and dissected. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% NaCl solution, frozen in liquid N_2 and stored at –80 °C until they were used for extract preparation. Mice were handled according to the norms stipulated by the European Community. The investigation was performed after approval by the Ethical Committee of the University of Córdoba (Spain). Entire organs of each type (lungs, livers, spleens, kidneys, brains, testicles, hearts and muscles) from 20 different animals were pooled. The weight of the brain in the pool was 3.265 g. After that a solution (3 ml g^{-1}) was added containing the following: 50 mM Tris-HCl buffer solution at pH 8, 1 mM DTT, 1 mM PMSF and protease inhibitors (100 µl ml^{-1}). Later, benzonase was added (500 U ml^{-1})

to the extracts, they were incubated for 30 min at room temperature and, finally, were centrifuged at 80,000 rpm for 1 h. Extracts were stored at –80 °C until analysis.

Results

The profiles of different elements, such as Cu, Pb, Zn, Ni, As, Mn were obtained with two SEC: High molecular mass (HMM) for a separation range of 3–70 kDa and low molecular mass (LMM) for a separation range below 10 kDa ref.[3].

This work focuses the profile of copper from different organs with the LMM column. In this figure, a differential peak between 2,126 to 7,000 Da was observed in the brain extract.

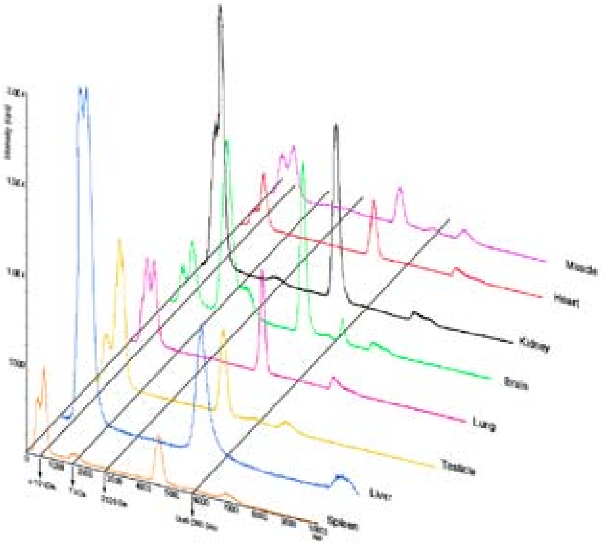

Fig. 1. **Molecular size distribution patterns of Cu obtained by SEC-ICP-MS with the LMW column**

For this reason, the copper fraction from brain was isolated for a preliminary study and was used a second chromatographic separation to purify the fraction previously mentioned by using (RP)-HPLC and ICP-MS.

In Fig. 2., we can observe the two peaks obtained.

The mass balance corresponding to both chromatography separation of this element was performed. A good recovery is obtained which de notes an acceptable isolation of the Cu molecules. For SEC the recovery of cooper from the total injected was 44.40 % and for (RP)-HPLC 76.5 %. The first peak contains 44.43 % of cooper from the total recovery for (RP)-HPLC and the second one 32.07 %. The total concentrations of cooper have been reported elsewhere[3].

Conclusions

Previous works report the molecular size distribution patterns of elements in *Mus musculus* mice. In spite of Mus *musculus* is well known since a great number of proteomic and genomic studies have been performed (i.e., the genome is completely known), the metallome has not been reported until now. The most interesting fraction is that from Cu in brain extract which is not present in any other organ. This fraction has been latterly purified by RP-HPLC-ICP-MS. The pure extracts from RP-HPLC present only two peaks that can be related to Cu-biomolecules. Future works will be focused in the identification of these copper-compounds by organic mass spectrometry.

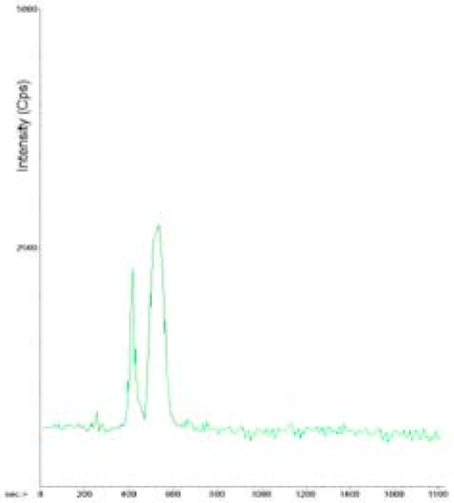

Fig. 2. **Elution profiles of Cu by (RP)-HPCL-ICPMS from the brain extract**

The ICP-MS constitutes a powerful technique to obtain the patterns of many elements in complex biological tissues and it is an excellent guide for further studies of the fraction of interest like further separations by orthogonal techniques, molecular mass-spectrometric elucidations of the metallo-species involved and so on.

This work has been supported by the projects CTM2006-08960-C01/02 and BFU2005-02896 from the Ministerio de Educación y Ciencia and by projects FQM-348 and RNM-523 from the Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía). M.G.-F. thanks the Ministerio de Educación y Ciencia for a predoctoral scholarship.

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P31 ANALYSIS OF FLAVOR COMPOUNDS IN FRUITS BY GC WITH TWO DIMENSIONAL DETECTION BY FID AND MS

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Introduction

The fruit juice industry has become one of the world´s major agricultural businesses with world trade in fruit juices annually exceeding 10 billion $[1]. A key characteristic of this product is the flavour.[2–7] Sweet orange (*Citrus sinensis*) typical aroma is attributed to alcohols, hydrocarbons, esters and aldehydes considering solely the number of compounds involved. Among these compounds, citral, limonene, linalool, α-pinene, ethyl butanoate, acetaldehyde and octanal have been identified as most contributing to orange flavour, and can be used in orange juice authentication. In addition, some orange juice off-flavours can be developed such as α-terpineol that is a well known off-flavour compound present in stored citrus products formed from d-limonene or linalool[8].

In this work, a method based on liquid-liquid extraction with ethyl acetate and later preconcentration has been optimised for the extraction of about twenty flavour compounds in orange juice. The method is complemented by a chromatographic separation (GC) for the identification of different compounds based on the retention times obtained by gas chromatography.[4–9] with flame ionization detector (GC-FID) and the mass spectra of each analyte (GC-MS). Quantification data was obtained with both detectors. The levels of the flavour compounds have been used to enhance the quality of oranges for industrial juice production and authentication purposes.

Experimental

Materials

The gas chromatograph used for this study is an Agilent 6890N.

Chromatographic parameters:

- Column: HP-5MS. 30 m in length 0.25 mm ID. 0.25 μm film.
- Stationary phase (5% phenyl)-methylpolysiloxane
- Injector Temperature: 250 °C
- Oven Programme (Table I):

Table I
Oven program for the chromatographic separation

Temperature [°C]	Rate [°C min^{-1}]	Hold [min]	Total [min]
50		5	5
225	3	10	73.33

- Detector temperature: 300 °C.
- Column flow rate: 1 ml min^{-1}
- Carrier gas: Helium
- Injection volume: 5 μl

Analytical Methods

Sample preparation

An aliquot of 30 ml of juice, previously tempered and homogenized by manual or mechanical shaking, was measured using a test tube of 50 ml Class A and transferred using a funnel tapered to a 100 ml separating funnel, provided with a Teflon key.

After that, 20 ml of juice was extracted with a mixture of ethyl ether: methanol: ethyl acetate (18.5 : 1 : 0.5) and centrifuged to 5 °C and 4000 rpm for 5 minutes. Supernatant was withdrawn and passed to a 100 ml bottle with screw cap. The juice was extracted again with 20 ml of pentane and centrifuge to 5 °C and 4,000 rpm for 5 minutes. Supernatant was withdrawn and passed to 100 ml container together with the previous organic phase.

The third extraction was carried out with 20 ml of dichloromethane: methanol (19 : 1) mixture and centrifuged to 5 °C and 4,000 rpm for 5 minutes. The contents of the centrifuge tube were transferred carefully to a 100 ml separating conical funnel. After few minutes the organic phase (bottom) was separated in a vial. Anhidrous sodium sulphate was added and the content was stirred and centrifuged under the same conditions above described.

The total of all three phases was passed to a functional round-bottoned flask for evaporation with a rotary evaporator, keeping the bath temperature at 70 °C to eliminate the most volatile solvents (all of them with boiling point lower than 70 °C except ethyl acetate). The extract was transferred to an eppendorf tube and the internal standard (α-ionone) was added to achieve a final concentration 50 mg dm^{-3}. The extract was concentrated to a final volume of 1 ml.

Chromatographic analysis

5 μl of the extract with the internal standard were injected in the gas chromatograph with a FID detector. For the inequi-

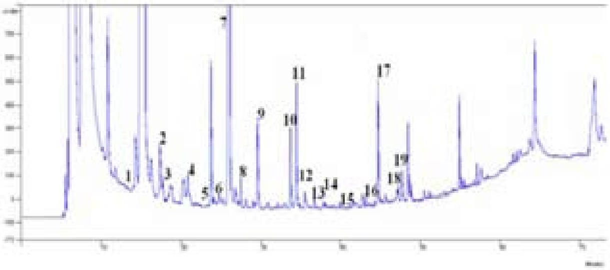

Fig. 1. **Chromatogram of a sample of orange juice using GC-FID: 1. Hexanal + ethyl butyrate, 2. Cis-3-hexen-1-ol, 3. Hexanol, 4. α-Pinene, 5. (-) β-Pinene, 6. Ehyll hexanoate, 7. Limonene, 8. γ-Terpinene, 9. L-linalool, 10. (+)Terpinen-4-ol, 11. S-α-terpineol, 12. Decanal, 13. Neral, 14. Carvone, 15. Geranial, 16. Dodecanal, 17. α-ionone (internal standard), 18. β-ionone, 19. Valencene**

vocal identification of some compounds in the sample, the mass spectrometer detector was used.

Results

Calibration and Detection Limits

Fig. 1 shows the typical chromatogram of a sample of orange juice. As we can see, 19 compounds responsible of the flavour were identified depending on their retention times using standards.

Presence of Volatile Compounds in Juices Studied

Fig. 2 shows the concentration of minority compouns present in the analysed oranges juices.

Fig. 3 shows the major flavour compounds found in the analysed samples. As we can see, the concentration of limonene is very high (with concentrations up to 72 ppm) and lower concentrations of S-α-Terpineol, a compound that comes from the degradation of limonene and is responsible of unpleasant odors at concentrations greater than 2 ppm.

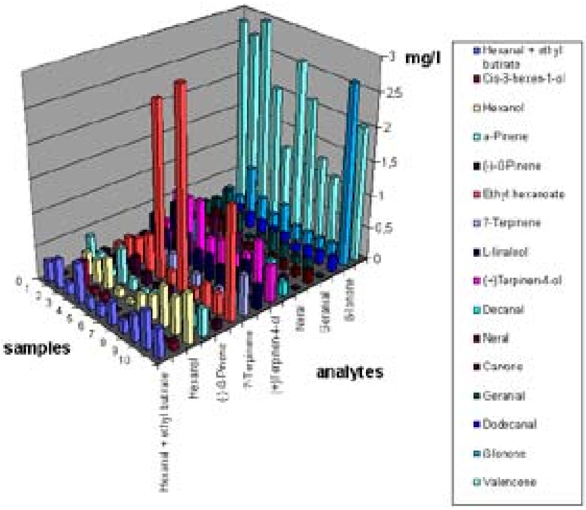

Fig. 2. **Three-dimensional bar chart that represents the concentration of the compounds responsible for the aroma minority ten samples of orange juice**

Conclusions

- It has been optimized a fast, accurate and sensible methodology for the quantification of volatile compounds responsible for the flavour of the orange juice.
- It has developed a procedure for extraction of these compounds with recoveries around 80%.

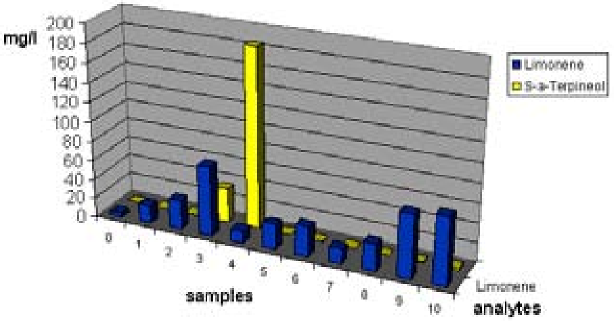

Fig. 3. **Three-dimensional bar chart that represents the concentration of the compounds responsible majority responsible for the scent of ten samples of orange juice**

- The methodology developed is transferable to the food industry and can contribute to the development of a new products of enhanced quality
- The method allows monitoring the process of maturation of the orange (*Citrus sinensis*) by assessing the concentrations of aromas.

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P32 CONTENT OF CADMIUM IN SELECTED MEAT PRODUCTS SUPPLIED ON THE SLOVAK MARKET

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Introduction

One of the most important aspects of environmental pollution to humans is that a significant intake of heavy metals occurs through diet. Food-producing animals are a rich source of food, but they are also a source of various contaminants. Heavy metals are natural components of the environment, but in recent years industrial, agricultural and zootechnic development has been responsible for the diffusion of these substances in the environment[1]. The toxicity and metabolism of metals may be influenced by number of factors, which vary in importance for different metals. Many factors, such as sex, age, metal interaction and diet, can affect the absorption and accumulation of these metals[2]. The cadmium levels in meat from muscle are of the order of 0.01 mg kg^{-1} for animal at slaughter, although they may be higher in older animals. Cadmium concentrations in liver, and particularly, kidney are substantially higher than that in muscle. In livers of calves, pigs and poultry, Cadmium levels range from 0.02 to 0.2 mg kg^{-1}, these levels may also be higher in older animals. Cadmium levels found in kidneys from calves and pork pigs from 0.05 to 0.5 mg kg^{-1}, while the concentration in ox kidneys may approach 1 mg kg^{-1}. Horse kidney and liver may have cadmium concentrations exceeding 10 mg kg^{-1}(ref[3]). The levels of cadmium and other elements were determined in beef and pork imported to Sweden from six different countries. All results for cadmium were below the detection limits of 0.001 mg kg^{-1} fresh weight[4].

Experimental

In this study we analysed 360 samples of meat products – sausages, hams and frankfurters. The 90 of samples descended from Slovak Republic, 90 of samples from Czech Republic, 90 of samples from Austria and 90 of samples from Germany. The samples were collected of the Slovak market in 2007.

Cadmium in food samples was determined by pressure mineralisation in the combination with atomic absorption spectrophotometry. The samples were separately packed in polyethylene bags and stored at –20 °C until the analysis. Samples were weighed in glass digestion tubes on a precision balance. Prior to analyses the samples were homogenised and then 1.5 g of sample was taken for pressure decomposition. A 10 ml high purity nitric acid (HNO_3) added to the tube and enclosed into pressure cooker, and stationed into pre-heat drier. The pressure decomposition insisted 3 hour at 150 °C. The measurements of cadmium were performed on an atomic absorption spectrophotometer VARIAN AA-175.

Results

We evaluated the content of cadmium in three kinds of the meat products: sausages, hams and frankfurters, which descended from different producers. The basic variance-statistical characteristics of cadmium in meat products are showed in Table I. The highest mean value of cadmium in sausages (0.0589 mg kg^{-1}) was found at products supplied from Austria. The lowest mean value of cadmium (0.0525 mg kg^{-1}) was found in Slovak sausages. The highest mean value of cadmium (0.0583 mg kg^{-1}) was found in Slovak hams, and the lowest average value of cadmium (0.046 mg kg^{-1}) was detected in Austria hams. The highest mean value of cadmium (0.0679 mg kg^{-1}) was found in Slovak frankfurters and lowest value of cadmium (0.0513 mg kg^{-1}) was found in Austria frankfurters. Variability content of cadmium in individual meat products was very different and varied from 69.32 % to 111.03 %. At sausages was the highest variability (111.03 %) at products made in Slovak republic, the lowest variability content of cadmium (70.28 %) was found in products made in Austria. At hams was the highest variability (96.77 %) of the content of cadmium at products made in the Slovak Republic, and the lowest variability at hams (69.32 %) was at products made in Germany. At frankfurters was found the highest variability of cadmium from Austria (107.5 %) and the lowest variability of cadmium was at frankfurters of Czech Republic (77.99 %). The number of samples for individual meat products and countries, which suitable respectively unsuitable applications of Food Codex Slovak Republic are shown in Table II. The number of unsuitable samples in the each group of meat products, ranged from 2 to 6. From total number 360 of samples were an excess of the allowable limit in 57 cases. Content of selected chemical element in the followed meat products was different. The origin of cadmium in the meat products is from raw material (meat and organs of animals), additive material (condiment) and water. Due to this, it has to be stated, that differences in the content of chemical elements are of reason. Meat from different animals, of different conditions and breeding was used for products. They were fed with different feed. Therefore content of selected metal in their muscle is different. Meat has been minced and mixed together, therefore content of cadmium in the meat products is dependent on the level of mince or homogenisation respectively. Different occurrence of studied chemical element in the meat products is dependent on the level of meat and organs contaminant.

Many of previous reports has followed the content of chemical elements in the muscle, kidney and liver. From the point of view of human exposure it is necessary to follow the content of toxic substance in meat products, as all slaughtered animals are not examined individually. Therefore monitoring of distribution and occurrence of chemical elements in the meat products is necessary.

The occurrence of cadmium in meat and meat products studied other authors; referred to toxic effects of cadmium[5,6,7]. The obtained results content of cadmium in meat products are comparing with the results of other authors[8,9].

Table I
Basic variance-statistical characteristics of cadmium in meat products

Parameters	x [mg kg^{-1}]	S. D* [mg kg^{-1}]	C. V** [%]	x_{min}	x_{max}
		Slovak republic			
Sausages	0.0525	0.0583	111.03	0.004	0.241
Hams	0.0583	0.0564	96.77	0.005	0.217
Frankfurters	0.0679	0.0636	93.67	0.008	0.247
		Czech republic			
Sausages	0.0544	0.0518	95.35	0.002	0.168
Hams	0.0534	0.0427	79.83	0.004	0.146
Frankfurters	0.0547	0.0427	77.99	0.001	0.174
		Austria			
Sausages	0.0589	0.043	73.0	0.006	0.147
Hams	0.046	0.0424	92.18	0.002	0.156
Frankfurters	0.0513	0.0552	107.5	0.002	0.241
		Germany			
Sausages	0.034	0.0375	70.28	0.002	0.141
Hams	0.047	0.0353	69.32	0.006	0.144
Frankfurters	0.0562	0.0459	81.63	0.004	0.212

Table II
Numbers of samples suitable and unsuitable by Food Codex in the Slovak Republic

Products	Suitable samples	Unsuitable samples
	Slovak republic	
Sausages	24	6
Hams	25	5
Frankfurters	24	6
	Czech republic	
Sausages	25	5
Hams	25	5
Frankfurters	25	5
	Austria	
Sausages	24	6
Hams	26	4
Frankfurters	26	4
	Germany	
Sausages	27	3
Hams	28	2
Frankfurters	24	6

Conclusions

We found, that meat product were contaminated by cadmium. From evaluated 360 samples (salami, hams and sausages) was 15.8 % (57 of samples) evaluated as over-limit in comparison with the highest allowable value (0.1 mg kg^{-1}) by Food Codex Slovak Republic. The highest values of cadmium were detected in sausages made in the Slovak Republic (0.247 mg kg^{-1} respectively 0.224 mg kg^{-1}). As primary source of cadmium in meat products can be regarding liver, kidney and meat. The high variability content of cadmium is given mainly thereby, that products descended from different producers.

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P33 CHIRAL SPECIATION OF IODINE IN THYDOID HORMONES BY USING HPLC/MS/MS AND HPLC-ICP-MS

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Introduction

Iodine is a key-element in the thyroid functions as active centre of the thyroid hormones, which activity depend on the number of iodine atoms into the molecule. These hormones are involved in the regulation of different important biological processes.

Thyroxine (T_4 or 3,5,3',5'-tetraiodothyronine) is the major hormone produced and secreted in the follicular cells of the thyroid gland by the protein thyroglobulin (Tg). Other compounds homologues have also been subsequently characterized.

In addition, thyroid hormones have enantiomeric forms which affect their biological activities[1,2]. Therefore, these species have to be determined separately to know their real metabolic behaviour and action on living organisms.

There are a number of problems associated with the analysis of thyroid hormones. The use of liquid chromatography/mass spectrometry presents several advantages over the currently used radio-immunoassay (RIA) and gas chromatography-mass spectrometry methods. The use of a UV detector to monitoring chromatographic separations allows a good sensitivity. There is an increasing interest for the use of HPLC coupled to MS, which increases the selectivity with acceptable sensitivity that is advisable for the complex biological matrices. However, the most sensitive analytical approach for iodinated hormones is HPLC-ICP-MS, which has been proposed by Michalke and other authors[3].

In the present study an analytical method for eight thyroid-related compounds has been performed. The coupling of HPLC-UV(PDA)-MS allows the identification and quantification of all the compounds and HPLC-ICP-MS the analysis of very low level of these hormones. A procedure for the analysis of these substances in human serum samples has been optimized and method reliability validated by recovery experiments.

Experimental

All the calibrants: L-thyronine – T_0; 3,5-diiodo-L-thyronine – L-T_2; 3,3',5-triiodo-L-thyronine – L-T_3; 3,3',5'-triiodo-L-thyronine – L-r-T_3; L-Thyroxine – LT_4; D-thyroxine – D-T4; 3,5-diiodo-L-tyrosine – DIT; 3-iodo-L-tyrosine – MIT were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Stock solutions were prepared at a concentration of 1 mg ml^{-1} in a mixture of MeOH and 10 nM NaOH (1 : 1 (v/v)).

Chromatographic Conditions and Instrumetation

A SpectraSYSTEM P4000 (HPLC) coupled in series with a SpectraSYSTEM UV6000LP photo diode array detector (PDA) and a LCQ Advantage mass spectrometer from Thermo-Finnigan (San José, CA, USA) with electrospray ionization (ESI) as ion source was used with a CHIRALPAK® QN-AX column. The mobile phase was 3% acetic acid in 40 % acetonitrile and 60 % water mixture; flow rate 0.7 ml min^{-1}.

HPLC-ICP-MS Coupling

An ICP-MS (HP 7600e, Hewlett-Packard, USA) was used with the same chromatographic conditions of previous paragraph. Iodine was determined at m/z 127. The operational conditions were the followings: forward power 1500 W, plasma gas flow rate 15.0 ml min^{-1}, auxiliary gas flow rate 1.0 ml min^{-1}, carrier gas flow rate 0.6 ml min^{-1}, optional gas (O_2) 6%, sampling depth 6.5 mm, sampling and skimmer cones of platinum. The dwell time was set to 0.3 s.

Hormones Extraction from Serum

A ClinChek®-Control serum (RECIPE, Chemicals+ Instruments GmbH, Munich, Germany) serum was used for hormone extraction. A volume of sample between 300 and 450 µl of serum was treated with a triple volume of 1% formic acid in acetonitrile. The mixture was vortexing for 5 min and centrifugated at 3,000 rpm for 10 min. The supernatant was filtered by 0.2 µm before to inject into the chromatographic column.

Results

The use of the in series coupling HPLC-PDA (UV-vis)-MS intents to combine the sensitivity of UV detector with the PDA system and the selectivity of MS. However, the very low levels of these hormones in healthy and hypothyroid people require more sensitive detection that can be reached with HPLC-ICP-MS.

A chiral separation of these hormones was performed using a *tert*-butyl carbamoylated quinine stationary phase.

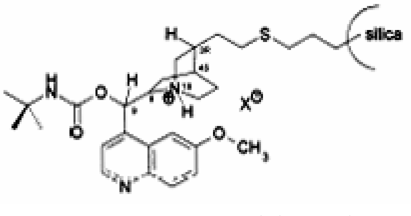

Fig. 1. **Tert-butyl carbamoylated quinine stationary phase**

Several solvent mixtures were tested as mobile phases. The optimum separation was obtained with 3 % of acetic in acetonitrile:water (40:60) at a flow rate 0.7 ml min^{-1}.

In Fig. 2 is shown a typical chromatogram obtained with the HPLC-(PDA)UV system for 20 mg dm^{-3} of each compound. The different compounds are well resolved but overlapping of T0 and L-MIT was observed. For this reason MS/MS mode detection was tested for selective extraction of the ion $[M+H]^+$ of To (m/z 274.1) against L-MIT (m/z 308.1) – Fig 3.

A similar chromatogram was obtained with HPLC-ICP-MS, but limits of detection decreased drastically and the mixtures of hormones were traced at 5 µg dm^{-3} (Fig. 4).

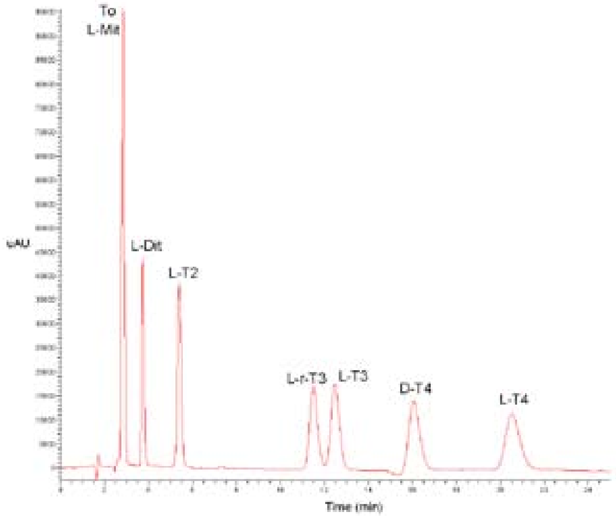

Fig. 2. **Chromatogram of thyroid hormones with HPLC-(PDA)UV**

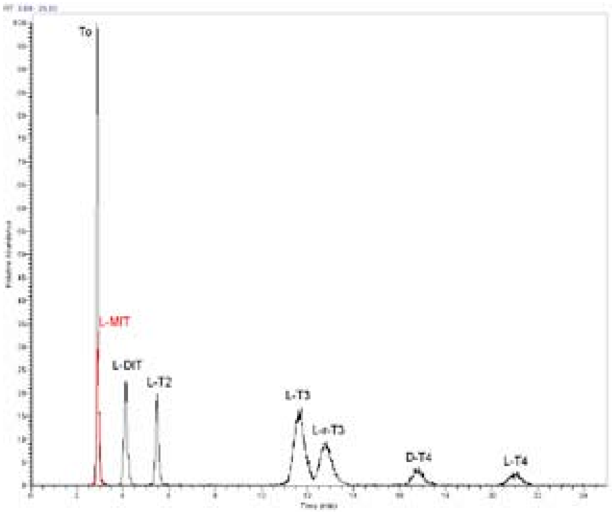

Fig. 3. **Chromatogram of thyroid hormones with HPLC-MS/MS**

Methods Performance

The method based on HPLC-(PDA)UV present a linear range between 1 and 25 mg dm^{-3}, with detection limits between 0.04 to 0.1 mg dm^{-3}.

For the HPLC-ICP-MS method the linear range was obtained between the detection limit and 100 µg dm^{-3}, and detection limits between 0.15 and 2.0 µg dm^{-3}, depending of compound considered.

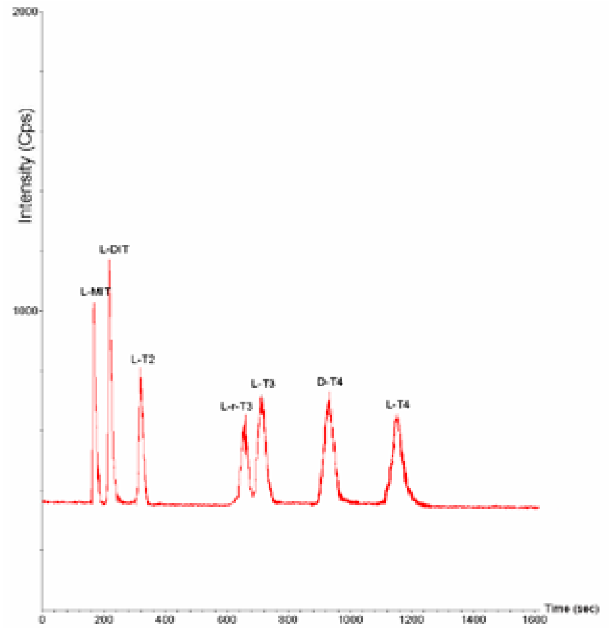

Fig. 4. **Chromatogram of thyroid hormones with HPLC-ICP-MS**

Hormones Extraction from Serum

Proteins present in the serum were eliminated by precipitation with 1 % of formic acid in acetonitrile.

The final approach were appplied to a control human serum (ClinChek®-Control serum), usually used in the hospital for quality control, with good recoveries.

Conclusions

The combination of HPLC-PDA-UV-MS and HPLC-ICP-MS allows identification and cuantification of thyroid hormones in human serum. The use t-BuCQN colunm allows chiral speciation of these hormones. Proteins elimination from serum is necessary for the analysis of thyroid hormones in human serum. The method is suitable for the analysis of these hormones in control serum samples. Further studies are necessary to check the applicability of the approach to real human serum.

This work has been supported by the Grant CTM2006-08960-C02-01(Ministerio de Educación y Ciencia-Spain and project FQM-348 from the Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía)

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P35 COMPARISON OF PROTEOME AND METABOLOME CHANGES IN STRESSED YEAST STRAINS *RHODOTORULA GLUTINIS* AND *RHODOTORULA RUBRA*

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Introduction

Exogenous stress and other environmental factors can induce many changes in cell composition. Some of these changes involved in stress response may lead to over- or underexpression of cell proteins. Identification of metabolic markers characteristic for certain events provides important insight into the metabolism control. Additionally, this information could be used to the biotechnological production of some industrially significant metabolites.

Carotenogenic yeasts produce high amount of lipidic compounds. Carotenoids are membrane-bound lipid-soluble pigments, which can act as effective antioxidants and scavenge singlet oxygen. In red yeasts they probably act as adaptive and/or protecive mechanism against exogenous oxidative stress and UV-irradiation. Carotenoids are produced by a specific branch of common isoprenoid pathway and accumulated in particular cell organelles.

In this work, protein and metabolic profiles of salt- and peroxide- stressed carotenogenic yeasts of the genus Rhodotorula were analyzed. Yeast cells *Rhodotorula glutinis* CCY 20-2-26 and *Rhodotorula rubra* CCY 20-7-29 were cultivated in glucose medium in presence of 2–5% NaCl and 2–5 mM hydrogen peroxide. In yeast cells carotenoids and ergosterol as specific stress metabolites were measured using HPLC/DAD. Proteins were separated by 1D and 2D electrophoresis. Some spots were identified by LC/MS/MS.

Materials and Methods

Strains

Industrial yeasts *Rhodotorula glutinis* CCY 20-2-26 and *Rhodotorula rubra* CCY 20-7-29 were used as tested strains. As a comparative strain *Saccharomyces cerevisiae* CCY 21-4-88 was used. Red yeasts were cultivated on glucose medium aerobically at 28 °C. Exogenous stress was induced by 2–5 mM peroxide and 2–5% NaCl.

Cell Fractination

Protein fractions of red yeast cells were obtained by gradually separation using combination of chemical (NaOH and detergents) and mechanical (glass beads; 100 μm) lysis. Protein fraction for 2D analysis was isolated mainly from lyophilized cells.

Carotenoid Analysis

Levels of carotenoids – lycopene, beta-carotene, torulen and phytoene were analyzed using HPLC/MS. Ergosterol was analyzed by RP-HPLC (280 nm).

1D Gel Electrophoresis

1D PAGE-SDS electrophoresis of proteins was carried out by common procedure using 10% and 12.5% polyacrylamide gels. Proteins were staining by Coomassie Blue and by silver staining. For comparison, microfluidic technique using 1D Experion system (BioRad) and P260 chips was used for yeast protein analysis too.

2D Gel Electrophoresis and LC-MS/MS

2D electrophoresis of proteins was optimized in cooperation with Laboratory of Functional Genomics and Proteomics, Faculty of Science, Masaryk University of Brno. 2D gels were obtained using protein preparatives isolated from lyophilized cells. After optimization of separation conditions proteomes from stressed *R. glutinis* and *R. rubra* cells were isolated, lyophilized and analyzed. Quantitative analysis was done using BioRad Laboratories 2D software. Identification of some spots was done using LC-MS/MS.

Results

Proteins from red yeast species were isolated with cell lysis combined with mechanical disintegration and protein profiles were obtained by 1D and 2D electrophoresis. Meta-

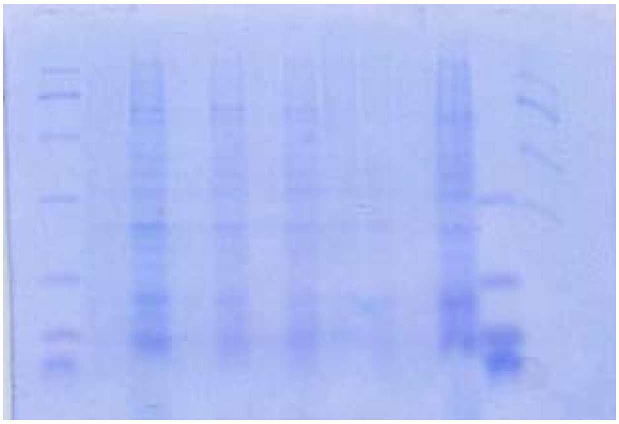

Fig. 1. **1D protein profiles – stressed *R. rubra* (NaOH); lane 1 – standard 6, 2 – proteome from lyofilized cells, 3 – proteome from non-lyofilized cells, 4 – proteome 2% NaCl, lyo, 5 – 2 % NaCl, non-lyo, 6–7 – 5 % NaCl, 8–9 – 2 mM hydrogen peroxide, 10–11 – 5 mM peroxide; 12, 13 – standards 4, 5**

bolome changes connected with proteome changes were observed. Presence of exogenous stress factor led to overproduction of carotenoids.

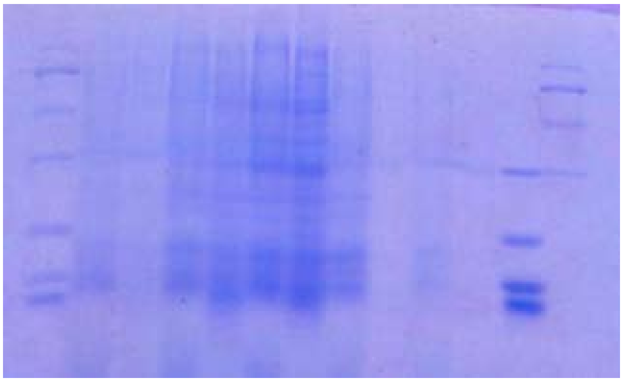

Fig. 2. **1D protein profiles – stressed *R. glutinis*; lane 1 – standard 6, 2 – proteome isolated by NaOH, 3 – proteome isolated by SDS, 4 – proteome 2% NaCl, NaOH, 5 – 2 % NaCl, SDS, 6–7 – 5 % NaCl, 8–9 – 2 mM hydrogen peroxide, 10–11 – 5 mM peroxide; 12, 13 – standards 4, 5**

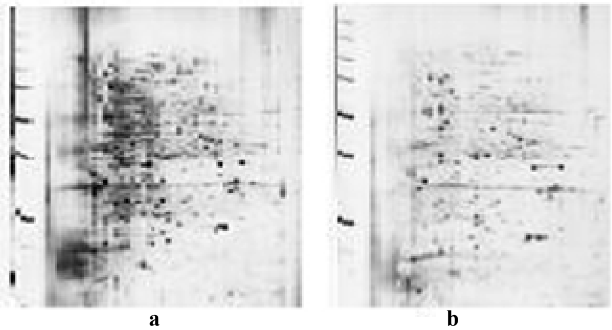

Fig. 3. **Protein profiles of *Rhodotorula glutinis* a) control b) peroxide stress**

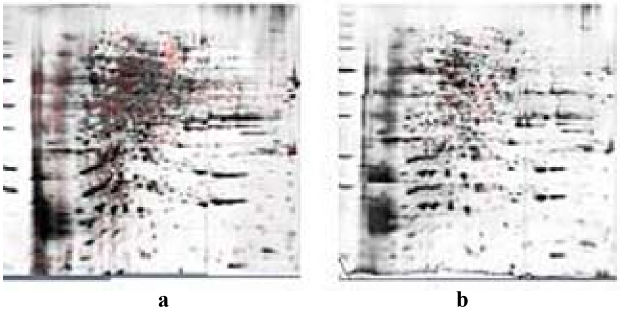

Fig. 4. **Protein profiles of *Rhodotorula rubra* a) control b) peroxide stress**

Conclusions

In this work proteome changes in two *Rhodotorula* strains grown under salt and peroxide stress was studied. Isolation of proteins from red yeast cells is extremely complicated by unusual character of cell wall. Combination of several glycosidases and glucuronidases was the most common procedure. However, this method can lead to high interference of lytic enzymes during electrophoretic separation. In this work isolation of proteome using detergent SDS or alcalic lysis (NaOH) combined by mechanical disintegration by glass beads was optimized.

1D electrophoresis PAGE-SDS exhibited relatively good separation of proteins. Better results were obtained by alcaline lysis.

2D electrophoresis was done using stressed yeasts *Rhodotorula glutinis* and *Rhodotorula rubra*. Proteomic profiles differed according to strain and stress type.

In *R. glutinis* proteome more spots were evaluated. Significant 2D proteome changes were observed mainly in peroxide stress. Relatively well-balanced response to stress influence was observed in presence of 2 % salt; it seems that *R.glutinis* cells could exhibit long-term adaptation to this stress factor.

In *R. rubra* proteome less protein spots were detected and changes in stress conditions were not such intensive as in *R.glutinis*.

Proteome changes corresponded to some metabolome changes in *Rhodotorula* cells. Presence of some exogenous stress factor led to important overproduction of beta-carotene and ergosterol. Both these industrially significant metabolites are induced by the same way through the common isoprenoid pathway. In presence of salt well-balanced adaptation mechanisms were probably activated and, thus, salt stress could be used industrially to enhanced production of beta-carotene enriched biomass. *R. rubra* produced substantially lower amount of beta-carotene, this production was quite independent on stress factor.

Further experiments are needed for detailed characterization of molecular changes connected with exogenous stress and for identification of proteins involved in stress response of red yeasts.

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P36 (1→3)(1→4)-B-D-GLUCAN: VARIABILITY AND FACTORS AFFECTING ITS CONTENT IN OAT GRAIN

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Introduction

β-D-glucan is major polysaccharide constituent of cell walls in the *Poales*, an order that includes cereals as well as other grass species. It is a linear and partially water-soluble polysaccharide that consists only of glucose linked through both β-(1→3)- and β-(1→4)-glykosidic linkages in various ratios. β-D-glucan plays an important role in cell wall architecture and plant development[1]. It has a positive impact on human health in terms of lowering cholesterol and blood glucose levels[2], increasing immunity against infection and positive influencing of bowel function[3].

Heritability estimates for β-D-glucan content have ranged from 0.27 to 0.58(ref.[4]). β-D-glucan content is affected by environmental factors. Genotype and the environment are significant sources of variation for its content; the ranking of genotypes is generally consistent over the environments[5].

Experimental

Mature grains of 99 oat genotypes were milled and passed through 0.5 mm sieve. The level of β-D-glucan was determined using Mixed-linkage Beta-glucan assay procedure, Megazyme (Ireland)[6]. Samples were suspended and dissolved in a 0.02M sodium phosphate buffer (pH 6.5), incubated with purified lichenase enzyme, and an aliquot of filtrate was reacted with purified β-glucosidase enzyme. The glucose product was assayed using an oxidase/peroxidase reagent. The measurement of the amount of β-D-glucan was performed in three parallel assays and expressed on a dry weight basis.

Results and Discussion

The β-D-glucan content ranged from 1.73 to 5.70 %, with 3.44 % as a mean value. The result is consistent with the determined values of β-D-glucan content in the set of European oat genotypes (3.90 %, 3.64 %, respectively)[7,8]. Our results show, likewise the literature[8], that naked genotypes dispose of higher levels of this metabolite (4.38 % on average) in comparison with the hulled oat, where the mean content was 3.26 % (Table I). The majority (90 %) of naked genotypes contain higher level of β-D-glucan than is the average in analysed oat set. The increased value of β-D-glucan is connected with the presence of gene *nud* for a hulless type of grain[9]. Naked oats are the feed that provide the largest welfare benefits[10] and their demand as a food crop is on a world-wide scale increasing[11]. Genotypes Terra, Avenuda, SV-5, Arnold, Neon, Izak, Salomon, Saul, Bandicoot, and Ábel with the highest level (> 4.00 %) observed can be therefore used as a convenient material in breeding and food industry.

In our work a relation between the oat glume colour (yellow, white, black) and the content of β-D-glucan was found. Our results indicate that the black genotypes show the lowest standard deviations in the content of evaluated metabolite. The degree variance of β-D-glucan level expressed as a coefficient of variation displayed the highest value in white hulled oat, next in yellow, and the lowest values were calculated for the genotypes with black glume colour (Table I). Because higher value of variation coefficient directly correlates with lower variance of metabolite biosynthesis within a set, we could state, that black hulled oat with low variation coefficient are characterized by markedly stable biosynthesis of β-D-glucan in the comparison with yellow and white types. Similarly, biosynthesis of other biochemical parameters (proteins, fatty acids, and lipids) also shows to by more stabile in black hulled oat[12].

Analysis of variance suggested that oat morphological and biochemical parameters depend on the environment and the genotype variously[13,14]. Test weight, groat percentage and weight, protein, and β-D-glucan content were about equally influenced by the environment and genotype[13], whereas oat lipids, total starch, and amylose content were more strongly influenced by genotype.

The content of monitored parameter according to the year of genotype registration is documented on the Fig. 1. The mean value of β-D-glucan in oats with the breeding date before the year 1960 was 2.53 %, moreover a stabilisation was observed during the period of years 1961–2000. At the beginning of the 21st century a gradually raising of β-D-glucan content in oat seeds was detected.

Table I
Mean values of the β-D-glucan content in naked and hulled oat and in individual groups according to the glume colour

Group of oat	Mean value [%]	Standard deviation	Variation coefficient
Naked	4.38^{***}	0.96	22.03
Hulled	3.26	0.60	18.34
Yellow	3.28	0.62	18.97
White	3.46	0.71	20.55
Black	3.31	0.46	13.89^{***}

*** significant at P ≤ 0.001

Conclusions

Naked genotypes dispose of higher β-D-glucan content in comparison with the hulled. Genotypes Terra, Avenuda, SV-5, Arnold, Neon, Izak, Salomon, Saul, Bandicoot, and Ábel with the highest level of monitored health beneficial polysaccharide can be used in breeding programs and seed processing in food industry as its donors. Genotypes with black colour of the glumes account significantly lower standard deviation and variation coefficients in the content of β-D-glucan, what indicates markedly stable biosynthetic mechanism of the studied metabolite.

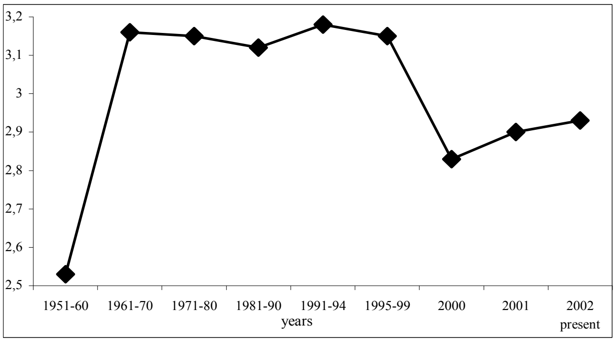

Fig. 1. **Mean content of β-D-glucan [%] according to the breeding date**

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P37 EXTRACTION AND DETERMINATION OF FLAVONOIDS IN PLANT MATERIALS

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Introduction

Flavonoids are one of the largest groups of secondary metabolites and they are responsible for coloration of plants. They are found in fruits, vegetables, beverages like tea, wine, beer, and large amount of flavonoids was also found in several medicinal plants. Flavonoids play important role in human diet because of their antioxidant properties, estrogenic action and wide range of antimicrobial and pharmaceutical activities[1].

Extraction of flavonoids from the samples is usually performed by maceration with aqueous methanol[2], solid-phase extraction[3], sonication[4], and supercritical fluid extraction (SFE)[5]. High-performance liquid chromatography (HPLC)[6], capillary electrophoresis (CE)[7], capillary electrochromatography (CEC)[8] or gas chromatography (GC)[9] are commonly used for their determination and quantification.

Since rapid and efficient extraction technique prior to chromatographic analysis is nowadays of primary interest, the liquid extraction at high temeperature and pressure – *Pressurized fluid extraction* (PFE), was introduced. PFE offers several advantages over conventional extraction procedures. The use of higher temperature increases the diffusion rate, solubility and mass transfer of the compounds and decreases the viscosity and surfance tension of the extraction solvent. These changes improve the contact of analytes with the solvent and enhance extraction, which can be achieved more rapidly with less solvent consumption as compared with conventional extraction methods. The elevated pressure not only maintains the extraction solvents in liquid state, but also improves the contact of solvent with analytes trapped in matrix pores. The absence of light and air significantly reduces degradation and oxidation of compounds during extraction[10].

In the last years, pressurized fluid extraction has been succesfully applied to the extraction of flavonoids from plant materials and foods. This application of PFE as powerful sample preparation step was recently reviewed by Mendiola et al.[11]

Recently, intense attention has been paid to *Stevia rebaudiana* Bertoni (Asteraceae) because of content of low-caloric high intensity sweet diterpene glycosides (50–400 times sweeter than sucrose)[12]. Besides sweet glycosides also flavonoids have been isolated from *Stevia*. Rajbhandari et al. determined that *Stevia* contains apigenin, luteolin, quercetin, kaempferol and quercitrin. The flavonoids were extracted by classical maceration and their structures were determined by standard methods of uv, nmr, and ms spectroscopy[13].

In this study, we focused on optimalization of liquid chromatograpfic conditions after extraction step for rapid and succesful determination and quantification of quercetin, luteolin, apigenin and kaempferol contained in *Stevia* leaves.

Experimental

Chemicals and Standard Solutions

Stevia leaves were obtained from Ukraine. Acetonitrile, methanol and formic acid, all HPLC-grade, were purchased from Riedel-de Haën (Prague, Czech Republic). Water was purified with a reverse osmosis system Ultra Clear UV (Barsbüttel, Germany).

Flavonoid standards quercetin, kaempferol, luteolin and apigenin were obtained from Sigma-Aldrich (Prague, Czech Republic). Stock solution of all flavonoids (1 $g\,dm^{-3}$ each) was prepared in methanol and stored in the fridge at 5 °C.

PFE of *Stevia Rebaudiana* Leaves

A static PFE of Stevia leaves was performed using a onePSE extractor (Applied Separations, Allentown, PA, USA). A portion (1 g) of leaves was placed into 22 ml extraction cell that contained inert material (glass beads (570–700 µm)) at the bottom of the cell. The PFE parameters were set as follows, temperature 60–120 ºC, pressure 15 MPa, extraction time 3 × 5 minutes, rinsing time 20 s, and nitrogen purge time 90 s after each cycle and 120 s after extraction run. After PFE run, the extract was cooled to 5 ºC and stored in the fridge until HPLC analysis.

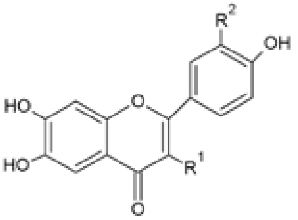
Chromatographic Conditions

HPLC apparatus was eqCipped with LC 1150 pump (GBC Scientific Equipment Pty Ltd, Dandenong, Australia), injection valve with 20-µl sample loop (Ecom, Praha, Czech Republic), column oven LCO 101 (Ecom, Praha, Czech Republic) and Linear UVIS-206 Multiple Wavelength detector (Linear Instruments, Fremont, CA). The wavelength was set at 360 nm. Mobile phase was composed of acetonitrile (A) and water (B) adjusted at pH 2.3 by formic acid. Several reversed-phase columns were tested: 1) 4.6 mm i.d., 150 mm long Symmetry C18 5 µm stationary phase (Waters, USA); 2) 3.0 mm i.d., 150 mm long Xbridge C18 3.5 µm stationary phase (Waters, USA); 3) 3.0 mm i.d., 150 mm long Atlantis T3 C18 3 µm stationary phase (Waters, USA); 4) 3.0 mm i.d., 150 mm long Xterra RP C18 3.5 µm stationary phase (Waters, USA); and 5) 4.6 mm i.d., 250 mm long Supelcosil LC-18 5 µm stationary phase (Supelco, USA). Gradient programme was employed at flow rate 0.5 $ml\,min^{-1}$: 0 min 10 % A; 20 min 70 % A. When Xterra C18 column was used, the flow rate was set to 0.3 $ml\,min^{-1}$ because of high backpressure. LC systems were connected to PC and controlled by Clarity software (DataApex, Czech Republic).

Results

Since luteolin, quercetin, apigenin and kaempferol have very similar structures (Fig. 1.), the main point of view of this study was to find optimal analytical conditions for their efficient separation.

Several single columns with different column lengths and different sorbent parameters were tested. The gradient of water/acetonitrile mixture was used as mobile phase. The replacement of acetonitrile with methanol as mobile phase component was also investigated, but the separations didn't show satisfactory results. After modification of mobile phase compositions by formic acid at pH = 2.3, significant peak shape improvement of the observed compounds was noticed. In all experiments, the flow rate of mobile phase was 0.5 ml min^{-1}, only when Xterra C18 column was tested, the flow rate was set to 0.3 ml min^{-1} because of the high column backpressure. The experiments were performed with flavonoid standard solution (c = 30 μg ml^{-1}). Injected amount of analyte was 20 μl.



compounds	R¹	R²
quercetin	- OH	- OH
luteolin	- H	- OH
apigenin	- H	- H
kaempferol	- OH	- H

Fig. 1. **Chemical structures of analyzed flavonoids**

The comparison of column efficiency for separation of the above compounds is shown in Fig. 2. First, two hybrid C18 columns with the same column parameters were compared: (a) 3.0 mm i.d., XBridge 150 mm long column, 3.5 μm particle size, (b) 3.0 mm i.d., Xterra 150 mm long column, 3.5 μm particle size. Both separations showed only three peak. In separation (a) very nice separation of apigenin and kaempferol was observed, while in separation (b) the separation of apigenin and kaempferol was slightly poor. However, the column performance of both columns was too poor to separate luteolin and quercetin, the peak coelution was observed. Nevertheless, neither the utilization of longer column, (c) 4.6 mm i.d., Supelcosil C-18 250 mm long column, 5 μm particle size, didn't show the improvement of luteolin and quercetin separation and also the separation of kaempferol and apigenin wasn't very acceptable. Further, (d) 3.0 mm i.d., Atlantis T3 C18 150 mm long column, 3.0 μm particle size was tested. The column separated not only apigenin and kaempferol but also luteolin and quercetin. However, although the column proved to separate single flavonoids, the peak shapes didn't match our ideas. The efficient separation of observed compounds was achieved by (e) 4.6 mm i.d., Symmetry C18 150 mm long column 5 μm stationary phase. The kaempferol and apigenin were separated to baseline and separation of luteolin and quercetin was sufficient for succesful determination and quantification of both compounds present in the sample.

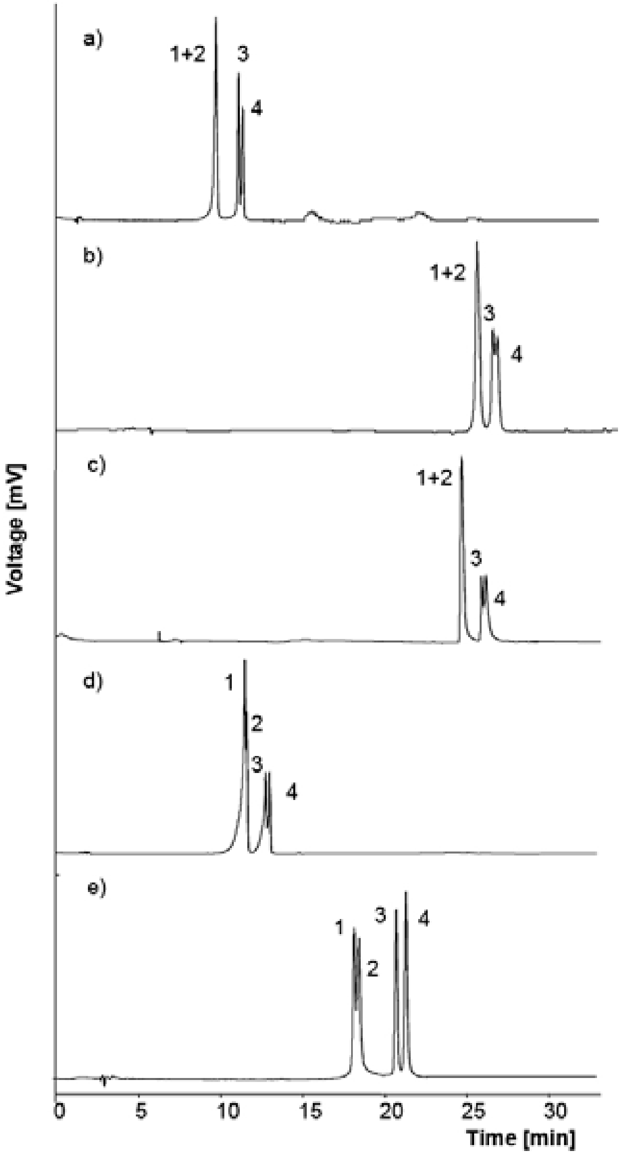


Fig. 2. **HPLC chromatogram of comparison column separation of flavonoid standards. (1) quercetin, (2) luteolin, (3) apigenin, (4) kaempferol. (a) Xbridge C18 column (3.0 × 150 mm length), (b) Xterra C18 column (3.0 × 150 mm length), (c) Supelcosil C18 column (4.6 × 250 mm length), (d) Atlantis T3 C18 column (3.0 × 150 mm length), (e) Symmetry C18 column (4.6 × 150 mm length). For chromatographic conditions see the experimental part**

Conclusion

The data presented in this report indicate that there are wide variations in the effectiveness of different reversed-phase HPLC columns when used to analyse flavonoids. High efficiency separation was obtained with Symmetry C18 column. Gradient elution with acetonitrile in water adjusted to pH 2.3 by formic acid facilitated the separation of luteolin, quercetin, kaempferol and apigenin. In the future, this procedure will be used for quantitative analysis of Stevia flavonoids after PFE and appropriate pre-cleaning step of the extracts prior to HPLC analysis.

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P39 *TRICHOSPORON CUTANEUM*: CELL ADHESION ON CELLOPHANE SURFACE

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Introduction

Recently we have faced the problem of increasing amount of solid cellulose wastes that come mainly from agriculture activities, food industry as well as from municipal waste[1,2].

Cellulose is considered to be a solid nontoxic pollutant; however, its recalcitrant nature causes many difficulties in removal of cellulosic waste. The microbial degradation is one of the possibilities how it could be solved.

Colonization of solid material by microbial cells is the crucial step in their biodegradation. Biofilm formation by cellulolytic microorganisms is not investigated enough. Therefore, establishing more efficient arrangement for technological solubilization of solid cellulosic wastes could be achieved using cellulolytic biofilms, formed by direct colonization of these wastes by cell populations. In this association, cellophane was chosen as a representative of solid cellulosic substrates (carrier) and the yeast *Trichosporon cutaneum* as a relatively little researched cellulolytic strain.

Experimental

The yeast *Trichosporon cutaneum* CCY 30-4-5 was obtained from Department of Genetics and Microbiology, Faculty of Science, Charles University, Czech Republic.

Inoculum was grown in complex medium and after 2 days it was replaced in minimal medium supplemented with 1% cellulosic substrates as a sole source of carbon. Sigmacell Type 101, carboxymethylcellulose – CMC, hydroxyethylcellulose – HEC, cellophane and filter paper was used as the representatives of these substrates. Minimal medium composition in g dm^{-3}: KH_2PO_4 – 1.7; $Na_2HPO_4 \cdot 7H_2O$ – 0.75; $(NH_4)_2SO_4$ – 5.0; $MgSO_4 \cdot 7H_2O$ – 0.02; $CaCl_2$ – 0.02; $FeSO_4 \cdot 7H_2O$ – 0.001; $MnSO_4 \cdot H_2O$ – 0.001, pH 5.8. The temperature was maintained at 28 °C. Cultures were harvested during the exponential phase; at 48–72 hr. Separated and rinsed cells were used for experiments.

FC 81 Flow Cell Description

The FC 81 Flow Cell in Fig. 1. is a flat plate flow cell designed for use with transmitted light microscopes and it was used for observation of biofilm formation by yeast *Trichosporon cutaneum*. The capability of the yeast cells to colonize cellophane as well as the effect of shear stress was investigated.

Experiment conditions:

- Cell suspension – $OD_{400\,nm}$ 0.1
- Temperature – 22 °C
- Flow rate – 2-20 ml min^{-1}
- Time period – 2 hr.

Fig. 1. **Photograph of the FC 81 Flow Cell (Biosurface Technologies, Corp., USA)**

Experiments were carried out as shown by Fig. 2.; Fig. 3. illustrates an emplacement of cellophane stripe in the flow cell.

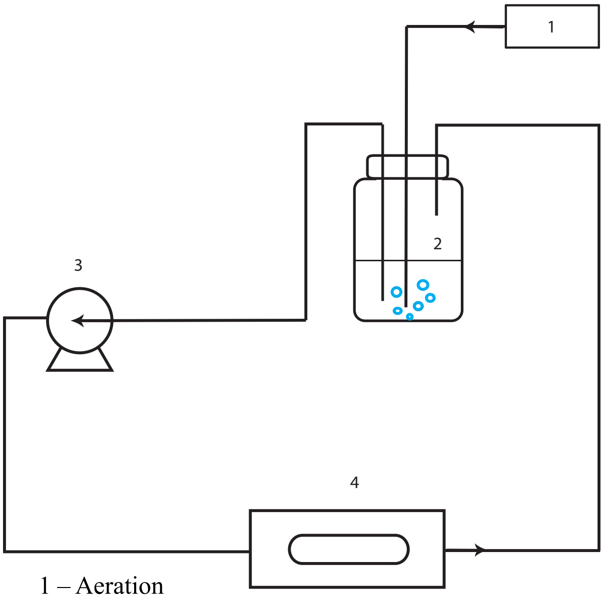
Hydrophobity of Yeast Cells

The yeast populations were prepared in cultivation medium supplemented with different types of cellulosic substrates. Hydrophobity of yeast cell surface was determined using MATH method[3].

Microscopy and Image Analysis

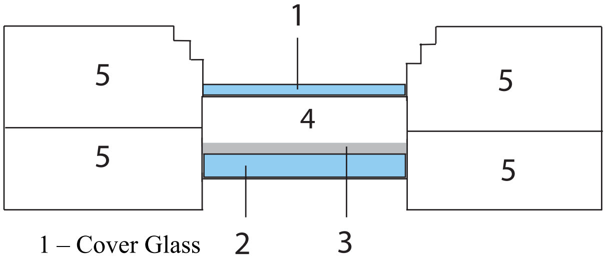
The colonization of cellophane was observed with transmitted light microscopy and analysis of the images was accomplished with Lucia (Laboratory Imaging, Ltd., UK). The areal parameters of objects (colonies, cells) such as length, width and colonized area were measured with image analysis4. The observation area of the flow cell was divided into three fields (inflow, middle, and outflow). Ten images were taken in every field.

- Microscope – NIKON Eclipse E400, Plan Fluor objective, 10×/0.30, Ph1 DLL, ∞/0.17 WD 16.0 (Japan)
- Filter – 45 mm, NCB11
- Camera and software – Canon PowerShot A620, ZoomBrowser EX 5.5



1 – Aeration
2 – Stock bottle with mineral medium
3 – Pump
4 – Flow Cell FC 81

Fig. 2. **Scheme of the FC 81 Flow Cell connection during the experiment**



1 – Cover Glass
2 – Slide
3 – Cellophane
4 – Space for medium flow
5 – Parts of the flow cell

Fig. 3. **Cross-section of the FC 81 Flow Cell**

Table I
Hydrophobity of cell envelopes in dependence on using different types of cellulosic substrates as sole of carbon

Cellulosic substrate	Cell hydrofobity [%]
filter paper	83.8
cellophane	51.8
carboxymethylcellulose	76.4
hydroxyethylcellulose	79.3
Sigmacell Type 101	9.6

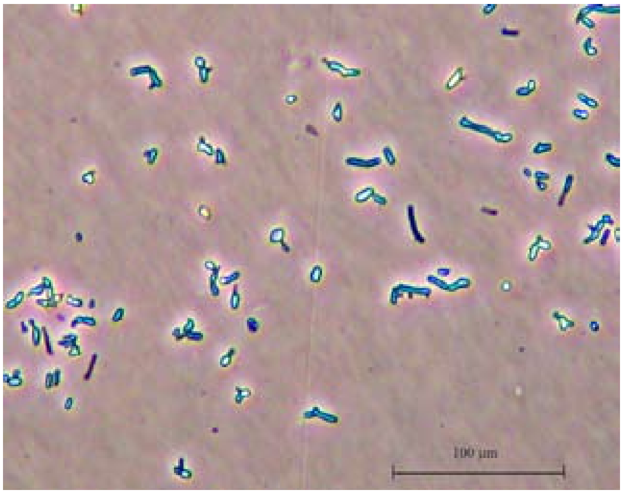


Fig. 4. **Cells of *Trichosporon cutaneum* attached on cellophane (phase contrast)**

Results

Character of Cell Envelopes of *Trichosporon Cutaneum*

The results obtained by MATH method are summarized in Table I. The cell envelopes of the population *Trichosporon cutaneum* grown in the presence of filter paper had the most hydrophobic character (83.8 %), whereas the population cultivated on Sigmacell had very hydrophilic cell surfaces. The hydrophobity of cellophane stripes (95.6 %) was determined at Department of Polymers, ICT Prague. We wanted to choose cells with similar surface characters as the cellophane stripes. Filter paper could not be used for biomass preparation because of low biomass concentration. For this reason HEC was used for following experiments.

Influence of Shear Stress on Cellophane Colonization

Low flow rates of medium are less favourable for the cellophane colonization (Table II). Also small areas

Table II
Influence of shear stress on the cellophane colonization and object morphology changes

Flow rate [ml min^{-1}]	Colonized area [%]	Objects in field	Area [µm^2]	Lenght [µm]	Width [µm]	Elongation*
2	4.86	12	180.98	27.92	5.94	1.76
4	4.42	12	176.64	24.00	6.33	1.81
6	16.07	27	194.27	29.56	5.40	1.76
8	8.49	40	80.42	15.02	5.01	1.69
10	4.25	21	97.32	15.69	5.50	1.74
15	2.38	15	86.32	15.79	5.25	1.97
20	6.08	24	117.22	20.90	5.65	2.18

(on an average 4 %) were colonized as long as the rates are higher than 8 ml min^{-1} (probably the influence of shear stress). 8–16 % area was colonized using the flow rates between 6 and 8 ml min^{-1}. The increase of shear stress had not significant influence on morphological parameters of objects – width and elongation with exception of length and area of objects.

Conclusions

The capacity of *Trichosporon cutaneum* cells to colonize cellophane (used as a carrier and the source of carbon) was proved (Fig. 4.). FC 81 Flow Cell appears to be suitable apparatus for microscopy study of biofilm formation. Image analysis is favourable tool for monitoring and evaluation of biofilm formation.

The flow rate 10 ml min^{-1} was appropriate for study of biofilm formation. However, for the purpose of cellophane degradation more colonized areas are required (6 ml min^{-1}).

The effect of shear stress is one of required information needed for optimalization of these processes. Nutrient starvation, cell suspension density, medium composition etc. are factors that could probably affect biofilm formation.

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P40 SEASONAL CHANGES OF RUBISCO ACTIVITY AND ITS CONTENT IN NORWAY SPRUCE EXPOSED TO AMBIENT AND ELEVATED CO_2 CONCENTRATIONS

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Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase, Rubisco (EC 4.1.1.39) is the most abundant protein on the Earth. This enzyme catalyses carboxylation of D-ribulose-1,5-bisphosphate (RuBP), the first step of the Calvin cycle in competition with oxygenation of RuBP that leads to the photorespiratory pathway. Rubisco is a key enzyme of photosynthesis regulation[1]. It must be reversibly activated with CO_2 and Mg^{2+} before catalysis can occur. The activation in vivo must be facilitated by the presence of a second protein, Rubisco activase[2].

Rubisco activity depends on the amount of enzyme and the activation state of its active sites and changes within several minutes depending on temperature, RuBP supply, irradiance, CO_2 concentration, inorganic phosphate content, and presence of inhibitors in the active site[3]. Rubisco content varies over the time scale of hours and days in dependence on specific saccharide contents (e.g. glucose, sucrose). Increased contents of these sugars lead, via a hexokinase-related signal, to the repression of Rubisco gene expression and subsequent decrease in the content of Rubisco protein[4,5]. Decrease in Rubisco amount may be also the result of non-selective decrease in leaf nitrogen content[6,7]. Many authors[8–10] present that Rubisco content reaches a maximum soon after full expansion of the leaf. Then Rubisco is gradually degraded and its nitrogen is translocated into growing organs during senescence.

Short-term exposure of higher plants to elevated CO_2 concentrations usually increases photosynthetic CO_2 uptake. Long-term growth in elevated CO_2 usually leads to decrease of content and activity of Rubisco. This biochemical adjustment, which is often termed acclimation or down-regulation, reduces photosynthetic capacity[11].

In this work we measured seasonal changes of Rubisco initial and total activities in vitro as well as Rubisco content in Norway spruce needles to answer following quesions:

- Is there a decrease of Rubisco activity and content in Norway spruce during the growing season?
- Is there a down-regulation of Rubisco activity and content in Norway spruce cultivated in elevated CO2 concentration?

Experimental

Materials

The experiment was conducted in 2007 at the experimental site Bílý Kříž in Beskydy Mts. Five-year old seedlings of Norway spruce (*Picea abies* [L.] Karst.) were grown under ambient (A = 375 μmol (CO_2) mol^{-1}) and elevated (E = 700 μmol (CO_2) mol^{-1}) CO_2 concentrations using the glass domes with adjustable windows. Last year needles were sampled between 11 a.m. and 3 p.m. at the following dates: 15th May, 23th July and 10th October. About 0.06 g of needles were weighed and the projected area of these needles was estimated according[12]. Needles were inserted into microtube Eppendorf and put into liquid nitrogen.

Methods

Rubisco activity assay. Needles from one microtube were homogenized in a chilled mortar with 0.02 g inert sand and 5 ml extraction buffer composed of: 50 mM HEPES, 5 mM Na_2EDTA, 5 mM dithiothreitol (DTT), and 1 % insoluble polyvinylpolypyrrolidone, all at pH 8.0. The homogenate was centrifuged at 10,000 × g for 30 s and an aliquot of the supernatant was used immediately for spectrophotometric Rubisco activity assay, based on the continuous measurement of 3-phosphoglycerate-dependent NADH oxidation in a coupled enzyme system[13]. The initial Rubisco activity was determined by adding 20 μl of extract to 100 μl of activation solution which contained 25 mM $KHCO_3$ and 20 mM $MgCl_2$ and 850 μl of assay solution composed of: 50 mM HEPES (pH 8.0), 25 mM $KHCO_3$, 20 mM $MgCl_2$, 5 mM Na_2EDTA, 5 mM DTT, 3.5 mM ATP, 0.35 mM NADH, 3.5 mM phosphocreatine, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase, and 80 nkat creatine phosphokinase. The reaction was started by the addition 30 μl of ribose-5-phosphate (R5P) at final concentration 0.4 mM and the changes in absorbance at 340 nm were immediately measured at 25 °C using Helios γ (Spectronic Unicam, UK) spectrophotometer.

The total activity was measured after 15 min incubating 20 μl of the extract with 100 μl of activation solution. 850 μl of assay solution were added, the reaction was again started by adding 30 μl of R5P at final concentration 0.4 mM and the changes in absorbance at 340 nm were measured.

Rubisco content determination. Needles from one microtube were homogenized in a chilled mortar with 0.02 g inert sand and 2 ml extraction buffer containing 62 mM Tris, 2% (w/v) sodium dodecyl sulphate (SDS), 65 mM DTT, and 10% (v/v) glycerol, all at pH 6.8. The homogenate was centrifuged at 10,000 × g for 2 min, 0.5 ml of the supernatant were added to 0.5 ml sample buffer composed of 3% (w/v) Tris, 5% 2-mercaptoethanol, 10% (w/v) SDS, 20% (v/v) glycerol, and 0.2% (w/v) bromophenol blue and the mixture was incubated 5 min at 100 °C.

Rubisco content was determined by SDS-PAGE with a Mini-PROTEAN 3 system (Bio-Rad). Resolving gels contained 10 % (w/v) acrylamide, 0.27 % (w/v) N,N′-methylene-bis-acrylamide, 0.37M Tris-HCl (pH 8.8) 0.1 % (w/v) SDS, 0.04 % (v/v) N,N,N',N'-tetramethylethylene-diamine (TEMED), and 0.1 % (w/v) ammonium persulphate. Stacking gels contained 5 % (w/v) acrylamide, 0.13 % (w/v) N,N´-methylene-bis-acrylamide, 0.19M Tris, 0.02 % (v/v) TEMED, and 0.1 % (w/v) ammonium persulphate.

A 5 μl of sample solution were loaded on polyacrylamide gel. Electrophoresis ran 60 min at the constant voltage 140 V. The large subunit of Rubisco was detected by staining with Coomassie brilliant blue and its identity was confirmed by co-electrophoresis of Rubisco standard (Sigma-Aldrich) (Fig.1). Quantification of individual bands was performed on HP Scanjet 5590P with programme Advanced Image Data Analyzer, ver. 3.23.001 (Raytest, Germany).

Results

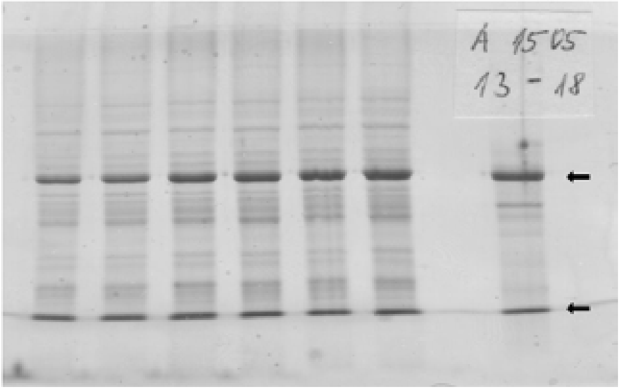

Fig. 1. **A representative SDS-PAGE gel with separated proteins from Norway spruce needles (six left columns) and Rubisco standard 1 mg ml^{-1} (the first column from the right). The upper arrow indicates the large subunit of Rubisco protein and the lower arrow indicates the small one**

Both initial and total activities in E were lesser than those in A with the exception of autumn. It is in good accordance with data of other authors[14–16]. Maximum of activities we measured in May and then they gradually decreased. Rubisco initial activity in A decreased about 57 % while in E dropped about 41 % during the growing season. Total activity decreased about 66 % in A and about 44 % in E. Rubisco content dropped about 55 % in A and about 50 % in E. The sharp diminution of Rubisco content in E in July could be caused by enormous drought in the first half of summer in combination with the phenomenon that elevated CO_2 concentrations accelerate the senescence of leaves[6,8,10]. Both initial and total activities as well as content decreased less in E than in A (Fig. 2.). We hypothesised that down-regulation in E – if there was any – proceeded in the first year of needles life.

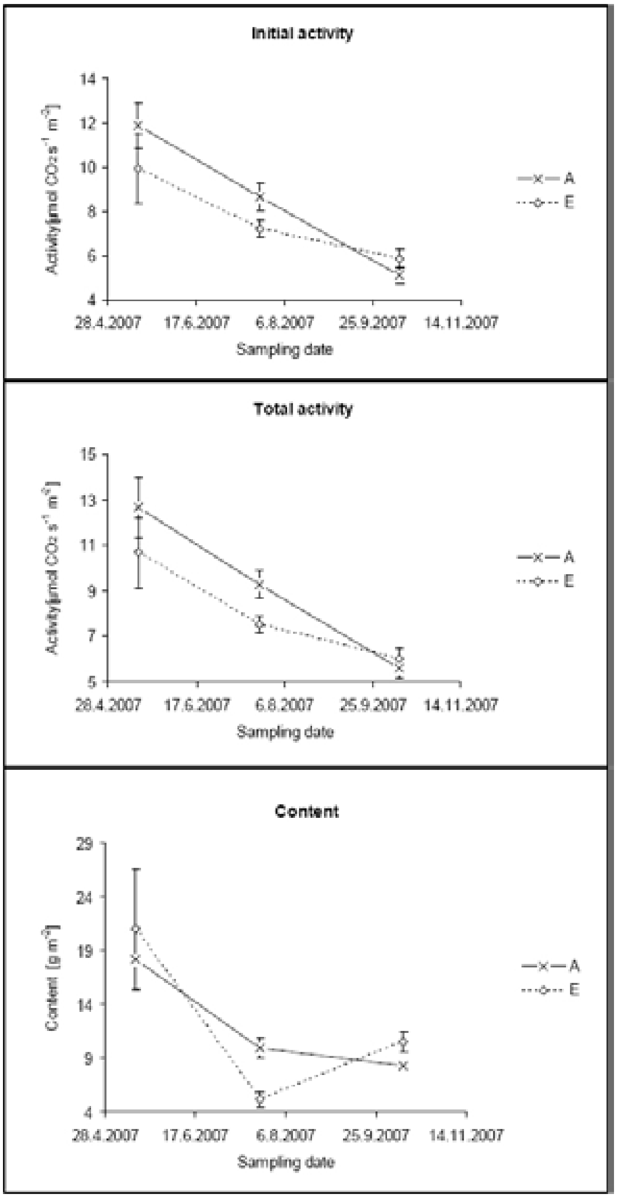

Fig. 2. **Rubisco activity and content in Norway spruce, A – ambient [CO_2], E – elevated [CO_2], n = 10**

Conclusions

We confirmed a decrease both Rubisco initial and total activities as well as Rubisco content in Norway spruce during the growing season. According to our measurements, there is not down-regulation of Rubisco activity and its content in last year needles in Norway spruce during the growing season.

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P41 LIPID DEGRADATION AND ITS APPLICATION TO LIPID-CONTAINING WASTEWATER

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Introduction

Food industry and restaurants produce each year about 15 % of the total wastewater and 50 % of the organic pollution. Character and composition of wastewater depends on manufacturing technology and current raw material. Wastewater from food industry and restaurants contain lipids. These lipids are present in the wastewater. They are difficult to remove and degrade, because they are difficult to dissolve in the water[1].

For facilitation of production unit and treatment wastewater fats and oils, enzymatic preparations (EP), which are used, they are accelerated decomposition of organic material lipids matter. These EP mix of lipases, proteases, amylases and cellulases, some of them contain suitable combination of bacteria, and they contain detergents and surfactants, as well.

In this study was evaluated 5 EP for their ability to degraded lipids in the wastewater. It was made isolation of lipid-degrading bacteria and the proof of lipolytic bacteria. They investigated their lipolytic activities at agar plates by aerobic and anaerobic conditions. Further ability of lipid degradation and fatty acids utilization at submerged cultivation and chemical oxygen demand was followed.

Experimental

Enzymatic preparations are mixtures of enzymes and bacteriological cultures. These preparations were inoculated agar plates with tributyrin agar (tributyrin agar base). Plates were incubed for 7 days at 27 °C for aerobic and anaerobic conditions. The growth colonies were used for determination of the lipolytic activity.

Samples for determination of lipid degradation and utilization fatty acids were taken from submerged cultivation The medium for submerged cultivation contained per liter distilled water 1.12 g K_2HPO_4; 0.48 g KH_2PO_4; 5 g NaCl; 0.1 g $MgSO_4 \cdot 7H_2O$; 2 g $(NH_4)_2SO_4$ and 100 µl EDTA. Medium was autoclaved at 121 °C for 20 min and then the medium was supplemented with 1 ml olive oil, as the natural substrate. In the end, the medium was inoculated by enzymatic preparations. Medium were incubated under aerobic conditions at 27 °C and agitated at 160 rev min^{-1} for 14 days in Erlenmeyer flasks in a shaker. The % free fatty acids were determined as an indication of the olive oil degradation by the tested enzymatic preparations[1].

Spirit Blue Agar (Detection of Lipolytic Activity)

Microoragnisms was grown on spirit blue agar plates, to which tributyrin and tween 80 were added as a lipase substrate in ratio 1 : 1. Plates were incubated at 27 °C. Lipolytic activity was identified the plates as a transparent halo around the colonies after 7 days of incubation[2].

Enzyme assay

The activity was determined by using culture in 0, 65 ml 0, 05 M phosphate buffer (pH 7.2) a 0.1 ml 0.025M pNP-laurate in ethanol. The hydrolytic reaction was carried out at 37 °C for 30 min, after which 0.25 ml 0.1M Na_2CO_3 was added. The mixture was centrifuged and the activity determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme which liberates 1 µg p-nitrophenol from p-NP-laurate, as a substrate in 30 min under assay condition[3,4].

Determination Lipid Degradation and fatty Acids Utilization

From each Erlenmeyer flasks 20 ml culture medium was aseptically drawn and transferred to a separating funnel, where it was mixed with 20 ml of hexane. The mixture was agitated for 2 min and the upper layer was put to the clean and weight beaker. The lower layer was re-extracted by a fresh 20 ml of hexane and the upper layer after the extraction was collected to the beaker again. The extract in the beaker was evaporated by heating at 100 °C. Then the dry extracted lipids were weight and dissolved in 50 ml of alcohol in the presence of phenolphethalene indicator. The solution was titrated with 0.1M KOH until the developing of pink color. The same procedure was repeated within 2, 6, 8, 10 and 14 days. The free fatty acids % in the sample was indicated lipid degradation and fatty acids utilization, which was calculated according to this equation:

$$\%\text{free fatty acids} = \frac{V_{\mathrm{KOH}} \cdot c_{\mathrm{KOH}} \cdot M \, . \, 100}{1000 \, . \, m}, \qquad (1)$$

where V_{KOH} is the volume of 0.1M KOH at the end point, c_{KOH} actual concentration of the 0.1M KOH, M is the molecular weight of the oleic acid and m is the weight of the dry extract[1].

Chemical Oxygen Demand (COD)

Chemical oxygen demand (COD) is a measure of the capacity of the water to consume oxygen during the decomposition of organic matter and the oxidation of inorganic chemicals such as ammonia and nitrite. COD measurements are commonly made with samples of wastewater or natural water, contaminated by domestic or industrial waste.

COD was determined by the standard potassium dichromate method, descriebed in the Methods for the Examination of Waters and Associated Materials (2007).

At 80 ml of water, 2 ml of the oil and 50 µl enzymatic preparations was added in to the separatory funnel. One of the separatory funnel was used as a control. The volume of the separatory funnel was mixed and within 20 min the sample of the water phase was taken to determine chemical oxygen demand (COD).

Results

Spirit Blue Agar (Detection of Lipolytic Activity)

The lipolytic microorganisms metabolized the lipid in the medium and form colonies with halos indicating lipolysis. It was found out, that in all tested microorganism preparations were presented and formed colonies with halos indicated lipolysis.

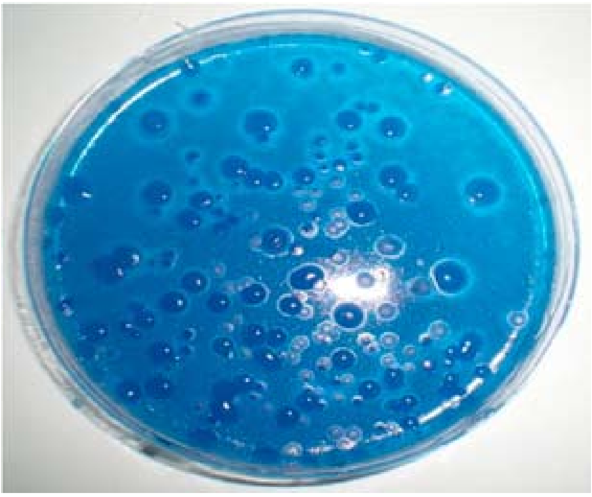

Fig. 1. **Bacterial colonies with halos indicating lipolysis**

Lipolytic Activity

Cultivation on agar plates. The highest value of the lipolytic activity was determined at EP 1, the lowest lipolytic activity was determined at EP 3.

The highest calculated value was found in supernatant of EP 1 by anaerobic conditions, value of the activity was $a = 9.16 \times 10^{-3}$ mmol min^{-1}, the lowest value was in sediment of EP 3 by aerobic conditions, ($a = 1 \times 10^{-4}$ mmol min^{-1}). Lipolytic activity was detected at all five enzymatic preparations.

Table I
Lipolytic activity in 5 of enzymatic preparations (expression $a \times 10^{-3}$ mmol min^{-1})

EP	aerobe		anaerobe	
	supernatant	sediment	supernatant	sediment
1	7.11	1.96	8.79	2.95
2	6.59	1.40	8.51	2.86
3	5.04	0.10	7.25	1.77
4	5.44	1.19	7.42	2.48
5	8.52	2.32	9.16	3.28

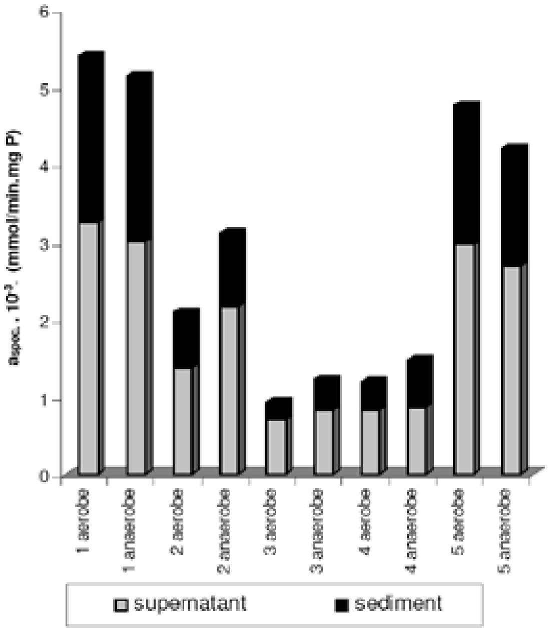

Fig. 2. **Comparsion values of the lipolytic activity for the individual enzymatic preparations**

Submers cultivation. The highest activity was measured on the 10th day of the cultivation. The decrease of activity was registered on the 10th day. The highest activity had the enzymatic preparation 5 and the lowest had the enzymatic preparation 3.

Table II
Relativic activity a_{rel} [%] in enzymatic preparations during submers cultivation

EP [days]	1	2	3	4	5
2	62.97	53.09	46.06	50.32	73.56
6	83.26	74.35	66.98	68.69	85.46
8	84.73	78.74	75.54	77.82	94.15
10	100.00	100.00	100.00	100.00	100.00
14	75.52	68.73	62.23	63.50	90.00

3.3 Lipid Degradation and Fatty Acids Utilization

Results of the determination demonstrate at Table III and at Fig. 3.

Table III
% free fatty acids of EP during submers cultivation

EP1	EP2	EP3	EP4	EP5	days
9.69	10.07	14.87	3.68	3.72	2
13.04	37.74	19.02	4.75	25.43	6
48.91	48.42	33.28	7.13	39.02	8
66.57	56.5	40.75	37.66	48.04	10
20.1	22.83	26.49	20.38	26.15	14

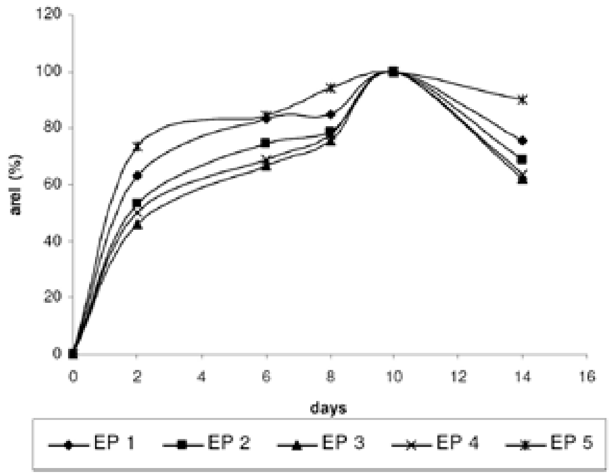

Fig. 3. **Comparsion individual EP during 14 day's submerged of cultivation**

Determination of COD

Results of determination of COD show Table IV, the best values were reached at the EP 1 and EP 5.

Table IV
Chemical oxygen demand

EP	1	2	3	4	5	control sample
c [mg O_2 dm^{-3}]	61	89	143	94	64	31

Conclusions

There were tested 5 commercial preparations with aim to find out, if they have lipolytic activity and if they have ability to degrade fats. Samples were cultivated at the agar plates and submerged cultivation in Erlenmeyer flasks. The comparison results of lipolytic activities of both cultivations were followed. The submerged cultivation, after 2 days of EP demonstrated approximately the same value of activities as cultivation on agar plates after 7 days.

All tested EP had ability to degraded olive oil and released fatty acids. The best degrading ability had EP 1, the lowest ability EP 4.

It was made a laboratory verification of function 5 EP for liquefaction and decomposition of fats and oils in the wastewater. On the basis obtained by dates, it might be choosen the best EP for suggestion technology. Biotechnology effects were made out for EP 1 and 5, the lowest efficiency was made out by EP 4.

This work has been supported by Ministry of Education, Youth and Sports under project MSM 021630501.

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P42 GAS CHROMATOGRAPHY-MASS SPECTROMETRY CHARACTERIZATION OF SEA BUCKTHORN

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Introduction

Besides naturally growing plants, sea buckthorn (*Hippophae rhamnoides)* hybrids of different subspecies are cultivated in order to prevent desertification, since it forms strong root and it is resistant to extreme temperature, drought and poor soil. There is a long tradition in Asia for using sea buckthorn berries in folk medicine. It was demonstrated that the oils extracted from pulp and seeds have regenerating, anti-inflammatory, anti-ulcerogenic, hepatoprotective, cytoprotective properties.[1–4] Sea buckthorn berries are exceptionally rich in both lipophilic and hydrophilic bioactive compounds and represent an important resource for pharmacy and food industry. The pulp oil has a special composition with a very high content of unusual palmitoleic acid, up to 54 % of total fatty acids, while the seeds oil is rich in linoleic and α-linolenic acids. Other important compounds of sea buckthorn oil are sterols, tocopherols and tocotrienols and their content depend on the berries origin, growth condition and oil processing method.

The aim of this study was to develop a sensitive analytical method for determination of fatty acids, vitamin F and E and sterols in sea buckthorn oil, by gas chromatography/mass spectrometry (GC/MS). The method was applied for testing the lipophilic compounds after the treatment at different kGy doses with accelerated electrons.

Experimental

Materials and Methods

Standards of fatty acids methyl esters and acetyl chloride were from Fluka (Buchs, Switzerland), α-tocopherol standard was from Sigma-Aldrich (Munich, Germany). All other chemicals were from Comchim (Bucharest). The oil and plant samples were from Hofigal (Bucuresti).

GC-MS and HPLC

A Trace DSQ ThermoFinnigan quadrupol mass spectrometer coupled with a Trace GC was used. The methyl esthers of the fatty acids were separated on a Rtx-5MS capillary column, 30 m × 0.25 mm, 0.25 µm film thickness, using a temperature program from 50 °C (1 min) to 310 °C, at 8 °C min^{-1}; then kept 8 minutes at 310 °C. The conditions used for GC-MS were: transfer line temperature 250 °C, injector temperature 200 °C; ion source temperature 250 °C; Splitter: 10 : 1. Electron energy was 70 eV and emission current, 100 µA.

Extraction and Derivatization

Fatty acid methyl esters (FAME) were obtained by esterification reaction of known quantities of oil with 100 µl 3M HCl methanol at 100 °C for 60 min followed by evaporation to dryness under N_2 stream 0.1 mg of the internal standard C11 : 1 was added at 20 µl oil. Then the sample was solved in 100 µl DCM or ethyl acetate, and 3 µl were injected into GC-MS. Vitamin E and sterols were also determined in the different samples.

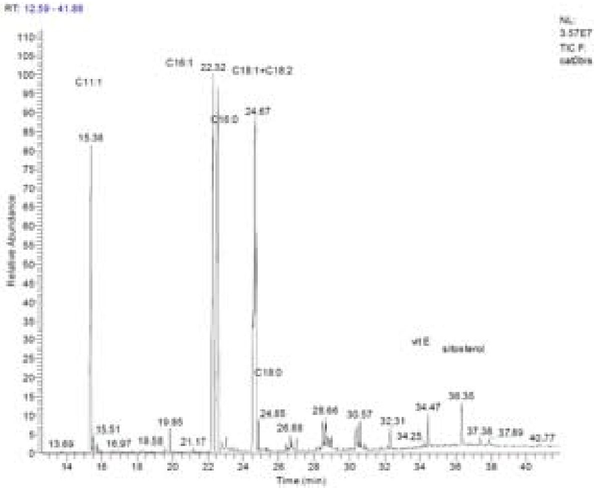

Fig. 1. **FAME, vitamin E and sterols separation in sea buckthorn oil**

HPLC Analysis of α-tocopherol

The sea buckthorn oil samples were injected into HPLC for vitamin E determination. The sea buckthorn oil was saponified with 60% KOH in ethanol, in the presence of ethanol and pyrogallol, at 75 °C for 45 min. The sample mixture

Table I
The compounds identified in sea buckthorn oil

Compounds	RT [min]	area [%]
Methyl myristate (C14 : 0)	19.85	0.79
methyl palmitoleate(C16 : 1)	22.36	30.68
methyl palmitate(C16 : 0)	22.63	24.53
methyl oleate +linoleat (C18 : 1 + C18 : 2)	24.73	37.18
methyl stearate (C18 : 0)	24.89	0.63
C20 : 1	26.83	0.55
methyl arachinate (C20 : 0)	27.01	0.3
C22 : 1	28.93	0.86
behenic acid methyl ester (C22 : 0)	29.03	0.22
C24 : 1	30.57	0.41
methyl lignocerate (C24 : 0)	30.89	0.14
C26 : 0	32.31	0.26
vitamin E	34.47	0.6
sitosterol	36.36	2.13
lupeol	37.41	0.42
urs-12-en-28-ol	40.8	0.31

was extracted with hexane: ethyl acetate (9 : 1). The organic layer was washed with 5% sodium chloride solution and then evaporated to dry and dissolved in methanol. HPLC system contained LC20 AT Shimadzu pumps and a Waters 990 PDA detector. A Supelco Discovery C18 (25 cm × 4.6 mm, 5 µm particle diameters) HPLC column was used. The mobile phase consisted in methanol: water (98 : 2) and the chromatogram was monitored at 292 nm. Tocopherol identification was based on the retention time of α-tocopherol standard (Sigma) and UV-VIS spectra. Quantitative analysis was done using a calibration curve with α-tocopherol (0.01–0.2 mg ml^{-1}), r = 0.989.

Results

The results obtained for the sea buckthorn oil by GC-MS are presented in Table I. The results obtained for sea buckthorn vitamin E at different doses are shown in Table II.

Table II
Vitamin E determination in sea buckthorn oil by HPLC at different doses [kGy]

Dose [kGy]	α-tocopherol [mg 100 ml oil^{-1}]
0	57.75
0.03	50.52
0.07	29.1
0.38	26.8
0.72	25.9
1.05	25.8

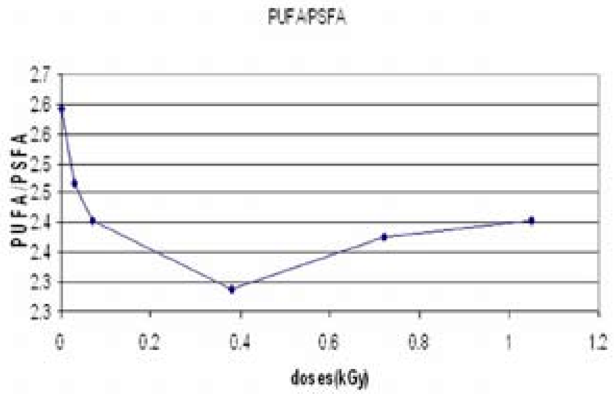

Fig. 2. **The ratio between unsaturated and saturated fatty acids evolution with doses [kGy] in sea buckthorn oil**

Vitamin E decreased two times at 1.05 kGy treatment (Table II). In sea buckthorn oil, at the highest dose applied, the ratio between unsaturated and saturated fatty acids has decreased at half value of control (Fig. 2.). Also the sterols and vitamin E values have decreased with irradiation more than two times, as presented in Fig. 4. Similar results have been obtained in juice of the fruits. In sea buckthorn leaves the changes are not significant (Fig. 3. and Fig. 5.).

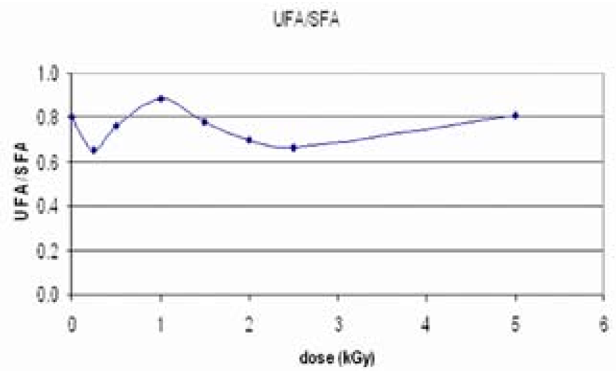

Fig. 3. **The ratio between unsaturated and saturated fatty acids evolution with doses [kGy] in sea buckthorn leaves**

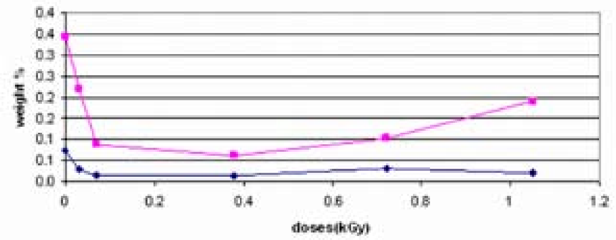

Fig. 4. **Vitamin E and sitosterol [% weight] versus applied doses (GC-MS determination)**

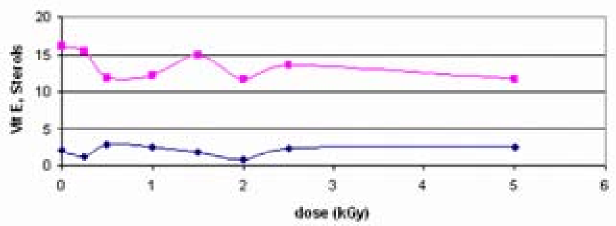

Fig. 5. **E vitamin and sterols [area %] with doses [kGy] in sea buckthorn leaves**

Conclusions

The GC-MS and HPLC methods developed showed significant changes of antioxidants as vitamins E, F and sterols with doses after the treatment of sea buckthorn oil, juice but not in leaves at different kGy doses with accelerated electrons. The treatment for decontamination needs to be optimized to avoid loses of its important nutrients.

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P43 RAPID AUTHENTIFICATION OF NATURAL JUICES BY GC-MS

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Introduction

A simple and rapid GC-MS method for the detection of adulteration of natural fruit juices is presented. The method consists of the analysis of sterol patterns and it is a useful rapid approach for the fruit juices control by GC-MS[1].

A GC-MS analytical method is described for some natural juices analysis. The fingerprint of sterols was used to characterize the natural juice.

Experimental

A rapid liquid-liquid extraction method was used. The fruit juices were extracted by using petrol ether as a solvent. The fruit juice extracts were separated on a capillary column, and identified with a mass spectrometer.

Apparatus

The sterols were separated on a Rtx-5MS capillary column, 15 m × 0.25 mm, 0.25 µm film thickness, in a temperature program from 50 °C for 1 min, then ramped at 15 °C min^{-1} to 300 °C and held for 15 min. Identification of sterols and their patterns were used for juice characterization.

A Trace DSQ ThermoFinnigan quadrupol mass spectrometer in the EI mode coupled with a Trace GC was used. The following conditions were used: ion source temperature 250 °C, injector temperature 200 °C, transfer line temperature 250 °C, splitter 10 : 1, electron energy 70 eV and emission current 100 µA.

Extraction Procedure

Ethanol, fruit juice and petrol ether 2 : 2.5 : 0.4, v/v/v, were mixed in a cap vial for 2 minutes. Then the mixture was centrifugated 2 minutes. 1µl was injected twice into the GC. Ethanol was used for pectine and emultion agents precipitation.

Results

Different compounds such as volatile aroma compounds, fatty acids and sterols have been identified into the cromatograms of the extracts of orange (Fig. 1.), grapefruit and pineapple juices. A NIST library was used for compounds identification from the mass spectra obtained after gas chromatographic separation. The sterols identified from the fruits juices were the following: cholesterol, campesterol, ergostanol, stigmasterol, beta-sitosterol, the main sterol, isofusterol and citrostadienol. Limonen was the main volatile compound extracted from citrus fruits.

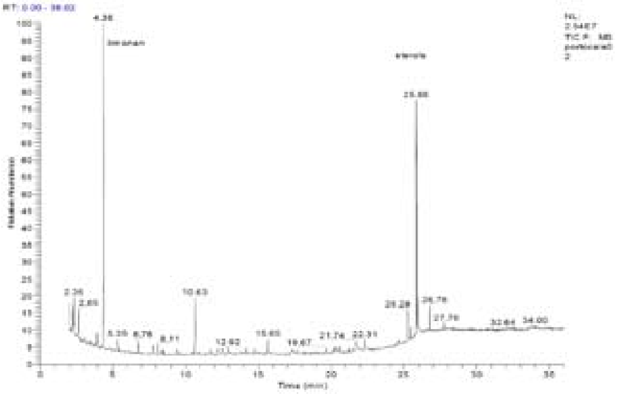

Fig. 1. **Compounds separation from orange extract**

Fig. 2. presents the separation of sterols from the fruit juice extract (orange). The fingerprint chromatograms of different fruits differ in the concentration of this sterols, but there are also few variation of the sterols identified in orange, grapefruit and pineapple juices In pineapple was identified stigmastenone at the retention time of citrostadienol (alpha 1 sitosterol).

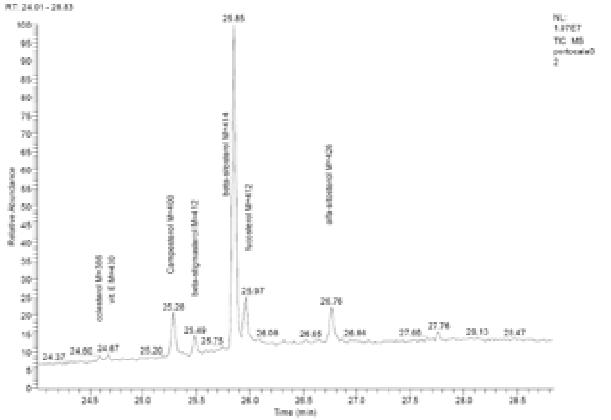

Fig. 2. **Separation of sterols from orange extract**

Table I
Sterols identified in orange juice extract

Tr [min]	Compound	M	m/z
11.3	Valencene	204	161
14.6	Nooklactone	218	147
25.3	Campesterol	400	400
25.4	Ergostanol	402	233
25.5	beta-stigmasterol	412	412
25.9	beta-sitosterol	414	414
25.97	isofucosterol	412	314
26.8	citrostadienol	426	285

Table I presents the compounds identified into the chromatogram of the orange juice extract.

Table II presents the important ions of the compounds identified in juices. The ratio of different peaks area from

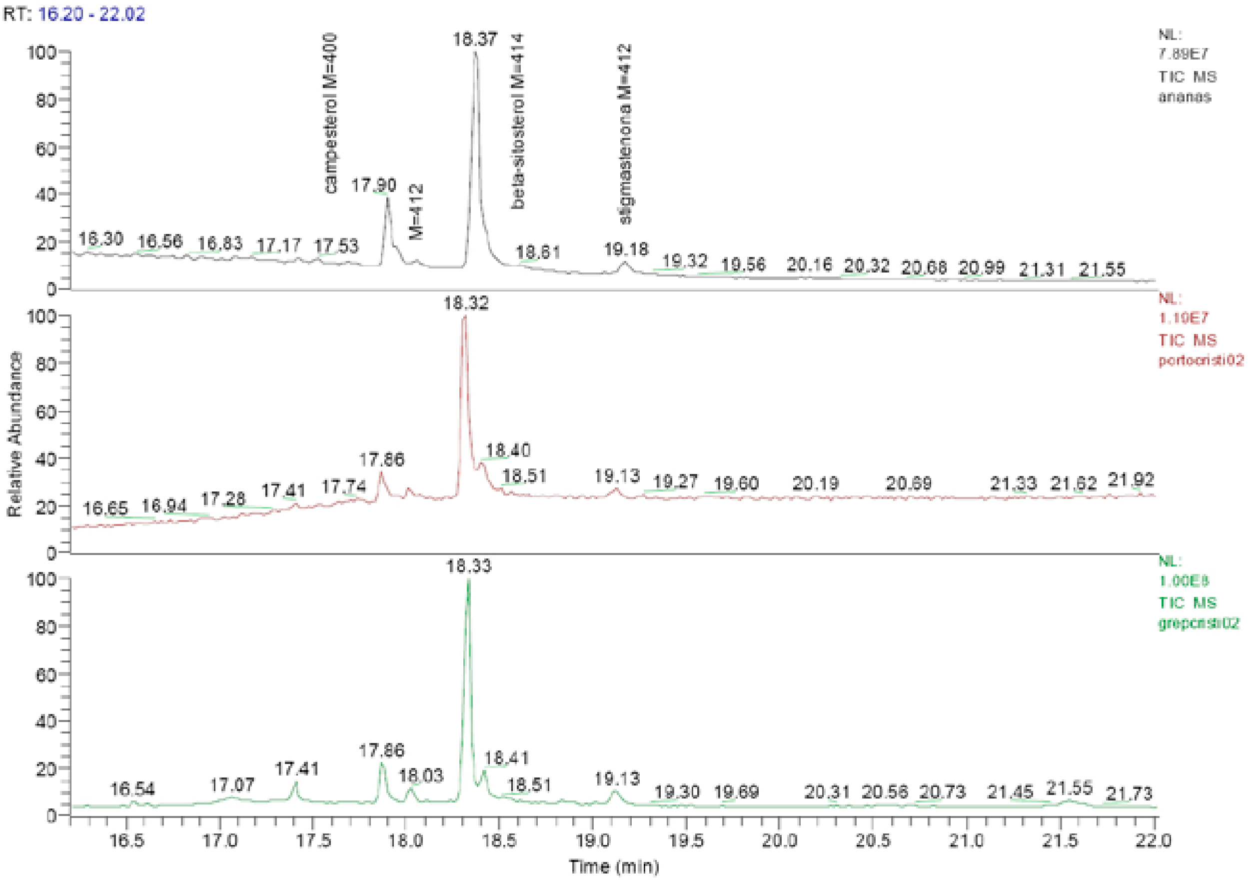

Fig. 3. **Comparison of sterol profiles from pineapple, orange and grapefruit extracts in a faster temperature program**

Table II
Relative intensity of the important ions of the sterols identified in orange juice extract

Compound	Base peak, m/z	Important ions m/z, rel. intensity [%]
Valencene	161	91, 93 (65); 105, 107 (60), 133, 204 (50); 147, 189 (37)
Nooklactone	147	79, 121 (84); 91, 133 (65), 105, 175, 203(56)
Campesterol	400	315, 382, 367, 289 (50), 145, 213, 255 (30)
Ergostanol	233	424(99), 203, 205 (20), 397 (30)
beta-stigmasterol	412	255, 271, 300 (50), 351 (33)
beta-sitosterol	414	329, 396 (50), 303, 381 (40)
isofucosterol	314	281 (34), 229 (26), 315(25)
citrostadienol	285	328, 286, 267 (35)

the mass spectrum can be used for a better evaluation of the differences among juices, the base peak but also some other important ions could be used for authenticity evaluation of the natural juice, as adulteration of grapefruit more expensive juice with orange juice.

The sterol profile is a useful approach for confirming the presence of juices of orange, grapefruit, pineapple and passion fruit in compounded beverages and for detecting of adulteration of fruit juices.

Conclusions

The developed method is very useful for fruit juices authentification. The method can be used in the control of adulteration of juices in the food industry. Sensitivity of the method could be 100 times improved in the SIM mode.

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P44 COMPARATIVE EXTRACTION METHODS OF SOME BIOLOGIC ACTIVE COMPOUNDS IN HERBS

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Introduction

Many studies are made for the optimization of the experimental design approach for obtaining the best recoveries, low solvent consumption and reduced extraction times. In the last years the number of procedures using extraction of organic compounds from different matrices has increased[1,2].

The aim of the present work is to present a comparison between some extraction methods, for qualitative characterization of flavors extracted from herb plants of different sources.

Experimental

A liquid-liquid extraction method (LLE), compared with two different solid-phase extraction methods (SPE- on 300 mg RP-18, C-18 silica bonded and 300 mg TCS), a microwave one (MWE) and an ultrasonic extraction one (USE) were compared using an aroma compounds standard mixture. The analytical method chosen were gas chromatography and gas chromatography – mass spectrometry (GC-MS).

Materials and Methods

A Hewlett Packard GC 5890 couplet with a MS engine 5989B in the EI mode was used for compounds identification. The GC was equipped with a HP-5MS capillary column 30 m × 0.25 mm diameter, 0,25 µm film thickness, in the temperature program: 50 °C for 2 min, then increased to 250 °C with a rate of 8 °C min^{-1}, helium flow rate 1 ml min^{-1}. Deactivation by treating the injector glass liner with 5% dimethyldichlorosilane in toluene was very important for a better sensitivity. The GC/MS interface line and the ion source were maintained to 200 °C, and quadrupol analyser at 100 °C. Electron energy was 70 eV and electron emission 300 µA.

Methanol, methylen chloride, hexane, ethyl acetate, acetone were purchased from Comchim (Bucharest, Romania). The standards were from Fluka, Sueden: 1. 3-Hepten-2-one, used as external standard (ES), 2. 1,8-cineol (eucalyptol) 3. linalool 4. geraniol 5. alpha-terpinyl-acetate 6. geranyl acetate 7. amyl salicylate 8. myristic acid (C14 : 0) 9. palmitic acid (C16: 0) 10. Stearic acid (C18 : 0) . FAME were from Polyscience Corporation, Evantson, Illinois, USA. The standards, 100 µl each, formed the *mixture M*, except of the external standard, 3-hepten-2-one, added separately before extraction. Cartridges of 300 mg RP-18 and TSC were obtained from Merck. A stock solution was obtained by diluting 100 µl of each compound. Working standard was prepared by diluting the stock solution to obtain a concentration of 3.3 % vol.

LLE Extraction

A mixture of three solvents (S) was prepared: ethyl acetate: hexane: methylen chloride (5 : 1 : 1, v/v/v). The LLE extraction procedure was: 30 µl *mixture M* in 1 ml solution distilled water: ethanol (1 : 1, v/v), or 1 ml hydroalcoholic flavour extract), 1 ml distilled water and 0.33 ml *solvent S*, (3 : 3 : 1, v/v/v) were mixed 1.5 minutes and then centrifugated 2 minutes. 1 µl 3-hepten-2-one, was added to the supernatant and then 1 µl was injected two times by using the autosampler injector.

SPE Extraction

The solid phase was conditioned with 3 ml methanol and 3 ml distilled water. After sample application, washing and drying 10 minutes at vacuum, the sample was eluted with 3 × 0.3 ml solvent. The solvent was *solvent S* in the case of RP-18 cartridges and chloroform-acetone (1 : 1, v/v), in the case of TSC cartridges. After adding of 1 µl of the external standard to the eluate, 1 µl was injected by using the autosampler injector. Each sample was injected twice.

MWE Extraction

The microwave extraction procedure was performed at 2,45 GHz for 4 sec, to a temperature of 60 °C, in a screw cap vessel. 30 µl *mixture S* was added to 1 ml hydroalcoholic solution, 1 ml distilled water and 0.33 ml *solvent S* were placed in the microwave funnel and extracted. Then 1 µl 3-hepten-2-one (ES), was added to the supernatant and analyzed twice.

USE Extraction

The ultrasonic extraction procedure was performed 1min, at a temperature of 60 °C. The ultrasonic probe was placed in the vessel containing 30 µl *mixture S* in 1 ml hydroalcoholic solution, 1 ml distilled water and 0.33 ml *solvent S*. After extraction, 1 µl 3-hepten-2-one (ES) was added to the supernatant and analyzed.

Table I
Recovery [%] obtained by different procedures

Component	LLE	SPE	SPE	MW	US
heptenone					
1,8-cineole (eucalyptol)	98.97	81.47	68.54	100.84	99.75
linalool	96.67	86.96	72.07	100.98	100.50
geraniol	94.31	87.54	77.91	111.48	112.12
alfa-terpenyl acetate	97.57	86.13	73.52	102.26	100.37
geranyl acetate	96.28	83.14	72.75	102.57	99.86
amyl slicilate	97.23	85.72	73.99	120.91	118.03
C14 : 0	100.82	86.24	75.07	98.02	95.04
C16 : 0	97.14	86.73	75.87	96.91	92.95
C18 : 0	96.93	85.54	74.68	95.74	92.07

Results

The average values resulted from two or three extraction procedures and two injections of each extract. The relative standard deviation values were bellow 3 % for each extraction procedure. The mean values for the recoveries found for each aroma compound studied are presented for different extraction modes in Table I. The best values for recovery were found for MWE (103 %) and USE (101 %), followed by SPE (85.5 %) on RP-18 and LLE (97.3 %).

Table II
LLE precision characterization for vermut extracts

Compound	mean	SD	RSD [%]
eucalyptol	8.86	0.14	1.58
fenchone	2.34	0.05	2.35
linalool	30.65	0.31	1.02
terpinen	2.96	0.06	2.16
geraniol	2.12	0.05	2.35
cinnamaldehyde	0.56	0.05	8.89
trans-anethole	4.2	0.09	2.22
thymol	1.55	0.06	4
alpha-terpenyl acetate	1.74	0.07	4.06
isobornyl isovalerate	2.07	0.14	6.76
eugenol	2.9	0.11	3.73
miristicin	1.14	0.05	4.8
elemicin	1.08	0.03	3.16
herniarine	0.93	0.05	5.24
bisabolol oxid A	1.04	0.03	3.17
myristic acid	1.24	0.07	5.7
ethyl miristate	1.39	0.14	9.78
lactone	0.78	0.05	6.37

Separation chromatogram is presented in Fig. 1. The LLE method (3 : 3 : 1, v/v/v) was used for studies on vermut, bitter and herbs aroma compounds. The relative standard deviation values found for four different extraction procedures injected twice were bellow 5 % in the case of vermut and also for bitter aroma compounds (n = 4).

Tables II and III present the precision obtained for different aroma compounds extracted by LLE from vermut and respectively from bitter (n = 4). The identification of the compounds was obtained by GC/MS.

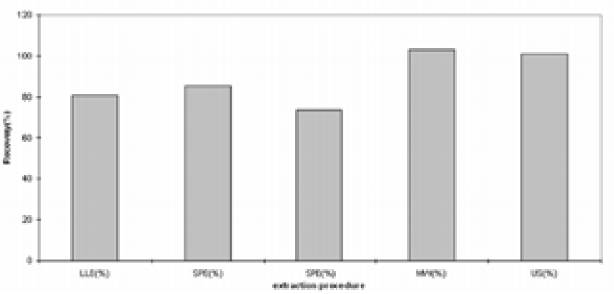

Fig. 2. **Recovery comparison for different extraction methods for the standard mixture**

Table III
LLE precision characterization for bitter extracts

Compound	mean	SD	RSD [%]
eucalyptol	0.34	0.001	0.29
fenchone	2.51	0.03	1.05
trans-sabinene hydrate	1.23	0.04	2.93
p-anis aldehyde	1.35	0.15	10.76
cinammaldehyde	1.61	0.05	2.79
trans anethol	8.87	0.42	4.68
eugenol	71.79	0.71	0.98
coumarin	0.86	0.05	6.15
miristicin	1.11	0.01	1.04

Conclusions

This work presents a comparison of different extraction methods for the characterization of some aroma compounds. The methods are suitable for the determination of trace amounts of aroma compounds in plant extract.

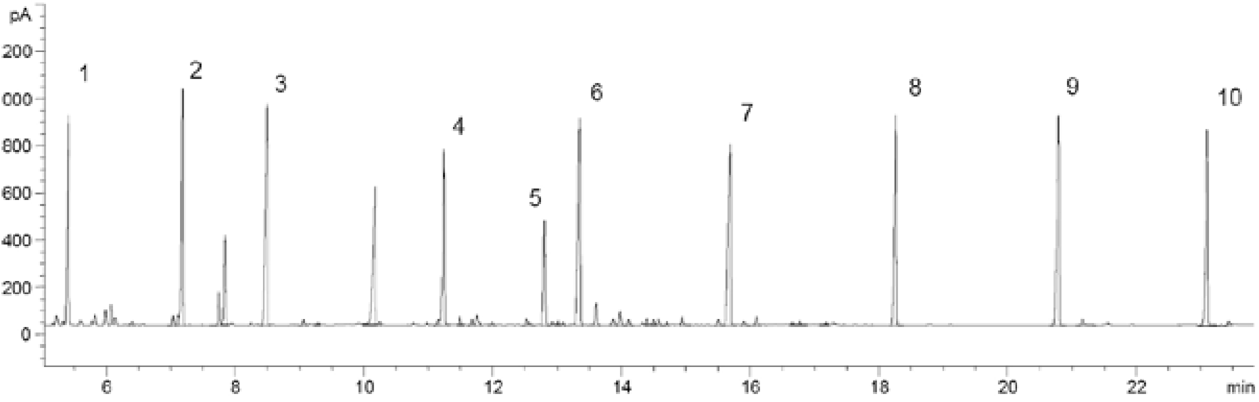

Fig. 1. **Separation chromatogram for the standard mixture**

Good recovery mean values were obtained by LLE (97 %) using an appropriate mixture of solvents comparable with the recoveries of the other extraction methods as SPE (86 %), MWE (103 %) and USE (101 %). The characterization of bitter and vermut aroma by LLE method showed R.S.D. lower than 5 %. The comparison of flavours of herbs from different sources showed qualitative and quantitative differences.

This work has been supported by the Romanian Scientific Research Agency (Grant ID_501 number).

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P45 BIOREMEDIATION OF BOTTON SEDIMENTS USING *BACILLUS MEGATERIUM* AND *BACILLUS CEREUS*

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Introduction

The water reservoir Ružín I. lies in an area, which for several centuries has been known for mining and metallurgical activities. The bottom sediments are contaminated with heavy metals, in the concrete with Hg, Cu, Mn, Zn, Ni and Cd, which were washed away into the water reservoir from locations of former mining activities (Cicmanova et al., 2003). Enhanced amounts of heavy metals preclude of direct utilization of sediments in agricultural and building industry and also in ground shaping (Brehuv, J., 2000).

The availing of bioleaching techniques on mobilization of heavy metals from sediments seems to be an appropriate manner of retreatment. In this attempt, besides indigenous microflora bacterial species *Bacillus megaterium* and *Bacillus cereus* was used. These bacterial species were izolate from the soil matrix of sampling place Hornád-inlet. Because heavy metals are increasingly found in microbial habitats due to natural and industrial processes, microbes have envolved several mechanisms to tolerate the presence of heavy metals (by either efflux, complexation, or reduction of metal ions) or to use them as terminal electron acceptors in anaerobic respiration (Senenska-Pobel S. et al, 1998). The objectives of this work were to determine the differences of influence *Bacillus cereus* and *Bacillus megaterium* on leachibility Ni and Cd ions from polluted sediments.

Experimental

Sediment samples were obtained from the botton of water reservoir Ružín I in Slovakia at differend depths (20 and 40 cm). Heavy metals composition is given in Table I.

Biological leaching of the sample material was carried out in conical flasks with 30 g sediment and 600 ml Ashby´s medium. The Ashby´s medium contained (per liter) 0.2 g urea, 1 g K$_2$HPO$_4$, 0.075 g NaCl. Glucosse (2 g dm^{-3}) was added as the organic substrate. The flasks were inoculated with *Bacillus megaterium* and *Bacillus cereus* cultures, originally isolated from the sediment of water reservoir Ružín I. in Slovakia. The two strains were purified and followed by streak plating on nutrient agar cultures. The isolates were identified with the BBL Crystal Identification System (Becton Dickinson, USA).

The flasks were incubated under static conditions for 6 months at 25 °C in the dark. Appropriate abiotic controls were included in these experiments. Changes in the chemical composition of solid and liquid phases were measured by atomic absorption spectrometry (Varian AA240-Z with GTA-120 a AA240-FS). The particle size distribution was measured by the laser radiation scattering using a model 22 Laser-Particle – Sizer Analysette (Fritsch GmbH, Idar-Oberstein, Germany).

Results

The Ni and Cd concentrations of sediments taken from 20 and 40 cm depth layers are shown in Table I. The amount of investigated heavy metals overrun the limit values given by Metodical Instruction ministry of Environment – Slovak Republic No. 491/2002. The established values give information about the potential risk of Ni and Cd releasing and about surrounding and outlying contamination ecosystems.

Table I
The results of surface layer analysis of sediments from 20 cm depth and their comparison with Metodical Instruction Ministry of Environment – Slovak Republic No. 491/2002

Element Depth		Ni	Cd
		[mg kg^{-1}]	
20 cm		87	1.2
40 cm		86	0.9
MIME-SR	MPC	44	12.0
No. 491/2002	TV	35	0.8

Table II
Effectivity of bioleaching process with application of *Bacillus megaterium* and *Bacillus cereus*

Element	Before BL [mg kg^{-1}]	After BL *Bacillus megaterium* [mg kg^{-1}]	After BL *Bacillus cereus* [mg kg^{-1}]
Ni	86.0	79.0	70.0
Cd	1.2	0.8	1.0

Fig.1. depicts the kinetic of the bioleaching process of Ni from sediment sample taken from the depth 20 cm. Ni concentration was observed during bioleaching in soluble form. The curves present the differences between the effectivity of bioleaching processes in two bacterial systems, *Bacillus megaterium* and *Bacillus cereus.* The above mentioned processes took 54 days. The maximum concentration of eluted Ni was achieved in the 21th day (*B. megaterium*) and by *B. cereus* in the 14th day. The maximum concentration Ni in *B. cereus* system achieved 1,247.3 ng ml^{-1} and in *B. megaterium* system 1,087.5 ng ml^{-1}. The results refer to the higher effectivity in the case of application of the bacterial strain *Bacillus cereus*.

Fig. 2. presents the kinetic curves of the bioleaching process of Cd from the sediment sample (20 cm depth). The Cd leaching processes were carried out under the same conditions as in the case of the Ni leaching. In the presence of Bacillus megaterium an increased concentration of Cd was detected. The medium in the 14th day contained

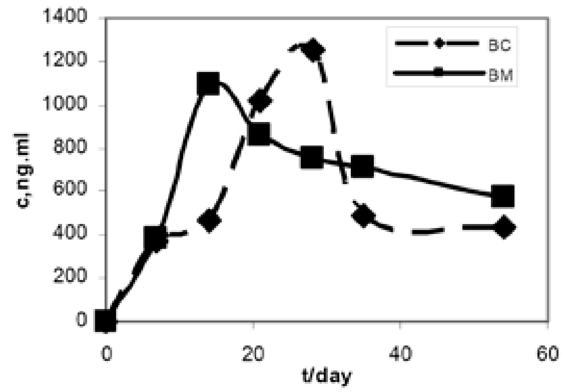

Fig. 1. **Basic kinetic of the bioleaching Ni, sediment depth – 20 cm, BM – *Bacillus megaterium*, BC – *Bacillus cereus***

14.2 ng ml^{-1} Cd. These experiments give a considerable evidence about the differences of metabolic activity of two bacterial strains, Bacillus cereus and Bacillus megaterium. The results show more effective application of Bacillus megaterium, because of the shorter time needed to Cd releasing into the solution. The concentration of Cd in the medium is approximately 3 times higher as in the case of obtained from the depth 40 cm were proved through the same experimental processes as the sediment from the 20 cm, but the behaviour and the results of bioleaching Ni and Cd were in both cases the same (Data not showed).

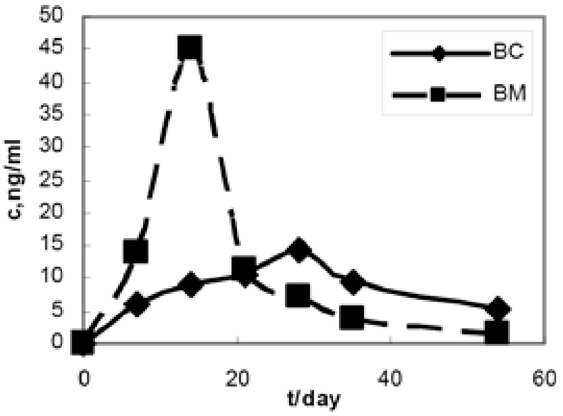

Fig. 2. **Basic kinetic of the bioleaching Cd, sediment depth – 20 cm, BM – *Bacillus megaterium*, BC – *Bacillus cereus***

pH of bioleaching system has a direct relationship to the mobility of metals relating to their solubility. The concentration of cations in sediment solution usually increase greatly under a low pH condition (Wu et al., 2006). The decrease of pH values was achieved through bacterial production of acetic acid, butyric acid, pyruvic acid, lactic acid and formic acid (Styriakkova et al, 99). Fig. 5 presented the decreasing of pH values in both *B. cereus* and *B. megaterium* bioleaching systems. The measurements indicate a markedly change in the pH solution during the first 20 days from pH 7 to 4.6 (*B. cereus*) and 4,9 (*B. megaterium*). In the next experiment a relatively constant level pH values are shown.

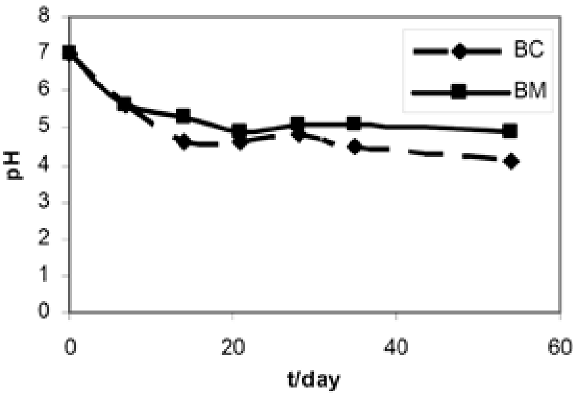

Fig. 3. **The decrease of pH in course of bioleaching. BM – Bioleaching system with the application of *Bacillus megaterium* inoculum BC – Bioleaching system with the application of *Bacillus cereus* inoculum**

Conclusions

Our study demonstrated that *Bacillus spp*. related to the species *Bacillus megaterium* and *Bacillus cereus,* presented and isolated from heavy metals contaminated sediments, are able to release Ni and Cd ions from polluted sediments. This kind of heterotrophyc microorganisms may pose positive impact on the availability and mobility of Ni and Cd and other heavy metals in sediments from water reservoir Ružín I . In this study Cd ions seems to be easier leached through *Bacillus megaterium* strain than *Bacillus cereus* according to their metabolic activity and adaptability to the Cd polluted environment. The Ni ions was easier leached with application of *Bacillus cerus* strain. In general, the bacteria innoculation enhanced the leachability of Ni and Cd. The possible mechanisms may also be responsible for the integrated effects of lowering pH through bacteria metabolism and production of organic acids.

This work has been supported by VEGA Agency supporting the projects No-2/0049/08.

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P46 BIOSORBENTS PREPARATION FOR HEAVY METALS REMOVING FROM WATERS

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Introduction

Heavy metals are very important pollutants in waters. They are non-degradable and therefore continue to accumulate in water bodies. Because of their toxic properties and the tendency for bioaccumulation in the food chain, it is imperative to take effective precautions and to reduce the concentration levels of heavy metals in waters[1].

Current methods in order to remove the toxic metal ions from wastewater are: chemical precipitation, ion exchange, extraction, adsorption, reverse osmosis techniques[2].

Adsorption is understood to involve the interphase accumulation or concentration of substances at a surface or interface. Absorption is a process in which the molecules or atoms of one phase interpenetrate nearly uniformly among those of another phase to form a solution with it[3]. A specific type of sorption based on the used solid phase that is derived from various types of biomaterials or biomass is biosorption[4].

Microbial biomass is frequently used for the bioremediation of heavy metals polluted waters as the biomass capable absorb, adsorb and in some cases precipitate the metals out of solution[5].

Sulphate reducing bacteria (SRB) are genera of dissimilatory bacteria, which utilise sulphate as a terminal electron acceptor and perform dissimilatory reduction of sulphate ions (SO_4^{2-}) to hydrogen sulphide (H_2S) gas. The hydrogen sulphide produced by the SRB, as part of their metabolic processes is capable to react with metal cations to form stable sulphide precipitates[6].

Microbial iron sulphide is well known as an adsorbent for the treatment of metallic ion polluted wastewater[5].

Material and Methods

Cultivating Medium

For sulphate-reducing bacteria cultivation was modified nutrient medium DSM-63 (ref.[7]) with the addition of $Fe_2(SO_4)_3 \cdot 9H_2O$ used. This medium creates optimal conditions for SRB growth genera *Desulfovibrio* and *Desulfotomaculum.*

Sulphate-Reducing Bacteria

Bacteria were isolated from a mixed culture obtained from potable mineral water collected at Gajdovka spring (Košice, Slovak republic) with pH 7.5 and H_2S odour.

Sorbents Preparation

The iron sulphides preparation was realized in the bioreactor filled with 500 ml of modified nutrient medium DSM-63 and inoculated with 100 ml of a culture of SRB during 21 days at 30 °C under anaerobic conditions. These conditions were generated by introducing an inert gas (N_2) and chemically with sodium thioglycollate. The pH of the medium was adjusted to the value 6.8 with sodium hydroxide.

Preparation was realized under 2 different modes. During first mode the bioreactor worked at 30 °C under anaerobic conditions without addition of fresh nutrient medium during 21 days. During second process of preparation the bioreactor worked 4 days in batch mode and then 3 days in continuous mode (i.e. fresh medium was supplied into reactor for more intensive SRB growth and iron sulphides production). We realized 3 periods (21 days).

Model Solutions

Model solutions were prepared by dissolving $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ analytical grade salts. For sorption experiments were solutions with contain of Zn^{2+} and Cu^{2+} 100 mg dm^{-3} used.

Sorption Experiments

Batch sorption experiments were realized in Erlenmeyer flasks filled by 100 ml of model solution of Zn^{2+} or Cu^{2+} and the sorbent. The sorbent dose was 0.1 g. For the best contact between sorbent and model solution were samples agitated by mechanical laboratory shaker at 250 oscillations per minute. Sampling was conducted during 90 minutes. The concentration of zinc and copper was determined by atomic absorption spectrometry.

Results and Discussion

The process of the iron sulphides preparation by SRB was successful in both modes, demonstrated by black precipitates in Fig. 1. and the sensorial detection of H_2S odour.

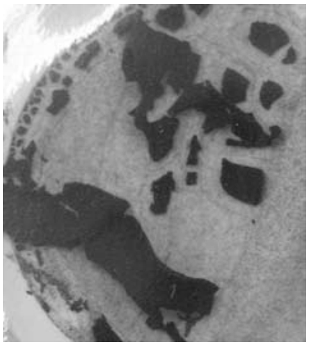

Fig. 1. **Bacterially produced iron sulphides**

Sorption of Cu and Zn cations from model solutions by biogenic sorbents prepared in batch-continuous mode was studied. Fig. 2. shows removal of Zn^{2+} and Cu^{2+} during 90 minutes. All copper ions were removed. In the same time only 50 % of zinc ions were removed.

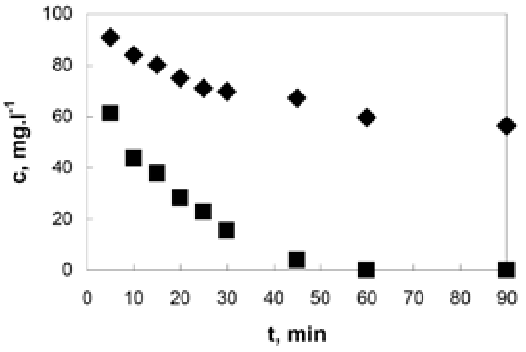

Fig. 2. **Removal of copper and zinc ions from model solutions. c – concentration of copper and zinc ions, ■copper, ◆ zinc, t – time**

In Fig. 3. are present quantities of copper and zinc ions that iron sulphides were able to capture from 100 ml of solution in calculation on 1 g weights of dry the sorbent.

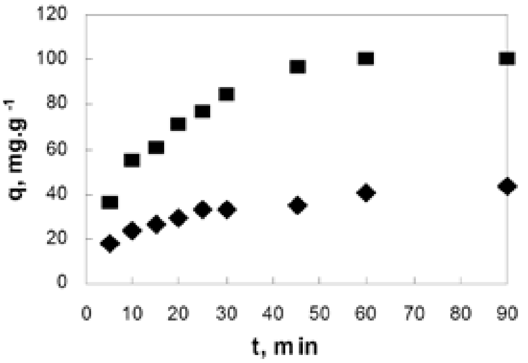

Fig. 3. **Sorption of copper and zinc ions from model solutions. q – sorption, ■copper,◆ zinc, t – time**

Fig. 4. shows sorption of zinc ions by iron sulphides prepared in batch-continuous mode (BCM) and in discontinuous mode (DM) from model solutions. We can see, that "batch-continuous sorbent", which production process is possible to see in Fig. 5., was able to remove more ions in the same times than "discontinuous sorbent".

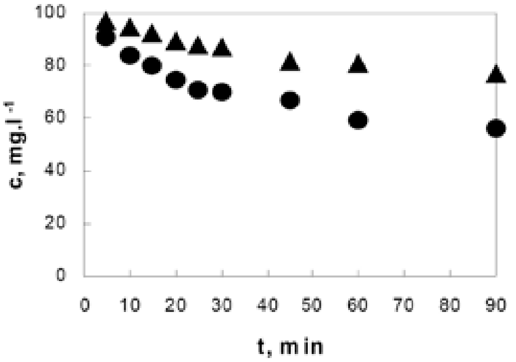

Fig. 4. **Removal of zinc ions using sorbent prepared in 2 different modes. c – concentration of zinc ions, ● batch-continuous sorbent, ⑦ discontinuous sorbent, t – time**

Fig. 5. **Bioreactor during continuous mode**

In Fig. 6. are present quantities of zinc ions that sorbents captured from solutions in calculation on 1 g weights of dry the sorbent.

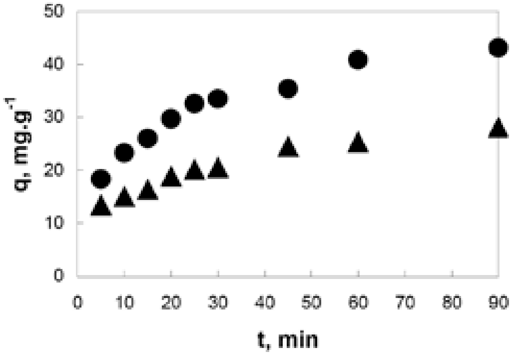

Fig. 6. **Sorption of zinc ions using sorbents prepared in 2 different modes. q – sorption ● batch-continuous sorbent ⑦ discontinuous sorbent, t – time**

Conclusions

This work was oriented on preparation of iron sulphides sorbents by sulphate-reducing bacteria for the heavy metals removing from waters and realization sorption experiments. From results it is clear that sorpion of copper ions was higher than sorption of zinc ions after 90 minutes of experiments. We can also conclude that different mode of iron sulphides preparation reflects in sortpive capability of sorbent, because batch-continuous prepared sorbent was more effective than discontinous prepared.

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P47 GENOTYPE VARIABILITY OF FATTY ACIDS IN CEREAL GRAINS

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Introduction

Cereals are important part of human and animal nutrition. From the view of lipids, cereal grains are quite low in fats (averaging 3.6 %) that consist of mainly palmitic, oleic and linoleic acids. Oat (*Avena* spp) includes many species of which *Avena sativa* L is the most commonly cultivated[1]. It is mainly grown for feed use, but due to its high nutritional value, much interest is now focused on expanding its food applications. Among cereal grains, oat has the best protein quality and quantity and is a valuable source of minerals and vitamins, especially thiamine and pantothenic acid[2]. Moreover, the lipid content of oat is high with very good balance between polyunsaturated and saturated fatty acids[3,4] and, therefore it is important crop from the nutritional point of view[5]. Significant levels of other lipid compounds, such as phospholipids, glycolipids, and phytosterols, may also find application in various pharmaceutical, medical, food, and industrial fields.

Buckwheat is annual melliferous crop. For many years cultivation of buckwheat declined, but recent interest in old, traditional foods have led to resurgence in its cultivation. Buckwheat belongs to pseudo-cereals together with amaranth and millet. These grains have been important food crops in various parts of the world and have potential for much greater and more widespread use[6].

Because lipids are essential compounds of cereal grains, this study was focused on genotype variability of fatty acid composition in following cereal grains: oat, millet, amaranth and buckwheat. The research in this field could significantly help in cereal classification.

Experimental

Oil content and fatty acid composition in following cereal grains have been determined in this study: oat (85 varieties), millet (29 varieties), amaranth (20 varieties) and buckwheat (10 varieties).

Total lipids were extracted from oat grains 2-times by 100 ml chloroform/methanol (2 : 1, v/v) for 60 min at laboratory temperature with occasional stirring. After extraction the mixture was filtered and 0.97% KCl (1.2-fold of total extract volume) was added. The mixture was stirred vigorously for 1 min and centrifuged (5 min, 3,000 g) to effect phase separation. The chloroform – lipid containing layer was filtered through anhydrous Na_2SO_4 and evaporated under vacuum[7].

Table I
Average amounts (AV), standard deviation (SD) and variation coefficient (VC) of lipid content and fatty acids in studied cereal grains

Cereals		Lipids	Fatty acids [%]				
(number)		[%]	C16 : 0	C18 : 0	C18 : 1	C18 : 2	C18 : 3
Oat (79)	AV	4.5	16.0	1.7	37.8	38.5	1.6
	SD	1.2	0.8	0.4	2.5	2.2	0.4
	VK	26.9	4.9	22.2	6.6	5.6	21.9
Millet (29)	AV	3.8	7.5	1.9	21.3	63.6	1.2
	SD	0.57	0.35	0.63	1.83	2.65	0.25
	VK	14.9	4.6	33.5	8.6	4.2	20.7
Buckwheat (10)	AV	2.3	13.3	2.1	36.3	35.2	1.9
	SD	0.54	0.52	0.07	0.97	0.53	0.07
	VK	23.5	3.9	3.4	2.7	1.5	3.5
Amaranth (10)	AV	5.2	17.1	2.7	23.6	43.0	0.7
	SD	0.79	2.50	0.29	4.34	6.34	0.11
	VK	15.1	14.6	10.8	18.4	14.8	15.3

Fatty acids of total lipids were analyzed as their methyl esters by gas chromatography (GC-6890 N, Agilent Technologies) using a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies) and a FID detector (constant flow, hydrogen 35 ml min^{-1}, air 350 ml min^{-1}, 250 °C) under a temperature gradient (130 °C for 1 min; 130–170 °C at program rate 6.5 °C min^{-1}; 170–215 °C at program rate 2.7 °C min^{-1}; 215 °C for 7 min; 220–240 °C at program rate 2 °C min^{-1}) with hydrogen as carrier gas (flow 2.1 ml min^{-1}, velocity 49 cm s^{-1}, pressure 174 kPa) and a split ratio of 1/50 (Inlets: heater 230 °C, total hydrogen flow 114 ml min^{-1}, pressure 174 kPa)[8]. The fatty acid methylester peaks were identified by authentic standards of C_4–C_{24} fatty acid methylesters mixture (Supelco, USA) and evaluated by ChemStation 10.1 (Agilent Technologies). The variation coefficient (VC) of individual fatty acids was calculated from the equation: VC = (δ_{n-1}/average value of selected fatty acid) × 100, where δ_{n-1} is a decisive deviation.

Results

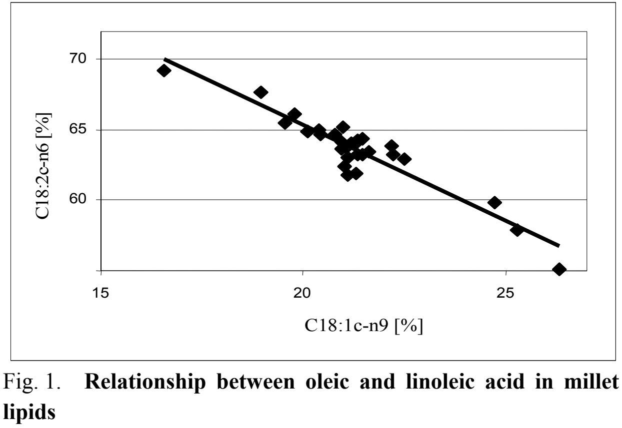

Fig. 1. **Relationship between oleic and linoleic acid in millet lipids**

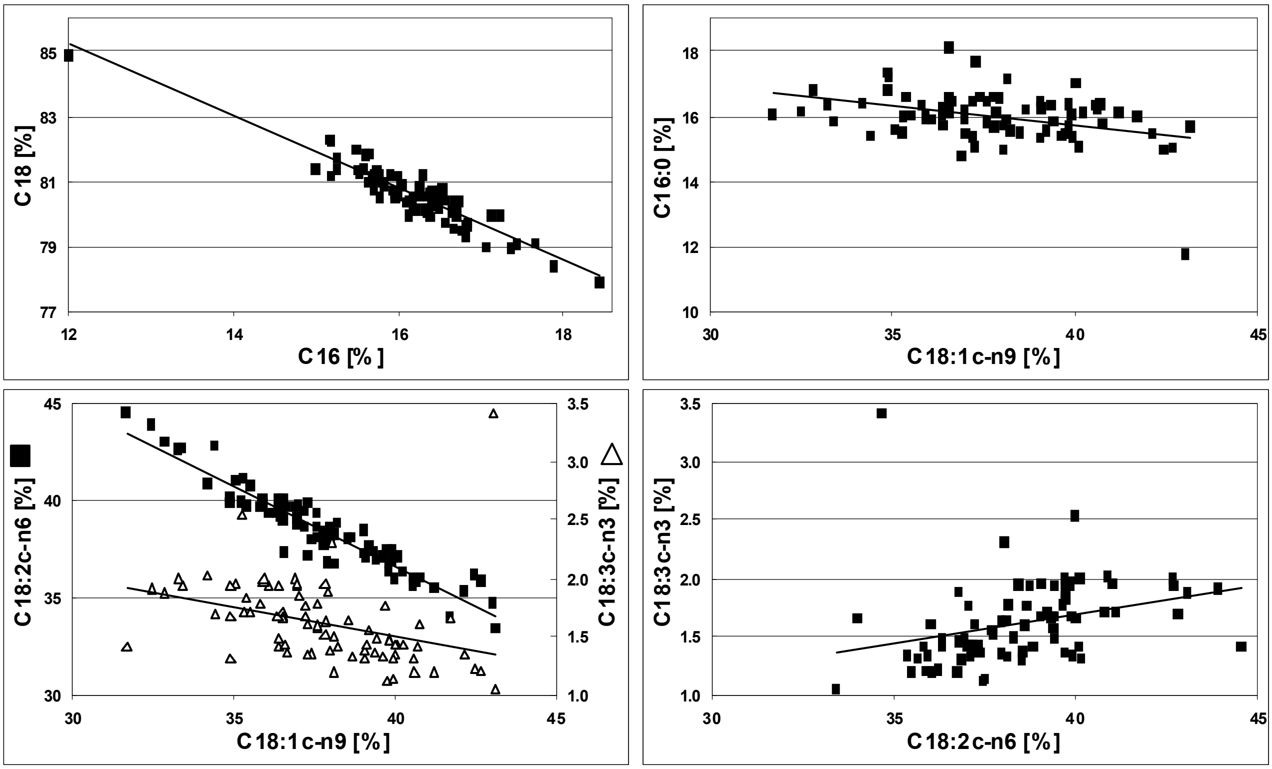

Fig. 2. **Relationship between main fatty acids in oat lipids**

Lipid amounts in investigated cereals varied between 1.5 to 8.2 %. Linoleic (C18 : 2c-n6), oleic (C18 : 1c-n9) and palmitic (C16 : 0) acids were the major fatty acids whereas α-linolenic acid (C18 : 3c-n3) and stearic acid (C18 : 0) were minor fatty acids (Table I).

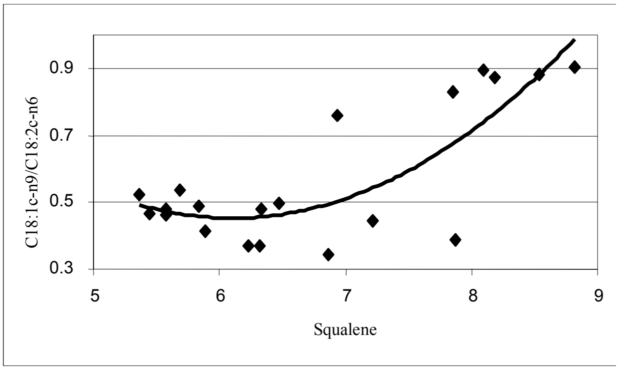

Fig. 3. **Relationship between C18 : 1c-n9/C18 : 2c-n6 ratios and squalene amounts in amaranthus grains**

Correlation between levels of oleic and linoleic acids indicated that activity of Δ^{12}-desaturase might be controlled genotypically (Fig. 1.). The results indicate that elevation amount of total C16 fatty acids is related with loss of amount total C18 fatty acids in oat lipids. Also decrease of oleic acid amount is accompanied with higher linoleic and α-linolenic acid amounts. Accumulation of linoleic acid amount is related with accumulation of α-linolenic acid amount as well (Fig. 2.). Variations in individual fatty acid levels also demonstrated deviation in biosynthesis stability of these compounds in selected cereal genotypes. Moreover, relationship between C18 : 1c-n9/C18 : 2c-n6 ratios and squalene amounts were found in amaranthus varieties (Fig. 3.).

Conclusions

Although fatty acid composition in all varieties of individual cereal type was similar, statistical analysis and calculation of various ratios among fatty acids clearly showed that fatty acid profile can be used for more precise description of single cereral genotypes. Oats containing lower lipids amounts were characterized by increased fatty acid unsaturation due to elevated concentrations of both linoleic and α-linolenic acids and reduced levels of oleic acid. Fatty acid composition together with calculated fatty acid parameters offers not only valuable taxonomic and physiologic information for classification of various cereal varieties, but also may be used by farmers and producers for aimed applications of cereals from the view of desired lipid attributes.

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P48 PRELIMINARY STUDIES ON THE ANTIFUNGAL ACTIVITY AND COMPOSITION OF THE HEXANE EXTRACT OF THE BRAZILIAN *CHENOPODIUM AMBROSIOIDES* L.

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Introduction

Extracts of several edible botanicals are reported to have antifungal activity. However, little work has been carried out to manage fungal deterioration of stored products by plant derived bioactive compounds.

Epazote (*Chenopodium ambrosioides* L.) is an herb native to South America and although there are a few reports on fungicidal properties of its dichloromethane extracts[1,2], no studies have been conducted on its chemical compositions. In this study, we report our preliminary results on antifungal activity of the crude hexane extract hexane extract (HE) of the Brazilian epazote against four fungi (*Aspergillus flavus*, *A. glaucus*, *A. niger*, *A. ochraceous*) and identification of volatiles in the hexane extract by gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC-MS).

Experimental

Plant Material and Hydrodistillation

The epazote leaves were harvested from shrubs in Viçosa, Minas Gerais, Brazil, and extracted with hexane (200 ml) for 12 h. The organic phase was collected, dried over anhydrous sodium sulfate; hexane was evaporated in a rotatory evaporator at 30 °C under reduced pressure, weighed and evaluated.

Antifungal Activity

The antifungal activity of the hexane extract was tested on potato-dextrose agar (PDA) with use of poison food assay. Percent growth inhibition was calculated by dividing radial growth in the treatment plates by growth in the control plates and multiplying by 100. The data were analyzed by ANOVA and the means compared by the Tukey test (p = 0.05).

Identification of Compounds

The volatiles in the crude hexane extract were identified by GC using RI (Kováts retention index) and GC-MS. The peaks were first identified by GC-MS library system based on similarity indexes (SI). RI was obtained for most GC peaks. The final identification was based on the best SI and RI fits

Results and Discussion

Depending upon the fungus an inhibition of 25–100 % was obtained (Fig. 1.). In this study, at concentration of 0.3 %, 100% inhibition of four important post-harvest fungi was obtained. The minimum concentration of 0.1 % was reported for complete inhibition of *Rhizoctonia solani*[1].

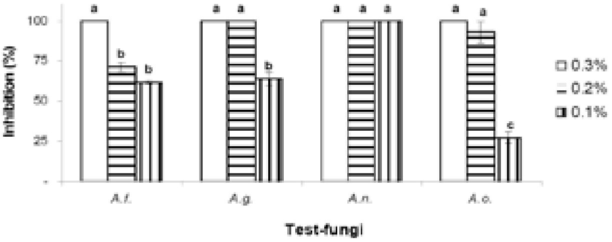
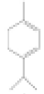
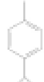
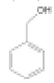
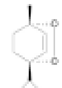
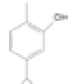
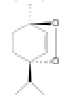


Fig. 1. **Percent radial growth inhibited by crude *Chenopodium ambrosioides* hexane extract after 6-day incubation (25 °C) at concentrations of 0.3 %, 0.2 and 0.1 %. Mean of three replications. For each concentration the histograms of different fungi, headed by the same letter do not differ at (p = 0.05). The bars represent the standard deviation within the treatment. A.f. = *Aspergillus flavus*, A.g. = *A. glaucus*, A.n = *A. niger*, A.o = *A. ochraceous***

Table I
Identification, based on gas chromatography (RIs-Kováts retention indexes) and gas chromatography-mass spectrometry and % composition of the crude hexane extract of the Brazilian Chenopodium ambrosioides

	Crude hexane extract			
Peak no	KI	%	Compound	Structure
1	1019	11.2	α-terpinene	
2	1026	6.0	*p*-cymene	
3	1031	0.4	benzyl alcohol	
4	1247	54.0	(*Z*)-ascaridole	
5	1287	2.3	carvacrol	
6	1305	17.3	(*E*)-ascaridole	
Other identified compounds		8.8	–	–

The composition of the hexanic extract has not been reported in the literature. About 91.8 % of the volatiles in the hexane extract were identified by GC and GC-MS (Table I,

Fig. 2.). In adddition, other non-volatiles such as mono, di and triacylglycerides etc., which would not be dectected under our GC conditions were presumably also present.

The % composition and antifungal activities were similar to those reported for the *C. ambrosioides* essential oil (EO) reported by us[3].

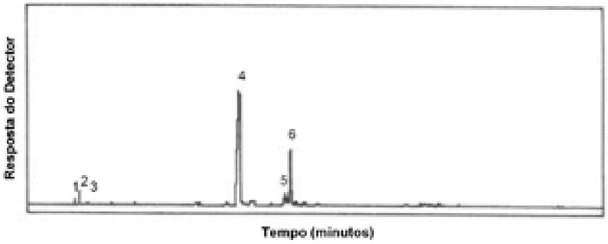


Fig. 2. **Reconstructed gas chromatogram obtained on analyses of crude Chenopodium ambrosioides hexane extract**

Conclusions

The antifungal activities of the *C. ambrosioides* essential oil[3] and hexane extract were very high and were attributed to terpene-like compounds. We are now conducting studies to isolate the active fraction of the hexane extract as well as extend our studies to other common storage fungus.

Acknowledgements This work was supported by the Brazilian govt agencies (CAPES- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CNPq-Conselho Nacional de Pesquisa and FAPEMIG- Fundação de Amparo de Pesquisa do Estado de Minas Gerais).

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P49 EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF NATURAL HONEYS

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Introduction

In general, the antioxidant capacity of honey appeared to be a result of the compounds including phenolics, peptides, organic acids, enzymes and other phytochemicals (Gheldof et al., 2002). The phenolic compounds contribute significantly to the antioxidant capacity in many studies. Reports on honey as antifungal agent were comprehensively reviewed by Molan (1992). The "inhibine number" is the degree of dilution to which a honey retains its antibacterial activity, representing a sequential dilution of honey in steps of 5 %, from 25 to 5 %. Allen *et al*., (1991) found many honeys with low activity and 36 % of samples with activity near or below detectable levels. In this study, antioxidant effects (by DPPH method) as well as the antifungal effects of natural honeys on five species of fungi collected, were tested.

Experimental

Honey was extracted from the hives of bees and kept at 4 °C away from direct light before use. Samples were derived from different plant species of Acacia (2 samples), Brassica (1 sample), Thyme (1 sample) and the rest of samples (5 samples) were of floral origin. Samples originated from different places of Slovakia and one sample came from Rodos

Antioxidant Activity

A method was adapted according to Sanches Moreno et al. (1998) and Brand-Wiliams et al. (1995). Honey (10 g) was dissolved in 100 ml of ethanol (90%), and 0.1 ml of solution was taken to 3.9 ml of DPPH methanolic solution (25 mg dm^{-3}) and mixed. Absorbance at 515 nm was measured at different time intervals. The blank reference cuvette contained DPPH methanolic solution. Results were expressed as % inhibition.

Antifugal Activity

All samples were prepared aseptically and handled protected from direct sunlight. Honey solutions were prepared in three fractions: 50, 25 and 10 % (by mass per volume). Five species of fungi were isolated from wheat samples collected from different locations in Slovakia: *Penicillium crustosum, P. expansum, P. griseofulvum P. raistrickii* and *P. verrucosum* by the agar well diffusion method. All fungal isolates were identified microscopically, and samples of each fungus were deposited in the fungal collection bank. The strains of fungi were maintained on Czapek yeast extract agar (CYA, HiMedia). All plates were incubated at 25 °C in the dark and inhibition zones were measured after 3 days. The statistical processing of the data obtained was implemented by means with SAS software.

Results

In term of antioxidant activity of honeys the best results were found at thyme honey from Rodos (11.84 %) and floral honeys from different places of Lučenec (10.09 %, 10.19 %). The lowest antiradical activity was determined at Acacia honey (7.615 %). Kücük et al. (2007) studied three different Turkish honeys and found high levels of polyphenolics and high antioxidant activity in three antioxidant assays. Radical scavenging assay was the highest at chestnut honey, then followed heterofloral honey and the least at rododendron one.

Table I
Antioxidant activity [%] of honey samples

Honey/Place of origin	Inhibition [%] ± SD
Acacia/Nitra	8.53 ± 0.08
Brassica/Nitra	9.89 ± 0.33
Floral/Smrečany	8.39 ± 0.11
Thyme/Rodos	11.84 ± 0.35
Floral/Nitra	8.98 ± 0.24
Floral/Hlohovec	9.96 ± 0.40
Floral/Lučenec	10.19 ± 0.53
Acacia/Lučenec	7.62 ± 0.56
Floral/Lučenec	10.09 ± 0.34
All	9.50 ± 1.25

Al-Mamary et al. (2002) showed a linear positive relationship with the extract concentration. The antioxidant activity of diluted honey samples increased with increasing the levels (50 μl, 100 μl, 200 μl) of honey samples. The total antioxidant activities of diluted samples varied from −6.48 % (prooxidant activity) to 65.44 % inhibition. In our case determined antioxidant activity (Table I) was low, varied from 7.62 % (Acacia/Lučenec) to 11.84 % (Thyme/Rodos).

The results obtained characterize honey samples as a product with a broad antimicrobial effect. The strongest antifungal effect was shown by honey samples of 50 % concentration against *Penicillium raistricki* strains. The least antifungal effect was shown by honey samples of 10 % concentration against to *Penicillium expansum* strains. No inhibitory activity was detected with any of the seven species with the pasture honey at any concentration up to the highest tested 50 % (v/v). Maughrabi (2003) found that wild honey was effective against *Alternaria solani* and *Phytophthora infestanns*, also against *Rhizoctonia solani* and *Fusarium oxysporum*, and to a less extent against *Stemphylium solani* and *Colletotrichum sp*.

Table II
Antimicrobial effect of honey samples against *Penicillium* strains

Microscopic fungi	Honey concentration [%] 10	25	50
Penicillium crustosum	11.67 ± 3.00	22.22 ± 8.38	28.67 ± 8.11
Penicillium expansum	7.22 ± 5.63	12.22 ± 4.79	24.22 ± 16.54
Penicillium griseofulvum	10.78 ± 1.92	15.11 ± 3.62	21.56 ± 5.53
Penicillium raistrickii	8.67 ± 2.87	16.22 ± 5.07	33.11 ± 14.59
Penicillium verrucosum	7.67 ± 1.94	12.78 ± 3.67	23.44 ± 9.84

Conclusions

Selected honeys showed low scavenging ability to DPPH radical, but can be classified as a product with a broad antimicrobial effect. The strongest antifungal effect was shown by honey samples of 50% concentration against *Penicillium raistricki* strains.

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P50 COMBINED TECHNIQUE LC/MS IN ANALYSES OF ANTIOXIDANT COMPOUNDS

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Introduction

Antioxidants constitute a large group of plant and microbial secondary metabolites (for exemple carotenoids, phenolics, oligopeptides, saccharidic derivates etc.) with major interest because of their anti-inflammatory effects and of their potential anti-atherogenic properities[1,2]. Many foods are good sources of antioxidants: vegetables, legumes, whole-grain cereals, tea extracts, honey etc. The best source of these physiologically active compounds with rapid effects on human health is represented by some sorts of fruit[1]. Especially citrus fruit contain high-levels of phenolics and/or ascorbic acid[3]. Widely consumed apples are rich on polyphenols too with the most abundant chlorogenic acid[4].

Phenolics in human plant nurtur play important roles in sensory properties of food, because of its influence on colour and/or flavour. Moreover major part of phenolic substances occure as physiologicaly active components with very hight antioxidative capability as well as effective protection against cancer growth and/or other degenerative diseases[3,4].

Analysis of phenolic substances could be realised using many different instrumental methods starting with TLC and ending with GC (some special applications). In the present the most preffered technique in separation of non-volatile compounds, such as phenolics are, is modern instrumental method LC/MS combining perfect separation with universal detection of individual sample components[5].

Experimental

Presented work was focused on characterisation of several substances (phenolics, carotenoids) with antioxidant activity containned in biologic materials and on their distribution in individual sample fractions (juice, pulp, peel).

Antioxidants were isolated *(i)* by ethylacetate-ether (phenolics) and/or aceton-ether (carotenoids) extraction, *(ii)* by SPE method with amide-2 (phenolics) and/or C18 AR columns (carotenoids).

Separation and detection of phenolics were optimalizated using followed conditions: Restek C18 Ultra aqueous column heated on 30 °C, gradient elution using 1% acetic acid: acetonitril in range 60-45 : 40-55 with flow of mobil phase 0.4 ml min^{-1}, UV-VIS detection (280 nm and/or 370 nm), MS tune file on chlorogenic acid (negative ion mode).

Identification of carotenoids was gone on column Polaris C18 A heated on 45 °C, using isocratic elution with LC/MS methanol (flow 0.25 ml min^{-1}), UV-VIS detection (450 nm) and/or MS detection (tune file on β-carotene in positive ion mode).

Standard compounds as well as food samples were determined by MS full scan and/or MS/MS full scan mode.

Results

Standards of antioxidants were analysed using off-line and/or on-line HPLC/UV-VIS/ESI-MS. However reported results were obtained by on-line LC/UV-VIS/MS, because of low response of off-line method.

Table I
Precursors (MS full scan) and fragments (MS^{2}full scan) of standards

Compound	m/z [M-H]$^-$ or [M]$^{+\bullet}$	Fragments (m/z)
β-carotene	537	–
epicatechin	289	217, 245, 271
catechin	289	227, 245, 271
quercetin	301	107, 121, 151, 239, 257, 273
morin	301	229, 257, 273
fisetin	285	163, 213, 229
kaempferol	285	151, 229, 257
acid gallic	169	–
acid chlorogenic	353	191, 309, 339
myricetin	317	179, 245, 289, 299
naringenin	271	–
rutin	609	301
procyanidin	577	–

In Table I determined parent ions (MS full scan) and/or typical fragments obtained by tandem mass spectrometry are resumed. MS full scan spectra of chosen phenolic standards are demonstrated on Fig. 1. and Fig. 2.

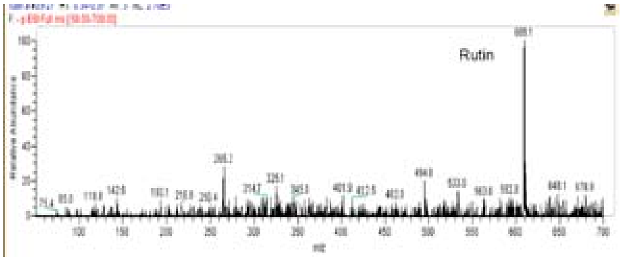

Fig. 1. **MS full scan spectrum of rutin**

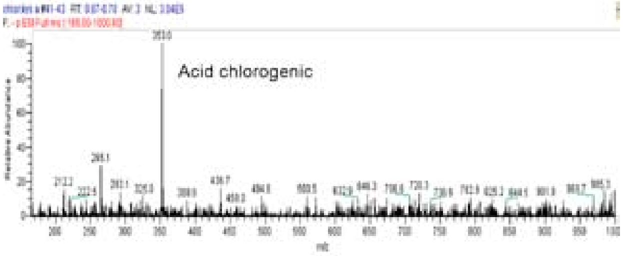

Fig. 2. **MS full scan spectrum of acid chlorogenic**

Compounds in food samples are identified in MS spectra according to m/z values of determined standards (Table I). Phenolic substances were analysed with higher sensitivity than carotenoids, because of its moderate polarity – general condition of electrosprey use[3,5].

Fig. 3. shows an exemple of chromatogram of several phenolics contained in orange.

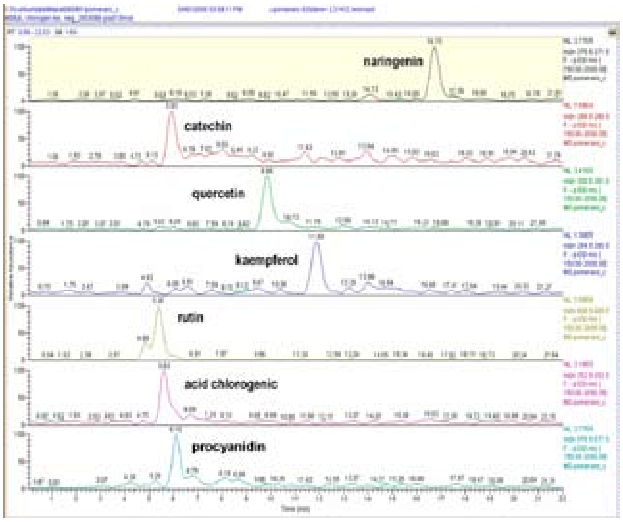

Fig. 3. **Phenolics in orange juice: naringanin, catechin, quercetin, kaempferol, rutin, acid chlorogenic and procyanidin**

Table II
Antioxidants in food samples

Sample	Total phenolics [mg 100 g^{-1}]	β-carotene [μg 100 g^{-1}]
orange	572 ± 13	264.3 ± 1.2
lemon	388 ± 12	218 ± 8
tangerine	148 ± 13	720 ± 25
grapefruit	172 ± 6	714 ± 36
apple	2181 ± 43	593 ± 70
carrot	1352 ± 46	5289 ± 123
tomato	13.4 ± 0.8	14.1 ± 2.1
sweet pepper	4.37 ± 0.03	4660 ± 27
onion	16.3 ± 1.0	40.1 ± 1.9
garlic	1.32 ± 0.04	–
potato	379 ± 16	2077 ± 127
gourd – seeds	1898 ± 28	–
sunflower-seeds	517 ± 10	–

Fruit samples were observed as the best and complex sources of phenolics (Table II). Amount of total phenolics was highest in apple, the mostly consummed fruit. But considerable yield was observed in orange as well as in sunflower-seeds. Very important food source of phenolics are also carrot and/or gourd-seeds.

Antioxidant capacity of carrot is increaced by high-level of β-carotene (carotenoids). Important concentration of carotenoids was observed also in sweet pepper and/or potato (Table II).

Studied samples contained rutin and/or acid chlorogenic as principal phenolics, their distribution in individual fractions of food is demonstrated on Fig. 4 and Fig. 5.

General part of phenolic substances is presented in fruit and/or vegetable juice, while higher amount of selected phenolics was observed in pulp.

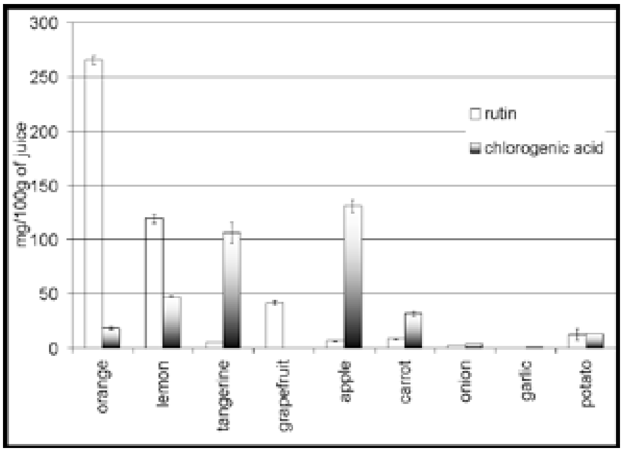

Fig. 4. **Occurrence of rutin and acid chlorogenic in juice**

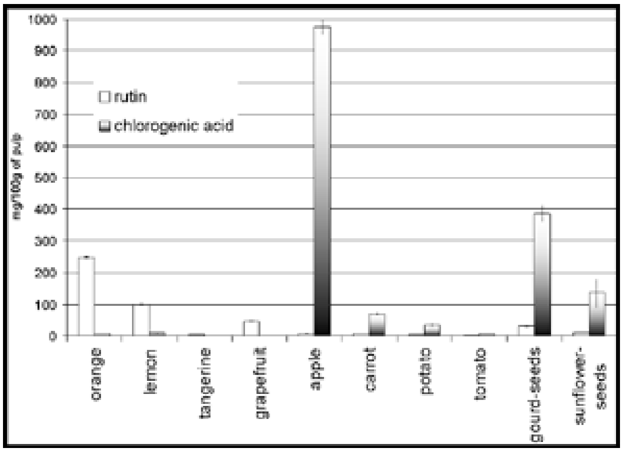

Fig. 5. **Presence of rutin and acid chlorogenic in pulp**

Peel was analysed in apple and potato only (data not show). This fraction was characteristic by higher amount of chlorogenic acid, while other analysed phenolics were presented in very lower concentration and/or was not determined.

Conclusions

Combined instrumental technique LC/ESI-MS is more suitable for analysis of phenolics than in identification of carotenoids. Carotenoid are lipid-soluble pigments, so ionisation process with ESI source is insufficient. More important method for carotnoid analysis is LC/APCI-MS.

Analysis of compound distribution is surely influenced by extraction method, however results demonstrate that fruit and/or vegetable should be used as whole product. The one fraction consummation (for exemple fruit juice only) reduces conciderable antioxidant yields contained in pulp as well as in peel of fruit and/or vegetable sources.

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P51 INFLUENCE OF CHEMICAL TREATMENT ON FUSARIUM TOXINS IN BARLEY HARVESTED 2005–2007

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Introduction

Mycotoxins are toxic secondary metabolites produced by certain toxigenic microscopic fungi in various crops used as raw materials for the production of foodstuffs and fodder.

Fusarium toxins have been shown to cause a variety of toxic effects (both acute and chronic) in humans and animals (vomiting, dermal irritation, haemorragic lesions, weight reduction, etc.).

The main objective of this study was to investigate the influence of key agricultural practices (resistance of barley cultivars, chemical treatment use, pre-crop and different growing locality) on *Fusarium* mycotoxins in barley. Field trials were realized in cooperation with the Agricultural Research Institute Kroměříž (harvest 2005, 2006 and 2007).

For the determination of the most important *Fusarium* mycotoxins, the high-performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was used. The trueness of the generated data was successfully demonstrated in proficiency test (Food Analysis Performance Assessment Scheme, FAPAS®) organized by Central Science Laboratory (CSL, York, UK).

Experimental

Mycotoxins

Characterisation of analytical method: isolation of the most important *Fusarium* mycotoxins from sample was carried out using an acetonitrile/water mixture for one hour by a shaker. Extract was analysed by LC–MS/MS (LCQ Deca, Finnigen, USA), on column Synergi Hydro RP (150 mm×3 mm×4 μm) after clean-up by solid phase extraction employing the MycoSep 226® cartridges.

Target mycotoxins:

- trichothecenes *type B* – deoxynivalenol (DON), nivalenol (NIV), fusarenon-X (Fus-X), sum of 13-acetyldeoxynivalenol and 15-acetyldeoxynivalenol (expressed as ADON)
- trichothecenes *type A* – HT-2 toxin (HT-2), T-2 toxin (T-2)
- zearalenone (ZON)

Pesticides

Characterisation of analytical method: Isolation of target pesticides from sample was carried out using a acetonitrile after addition 1% formic acid. Extract was analysed by LC–MS/MS (Quattro Premier XE, Waters, USA) on column Discovery C18 (100 mm × 3 mm × 5 μm).

Table I
Used pesticides

Active compound	Type	Product
Amidosulfuron	Herbicid	Secator
Mefenpyr-diethyl	Herbicid	Secator
Iodosulfuron-methyl-sodium	Herbicid	Secator
Fenpropimorph	Fungicid	Cerelux
Flusilazole	Fungicid	Cerelux
MCPA	Herbicid	Optica trio
Mecoprop	Herbicid	Optica trio
Tebuconazole	Fungicid	Falcon
Triadimenol	Fungicid	Falcon
Spiroxamin	Fungicid	Falcon

Growing Locality

Harvest 2005:
Žabčice

- chemical treatment (pesticide)
- no chemical treatment (pesticide)

Kroměříž

- no chemical treatment (pesticide)

Harvest 2006:
Kroměříž

- no chemical treatment (pesticide)

Branišovice

- chemical treatment (pesticide)
- no chemical treatment (pesticide)

Uherský Ostroh

- chemical treatment (pesticide)
- no chemical treatment (pesticide)

Harvest 2007:
Žabčice

- chemical treatment (pesticide)
- no chemical treatment (pesticide)

Kroměříž

- chemical treatment (pesticide)
- no chemical treatment (pesticide)

Examined Samples

Varieties of barley: Amulet, Bojos, Jersey, Malz, Prestige, Sebastián, Tolar and other

Harvest 2005:
6 barley samples
30 malt samples prepared from tested barley

Harvest 2006:
40 barley samples

Harvest 2007:
36 barley samples

Results

Table II

	Positive samples:	
	Toxin	[%]
	Harvest 2005	
Barley	DON (major mycotoxin)	100.0
	HT-2 toxin	86.0
	NIV	28.0
	T-2 toxin	17.0
Malt	DON (majority mycotoxin)	100.0
	ZON	13.3
	HT-2 toxin	23.3
	T-2 toxin	10.0
	sum ADON	10.0
	Harvest 2006	
Barley	DON (major mycotoxin)	100.0
	NIV	33.0
	T-2 toxin	65.0
	HT-2 toxin	22.5
	Harvest 2007	
Barley	DON (major mycotoxin)	94.0
	NIV	92.0
	HT-2 toxin	86.0
	T-2 toxin	58.0

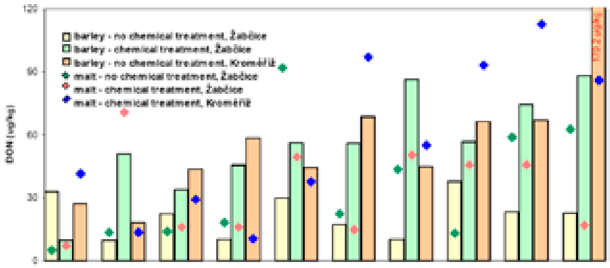

Fig. 1. **Changes of DON concentrations after malting process**

Conclusions

- Influence of pesticide treatment has not been documented. Highest and lowest incidence of DON were detected at "no - chemical treatment" samples.

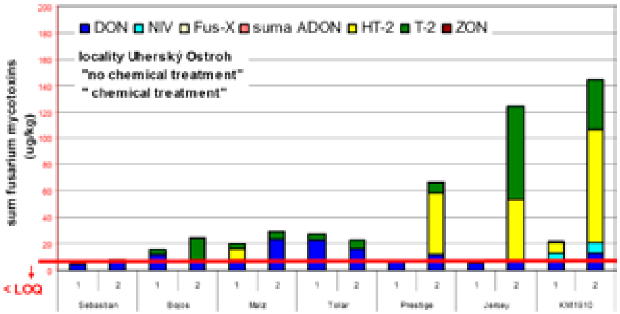

Fig. 2. **Influence of barley variety on trichothecene levels**

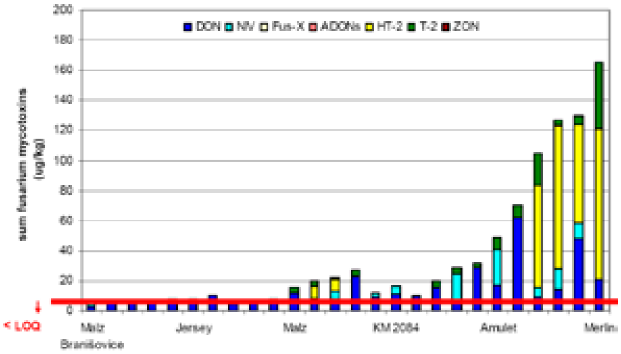

Fig. 3. **Influence of chemical treatment on production of fusarium toxins in barley samples**

- The differences among localities were significant, that could be caused by different weather conditions and different spectrums of trichothecenes indicated a contamination of barley by various fusarium fungi.
- Influence of barley variety was significant. Highest incidences of mycotoxins were detected at Amulet and Merlin varieties.

This study was carried out within the project RC No. 1M0570 and the project MSM 6046137305 granted by the Ministry of Education, Youth and Sports of the Czech Republic.

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P52 MONITORING OF ACRYLAMIDE IN FOODS IN THE CZECH REPUBLIC

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Introduction

Acrylamide is a substance that is produced, first of all (but not exclusively), in starch foods as a result of high temperature (> 120 °C) used during culinary processes, particularly during baking, grilling, frying or microwaving. Acrylamide can cause cancer in animals and experts assume that acrylamide can probably cause cancer also in humans[1,2].

The main mechanism of acrylamide formation in food is expected via a reaction between the amino acid asparagine and reducing sugars, e.g. glucose, under high temperature[1,3].

Methodology

Since 2004, content of acrylamide has been monitored within the framework of "The Project on Dietary Exposure of the Czech Population to Selected Chemical Substances" carried out by the Centre for the Hygiene of Food Chains in Brno with the objective to describe the dietary exposure of the Czech population to chemical substances.

Sample collection is designed to cover current diet composition and also various regions in the country. Important feature of the monitoring project is a fact that samples are culinary treated so that they could be analysed in the same stage as they are consumed[4].

The determination of acrylamide is conducted in starch-rich foods but also othercomm. commodities, where acrylamide was previously detected[5] (for example olives, coffee and so on) within the range of so-called "Food basket for the Czech population". Individual commodities were purchased four times per year.at a retail market of twelve towns in the CR[6].

The acrylamide is isolated from a matrix by the extraction to 100 ml demineralised warm water (60 °C). After 20 min. mixing the sample is centrifuged at 11,000 rpm for 10 min. 7 ml of supernatant is then transferred into a stoppered flask containing 2 g anhydrous potassium bromide. Derivatization (conversion of acrylamide into 2,3-dibromopropionamide) is a next step. 2.5 ml saturated solution bromine (100 ml H_2O + 3 ml bromine) were added into stoppered flask with sample. A product of bromination (over night reaction) was extracted with 2.5 ml ethylacetate and converted into stable 2-bromopropenamide by dehydrobromination with triethylamine. The ethylacetate extract is filtered and analysed by gas chromatography (HP 5890) coupled with mass spectrometry (HP 5972) employing $^{13}C_3$ acrylamide as the internal standard.

The m/z 149 and 151 were used in the method of quantification (SIM) of 2-bromopropenamide and the m/z 152 and 154 for labelled 2-bromopropenamide. The ions separated by two mass units are due to the contribution of the two isotopes of bromine. The ratio of 79Br to 81Br is 1 : 1, which enables us to choose between the above m/z in the process of quantification. The m/z value, which isn't influenced by interference, is chosen.

The method was validated and accredited according to CSN EN ISO/IEC 17025 (Czech Accreditation Institute). The quality control was implemented by participating in proficiency testing organised by Food Analysis Performance Assessment Scheme (FAPAS).

In addition, FAPAS samples of known acrylamide content were used as reference materials for internal testing.

Results and Discussion

In total 803 various samples were analysed during 2005–2007. The highest content of acrylamide was found in potato crisps (range 268–3,817 μg kg^{-1}), french-fries (range < 15–705 μg kg^{-1}), honey gingerbread (73–429 μg kg^{-1}), cocoa powder (56–567 μg kg^{-1}). Relatively high acrylamide levels were detected in spices (147–1,076 μg kg^{-1}).

Table I
Acrylamide content in food samples (2005–2007)

Commodity	n	μg kg^{-1}
Potato crisps	36	267.88–3817.48
French fries	33	<15–704.65
Ground paprika	12	147.39–1075.98
Pepper	12	182.06–456.48
Biscuits	36	50.19–1337.24
Savoury biscuits	24	68.70–708.38
Gingerbread	24	73.36–429.18
Cocoa powder	24	55.63–566.91
Wafers	36	22.00–420.94
Plain chocolate	12	61.11–246.86
Cornflakes	24	<15–299.48
Cake	24	15.59–190.24
Muesli	24	<15–195.36
Flaky pastry	12	22.54–146.93
Wheat-rye bread	24	19.84–83.58
Rye bread	36	<15–92.67
Cream cake	26	<15–97.51
Wholemeal bread	36	<15–87.09
Wholemeal rolls	36	<15–98.19
Pizza	12	<15–66.29
Chocolate sweets	36	<15–83.01
Chocolate bars	36	<15–123.22
Wheat rolls	24	17.78–70.26
French loaf	24	16.17–53.67
Milk chocolate	12	28.19–63.49
Coffee (infusion)	12	4.42–21.81
Peanuts	12	<15–48.97
Sponge biscuits	24	<15–34.79
Cocoa instant drink	12	<15–20.84

Concentration in peanuts, chocolate sweets, bread, rolls, sponge biscuits ranged from 15 μg kg^{-1} (LOQ = 15 μg kg^{-1}) to 100 μg kg^{-1}. The results in Table I summarize results of work at the Centre for the Hygiene of Food Chains during 2005–2007.

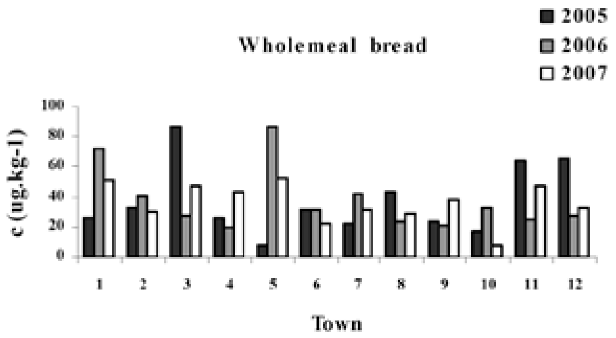

Fig. 1. **Acrylamide content in wholemeal bread**

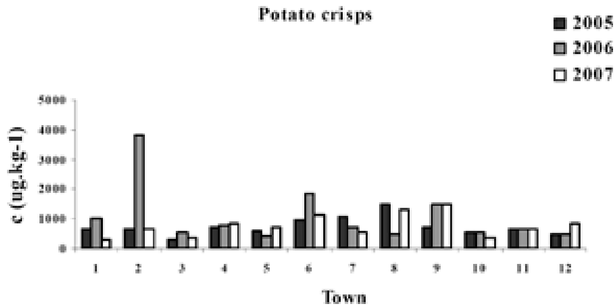

Fig. 2. **Acrylamide content in potato crisps**

The variation of acrylamide levels were recorded between individual samples of same commodities. It can be explained by fluctuation of acrylamide precursors (asparagine and sugars) in raw materials (potatoes, cereales – .), technology, storage conditions, etc. . For example Fig. 1. and Fig. 2. document variability of results for wholemeal breads and potato crisps.

Conclusion

The results will be used for both a point and probabilistic assessment of dietary exposure doses and consequently for health risk characterization. For more precise estimate of exposure doses more acrylamide concentration results are still required.

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P53 TRANS FATTY ACIDS IN DIET OF THE CZECH POPULATION DURING YEAR 2007

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Introduction

The topic "*trans* fatty acid" recently appears as a problem of growing interest. This is especially due to the relationship to negative health impact of these fatty acids.

Unsaturated fatty acids can have the same chemical formula but different chemical and consequently also physiological properties due to different geometrical configuration. The double bond of the unsaturated fatty acid can be in either *cis* or *trans* configuration. In nature most unsaturated fatty acids are in a *cis* configuration. This means that the hydrogen atoms are on the same side of the two carbons of the double bond. In the *trans* configuration, the hydrogen atoms are on the opposite side of the two double bond carbons.

Trans fatty acids (TFA) are formed in technological and microbiological processes by isomerization of *cis* double bonds to *trans* double bonds. Small amounts of *trans* fat are produced in the rumen of ruminants and then found in dairy and beef fat. *Trans* fats are predominately and not-intentionally produced commercially in large quantities through a process called partial hydrogenation used to protect foods from spoilage. The goal of partial hydrogenation is to add hydrogen atoms to unsaturated fats, making them more saturated. These more saturated fats have a higher melting point, which makes them attractive for baking and extends their shelf-life. These more stable fats are used in margarines and shortenings. Most of the *trans* fats are found in foods made with or fried in partially hydrogenated oils (margarines, high-fat baked goods, especially doughnuts, cookies, pastries and crackers). The *trans* fat content of these foods may be as high as 45–50 % of the fat.

High intakes of TFA may have an influence on total cholesterol. *Trans* fats increase levels of LDL (so-called "bad" cholesterol) and also lower levels of HDL (so-called "good" cholesterol). TFA increase the risk of coronary heart disease and of other chronic health problem (cancer, diabetes, obesity, interfility).

Several countries and the World Health Organization (WHO) have taken regulatory initiatives on the intake of TFA levels and proposed revisions to the criteria for nutrient content claims. The United States Food and Drug Administration (FDA) issued a regulation requiring manufacturers to list *trans* fat on the Nutrition Facts panel of foods and some dietary supplements. With this rule, consumers will have more information to make healthier food choices that could lower their consumption of *trans* fat as a part of heart-health diet. The WHO has tried to balance public health goals with a practical level of *trans* fat consumption, recommending in 2003 that trans fat be limited to less than 1 % of overall energy intake.[1–7]

The important input highlighting problems and questions around TFA in the Czech diet was an opinion of the Scientific Committee on Foods (VVP)[8,9]. This opinion was requested by the Czech Ministry of Health as a reaction on the open letter written by the group of Czech researchers criticizing content of TFA in some foods (shortening fat, cakes and biscuits, where TFA is main element – 50 % of all fatty acids content) placed on a market and requesting obligatory labeling similar to that used in the USA or in Denmark. The VVP repeated some ideas of EFSA and measures which can help to reduction the amount of TFA in foods. The VVP also recommended to analyse food samples representing current Czech diet to estimate main dietary sources and their influence on the total TFA intake[8,9].

The National Institute of Public Health in Prague, Centre for the Hygiene of Food Chains in Brno, is responsible to organize and perform longitudinal monitoring project named "Dietary Exposure to Selected Chemical Substances". In the framework of this project fatty acids have been monitored since 2005[10].

Material and Methods

Fatty acids were determined in food samples representing so-called food basket of the Czech population. 268 various food samples were collected for analyses of FA in 2007 (food samples are collected in 12 places which create 4 regions in the Czech Republic). Samples were culinary treated so that they could be analysed in the same stage as they are consumed. The result of the preanalytical treatment is a homogenous sample of cooked foods, which is then analysed[11]. One sample (mixture of samples from 3 places) represents composite sample for each region (A, B, C, D).

The analytical procedure consists of the three consequent steps - extraction of the triacylglycerols from the matrix, saponification and ester interchange with methanol and finally analyse methylesters of fatty acid using the GC – FID method. After culinary treatment and pre-homogenisation the samples were homogenized and extracted with mixture of petroleum ether/acetone (ratio 2 : 1) as a solvent by homogenizator (high frequency of revolutions) or by using of a hot solvent. Triacylglycerols of the extracted fat were saponified and ester interchanged with methanol. Methylesters of fatty acid were shaken out with hexan, dried, filtrated and then were analysed by gas chromatograph (TRACE GC) with flame ionization detection, split/splitless injection and capillary column (SP™ 2560, 100 m × 0,25 mm, thickness of film 0,2 μm). Standard Supelco 37 Component FAME Mix was used.

The method was validated using EffiValidation 3.0 software. The accuracy of the method was confirmed by analysing CRMs. Analytic quality control was implemented by participating in proficiency testing organised within Food Analysis Performance Assessment Scheme (FAPAS) of the Central Science Laboratory York (UK). Used method is

Table I
Results of the monitoring of fat and TFA in 2007 in food samples collected in 4 regions (n = 4)

Name of sample	Fat [%]	TFA [%]
Gingerbread	8.2–16.6	24.8–32.51
Wafers	26.6–30.9	0.23–12.14
Chocolate sweets	16.1–31.3	0.25–14.34
Biscuits	14.8–20.1	0.85–5.73
French fries	4.9–6.7	0.77–1.12
Potato crisps	29.3–36.7	0.93–1.10
Wheat-rye bread	0.2–0.3	0.14–0.45
Rye bread	0.6–2.1	0.27–0.34
Wholemeal bread	1.5–5.7	0.21–0.33
Wheat rolls	2.6–3.4	0.09–1.73
Sponge biscuits	3.0–10.6	0.34–4.26
Flour	0.7–0.7	0.08–0.12
Milk	0.8–1.1	1.95–2.41
Condensed milk	7.4–10.5	1.73–2.25
Infant milk formula	15.1–21.9	0.74–1.05
Butter	81.3–82.4	1.94–2.20
Butter spread	28.2–32.6	2.0–9.87
Margarines	41.2–59.3	0.13–1.15
Milk pudding	1.4–1.7	0.97–1.43
Whole milk yoghurt	4.9–5.6	1.65–1.78
Curd dessert	1.0–4.4	1.76–1.98
Whipping cream	24.8–31.3	1.40–1.70
Cream	10.2–11.2	1.09–1.50
Sour cream	11.5–14.0	2.03–2.27
Ice cream	5.7–8.0	1.24–7.13
Cream dessert	11.3–15.3	1.94–2.24
Processed cheese	15.0–19.5	1.90–4.15
Blue cheese	25.2–39.0	1.64–1.81
Camembert cheese	18.7–24.1	1.54–2.0
Hard cheese Edam	14.0–21.0	1.51–2.87
Eggs	7.6–9.4	0.24–0.47
Cocoa	2.2–3.3	not detected
Semolina	0.2–0.4	0.07–1.11
Soya products	1.4–2.2	0.05–0.05
Smoked fish	15.8–23.0	0.17–0.20
Freshwater fish	11.1–16.5	0.49–0.66
Canned fish	15.2–18.8	0.09–0.92
Sea fish	0.29–0.29	0.07–0.07
Marinated fish	4.2–12.6	0.06–0.07
Poultry offal	3.3–7.9	0.09–0.18
Pork liver	3.5–5.1	0.10–0.14
Sausages	14.8–22.2	0.14–0.24
Black and white pudding	12.8–24.7	0.19–0.21
Beef	10.7–14.2	1.46–2.70
Rabbit	3.0–7.9	0.24–0.30
Turkey	1.1–1.6	0.38–0.47
Chicken	12.3–20.0	0.22–0.24
Minced meat	22.5–29.8	0.76–1.14
Hen	7.7–11.4	0.13–0.23
Smoked meat	10.1–19.8	0.22–0.89
Pork	9.7–13.3	0.17–0.22
Pork flank	22.2–35.8	0.15–0.22
Frankfurters	14.7–38.9	0.51–1.0
Liver sausage	19.6–28.6	0.17–0.21
Cooked salami	14.7–23.2	0.13–0.59
Dry salami	25.1–48.9	0.21–0.32
Bacon	45.7–62.1	0.15–0.22
Poultry specialities	6.8–17.2	0.14–0.25
Knackwurst	31.0–34.4	0.26–0.41
Pork ham	2.9–4.3	0.10–0.60
Head cheese	17.0–24.7	0.30–0.44
Lard	86.2–97.9	0.15–0.18

accredited according to EN ISO IEC 17025 by the Czech Accreditation Institute.

Results and Discussion

Fatty acids were determined in food samples representing current diet consumed by the Czech population. The highest concentrations of TFA have been found in gingerbread, wafers, chocolate sweets and biscuits. Relatively high concentrations of TFA were also determined in butter spread, sour cream and ice cream. Very low concentrations of TFA have been found in poultry offal, pork liver, marinated fish, sea fish, flour and cocoa. In the Table I the name of sample, the ranges of content of fat and TFA are shown.

In total 268 individual food samples were analysed in 2007 (food samples are collected in 12 places which create 4 regions A, B, C, D) in the Czech Republic. In most of them the content of TFA did not exceed concentration 1 % TFA in fat (in 65 % of samples). The TFA concentration > 10 % in fat was observed in 2 % of samples. (Fig. 1.).

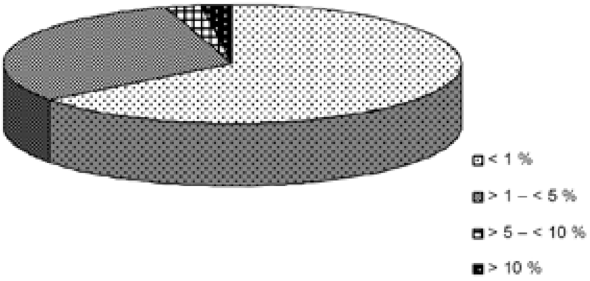

Fig. 1. **The content of the TFA as % of fat in samples collected in 2007**

In Fig. 2. an example of the comparison four kinds of sweet food samples from 4 regions (A, B, C, D) is shown. In this samples were measured the highest concentrations of TFA; it is possible to see differences between individual measures.

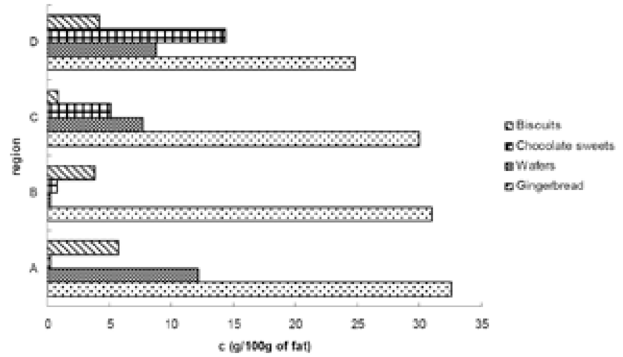

Fig. 2. **The content of the TFA in samples with the highest measured concentrations of TFA in 2007 from 4 regions**

As an example the average content of the TFA in meat (pork, hen, chicken, turkey, rabbit and beef) samples collected in 2007 from 4 regions is shown in Fig. 3.

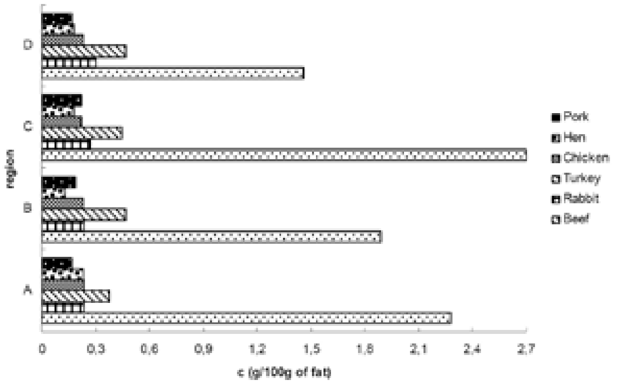

Fig. 3**. The content of the TFA in selected meat samples collected in 4 regions**

Conclusions

The topic "*trans* fatty acid" has importance for public health protection and promotion.

The content of TFA in the samples representing food basket for the Czech population was monitored in the year 2007. The highest concentrations of TFA were found in gingerbread, wafers and biscuits. Very low concentrations of TFA were found in pork liver, sea fish, flour and cocoa.

Ongoing monitoring program can cumulate new data which could be used for point and also probabilistic assessment of usual intake of FA/TFA. Results can considerably contribute to open public discussion focused on this important area of human nutrition.

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P54 DIETARY EXPOSURE MONITORING OF PERSISTENT ORGANIC POLLUTANTS FOR THE CZECH POPULATION

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Introduction

Persistent organic pollutants (POPs) are organic compounds that are resistant to environmental degradation through chemical, biological and photolytic processes. They are highly persistent, lipophilic and bioaccumulative industrial contaminants. POPs accumulate in fat of organisms. Some of them are classified as possible human carcinogens. Therefore POPs have been controlled as contaminants in the Czech environment for many years.

The Centre for the Hygiene of Food Chains in Brno guarantees "the Project on Dietary Exposure to Selected Chemical Substances" whose objective is to describe dietary exposure to selected chemical substances for the Czech population. In the framework of this project 26 OCPs – *p,p´*-DDD, *o,p´*-DDD, *p,p´*-DDT, *o,p´*-DDT, *p,p´*-DDE , *o,p´*-DDE, endosulfan I + II, endosulfan sulfate, HCH(alpha-, beta-, gamma-, delta-,), aldrin, endrin and its metabolite endrinketone, dieldrin, methoxychlor, heptachlor and its metabolites heptachlorepoxide (A + B), HCB, alpha and gamma chlordane, mirex and the seven most significant indicators of PCBs (28, 52, 101, 118, 153, 180) have been monitored since 1994. Cis- and trans- chlordane, oxychlordane and mirex were quantified since 2002.

Experimental

PCBs and OCPs were determined in samples which represent more than 95 % of composition (by weight) of current diet for the Czech population. First of all the samples bought on the Czech market were subjected to culinary treatment so that they could be analyzed under the same conditions as they are consumed. The result of the preanalytical treatment was a homogenous sample, which was then analyzed. The analytical procedure consisted of: *(i)* isolation (extraction) of the analytes from the matrix, *(ii)* removal of co-extracted compounds of the matrix and *(iii)* analysis using the GC method. The procedure was optimized as a multiresidual analysis for determination of polychlorinated biphenyls (PCBs) and organo-chlorine pesticides (OCPs).

Standards

We used a single standard solution of PCBs and OCPs (concentration 10 ng μl^{-1}, Dr. Ehrenstorfer) for preparation of calibration standard. Internal standards (PBB1, PCB 209 and 2,4,5,6-Tetrachloro-m-xylene, concentration 10 ng μl^{-1}, Dr. Ehrenstorfer) were used to determine the extent of recovery of the analytical procedure.

Sample Preparation

The amount of the food samples for the analysis was 50–200 g. Samples were ground with anhydrous sodium sulphate and spiked with PBB1, PCB 209 and 2,4,5,6-Tetrachloro-m-xylene as recovery standards. The first step of the preparation is the extraction. The type of extraction depends on the matrix of sample. The fat samples were extracted on automatic extraction device (Buchi Extraction System B-811) by hot solvents, non-fat samples were extracted at high speeds homogenizator (Polytron PT 3100) and the liquid samples were extracted in the liquid/liquid system. The mixture of petroleum ether/acetone (ratio 2 : 1) or dichloromethane was used as a solvent for liquid samples. The extracts were evaporated to constant weight in a rotary evaporator and the lipids were determined gravimetrically. The sample extracts were purified by a gel permeation chromatography (GPC) on column "Envirogel GPC cleanup column" fy Waters and by column chromatography on Florisil.

GC Analysis

The cleaned samples were analyzed by gas chromatograph (GC-Hewlett-Packard 5890) with two column system using different stationary phases and ECD detectors. For analyses were used following conditions:

column: DB 5 (30 m × 0.25 mm × 0.25 μm)
column: DB 17(30m × 0.25 mm × 0.25 μm)
initial temperature: 90 °C
initial time: 2 min
temperature rate 1: 15 °C min^{-1}
final temperature: 180 °C
temperature rate 2: 3 °C min^{-1}
final temperature: 220 °C
temperature rate 3: 1 °C min^{-1}
final temperature: 250 °C, final time 0.67 min
run time: 52 min

The method for the determination of POPs is accredited by the Czech Accreditation Institute by the CSN EN ISO/IEC 17025 Standard. Quality control of the results was conducted by the means of testing materials (TM) and certified reference materials (CRM). Certified reference material for PCBs was BCR 350 (mackerel oil) and certified reference material for OCPs was BCR 598 (cod liver oil) and BCR 430 (pork fat). Limits of quantification, depending on the type of the matrix, ranged between 0.002 and 0.05 μg kg^{-1}.

Results

Every year from 1994 to 2007 the content of 37 POPs was determined in the samples of foodstuffs. 110 samples of foodstuffs are analyzed per year. The highest content of POPs was observed in fishes, meat products, butter and milk products.

The results showed that the most abundant of all the measured analytes were *p,p´*-DDE and PCB 138, 153,180. The highest concentrations of these analytes were repeatedly found in freshwater fish and fish products. Their occurrence was also observed in meat. A common source of them is also milk fat. Selection of the highest sums of analytical findings in 2007 (n = 110) is shown in the Fig. 1. and Fig. 2. The non-fat samples are logically less contaminated groups concerning these analytes. The data were used for assessment of dietary exposure of the population of the Czech Republic (Fig. 3., Fig. 4.).

Conclusions

As the PCBs can accumulate in animal tissues, the foods of animal origin are one of the most significant sources of exposure. Higher exposure doses can be particularly expected in persons with high intake of animal fats. Hence, the decrease in consumption of animal fat can significantly contribute to lowering exposure doses.

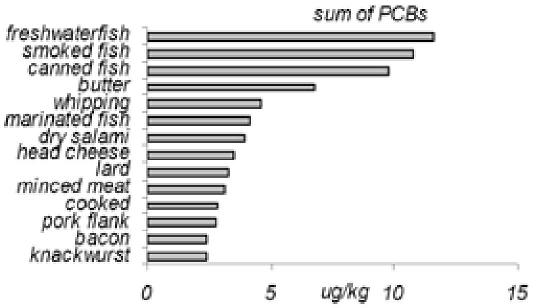

Fig. 1. **Selection of foods with the highest concentrations of PCBs (sum of PCB 28, 52, 101, 118, 138, 153, 180) in 2007**

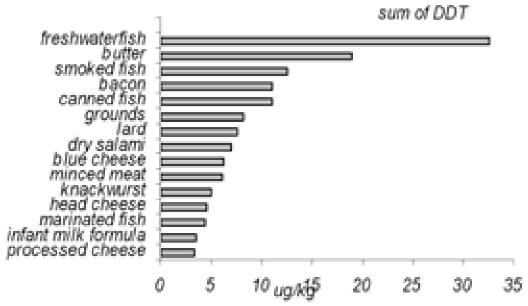

Fig. 2. **Selection of foods with the highest concentrations of sum of DDT + DDE + DDD in 2007**

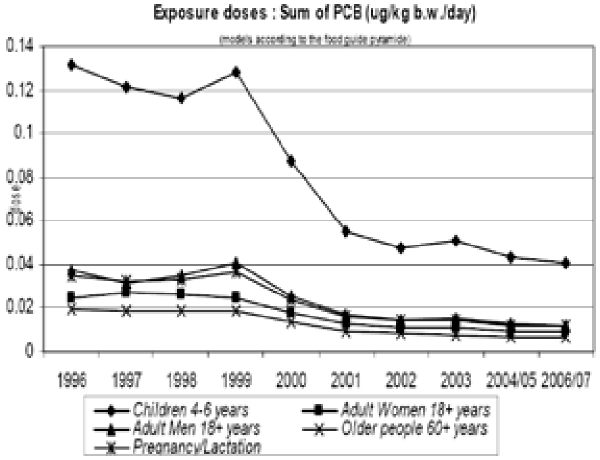

Fig. 3. **The trend of exposure doses for a sum of 7 PCBs (28, 52, 101, 118, 138, 153, 180) since 1996 to 2007**

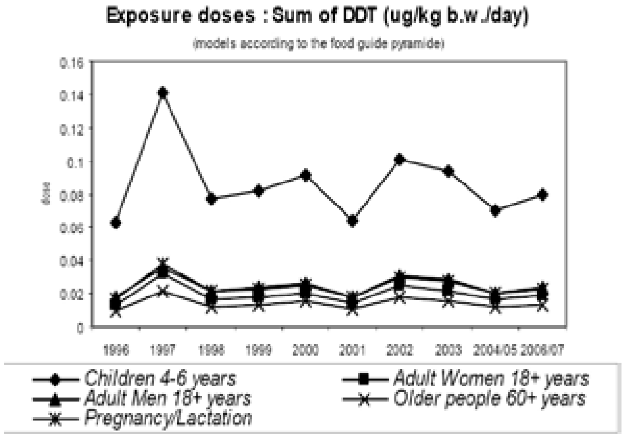

Fig. 4. **The trend of exposure to sum DDT (= DDT + DDE + DDD) since 1996 to 2007**

The trends in population exposure to DDT isomers and its analogues (DDD, DDE) and PCBs have been investigated since 1994. The found results expressed as the dietary exposure for the average Czech population (considering food consumption and the culinary factor) did not even reach the acceptable daily intake for any monitored POPs. The found exposition doses of individual POPs probably do not present a serious health risk for the average population of the Czech Republic. Detailed information about the monitoring can be found in publications of the National Institute of Public Health in Prague, describing human dietary exposure in the Czech Republic.

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P55 OCCURRENCE OF FUSARIUM MYCOTOXINS AND THEIR CONJUGATED FORMS IN CEREAL-BASED FOODS

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Introduction

Cereal-based foodstuff such as bread and beer belong in many countries to the most important items in markets´ basket. Trichothecene mycotoxins, the toxic secondary metabolites produced by species of *Fusarium* genus, are the common "natural" contaminats of cereals worldwide. Furthermore, these toxins are relatively stable, surviving household/industrial processing, thus transferred into the final food products[1,2,3].

Deoxynivalenol (DON) is one of the most abundant *Fusarium* mycotoxin. Recently, it has been shown, that besides the native form, DON also occures in cereals conjugated to glucose[1]. This "masked" DON-3-Glucoside (DON-3-Glc) is produced by metabolism of living plants and therefore, the consumers are exposed to both these mycotoxins that occur in food. The recent studies have shown that, alike the DON, "masked" forms can be transmitted thoughout the processing to food products[3]. With regard to the above mentioned, the special attention should be paid to control maximum limits established for toxins in unprocessed grains as well as food products.

Several studies concern with mycotoxin contamination of cereal-based commodities such as bread or beer, have been published up to now[4,5], however, only very limited information is known about the occurrence of conjugated forms of trichothecenes, especially DON in processed cereal-based foodstuff. The current study concern with analysis of DON-3-Glc in bread and beer is aiming at fillig the gap in this area. The aim of study was to determine: *(i)* the influence of baking technological times on levels of mycotoxins in bread and *(ii)* the average contamination of commercially available beers.

Experimental

Chemicals and Reagents

Pure crystalline standards of analysed mycotoxins deoxynivalenol (DON), DON-3-Glucoside (DON-3-Glc), 3-acetylDON and 15-acetylDON (ADONs), nivalenol (NIV), fusarenon-X (Fus-X) HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenone (ZON) were purchased from Biopure (Austria). Organic solvents in HPLC grade used for LC-MS/MS analysis were products of Sigma-Aldrich (Germany). Cellite for beer samples purification was obtained from Sigma-Aldrich as well. Ultra-pure water was produced by Milli-Q systém. Working composite standards stock solution prepared in acetonitrile at concentration 1,000 ng ml^{-1} was stored at 4 °C. Matrix-matched standards used for analysis were prepared by procedure described below (see sample preparation) at concentration range 1–1,000 ng ml^{-1}.

Samples

Bread samples (n = 9) were prepared in laboratory scale from whole-wheat flour, which was ground from naturaraly infected wheat grains. The loaves of bread were prepared in 3 variants differing in times of proofing, fermentation and baking (variant 1: 40 min. proofing and fermentation, variant 2: 45 min. proof. + ferment., variant 3: 50 min. proof. + ferment). For the dough-making process flour (300 g), yeasts (5.4 g), salt (4.5 g), saccharose (6 g), vegetable oil (4.5 g) and distilled water (150–165 ml) were used. Immediately after farinographic kneading the dough was placed into the laboratory thermostat for 40 or 50 min. After the proofing the dough was divided into three pieces that were allowed to ferment for 40 or 50 min. The bread loaves were baked in an electric laboratory oven at 240 °C for 15 or 20 min.

23 light and 7 dark beer samples, which were collected in Czech retail market in the year 2008. All of beers analysed within this monitoring study were derived only from barley malts and contained 4–6 volume % of alcohol.

Sample Preparation

Whole-wheat flour and bread samples after the drying were processed as follows: 12.5 g of homogenised samples were extracted with 50 ml of acetonitril-water mixture (84 : 16, v/v) for 30 min using an automatic laboratory shaker (IKA Laboratortechnik, Germany). Crude extract was then filtered (Filtrak No.390, VEB Freiberger, Germany) and 4 ml aliquot were evaporated to dryness, the residue was transfered into 1 ml of mobile phase in HPLC grade, consisted of water-methanol (1 : 1, v/v) and passed through a 0.2 μm microfilter (Alltech, USA).

The extraction step of beer samples was carried out by analogous procedure. To 16 ml of degassed beer were added 3.2 g of Cellite and 84 ml of acetonitrile. This heterogenous mixture was shaken for 30 min and then filtered. 5 ml of extract were evaporated to dryness and residue again redissolved in mixture of methanol-water (1 : 1, v/v).

LC-MS/MS Mycotoxins Analysis

Separation and quantification of target analytes were carried out by means of procedure described in detail in our previous study[2]. Briefly, HPLC separation (HP 1100 LC system, Agilent Technologies, USA) with reversed phase hyphenated to tandem mass spectrometer (Finnigan LCQ Deca, USA) were used for chromatographic separation of analytes. Mobile phase consisted of methanol and water acidified with ammonium acetate. Further MS/MS identification and quantification of analytes was carried out by ion trap analyzer with APCI ion source in both positive and negative mode.

Quantification and Quality Assuarance

The sample analysis was carried out in three repetitive. Matrix-matched standards of samples were prepared as described above with the only one difference, residue after crude extract evaporation was dissolved in toxins standard solution (methanol: water, 1 : 1, v/v). By means of described analytical method the limits of detection were achieved in range 0.5–5 ng ml^{-1} for measured mycotoxins.

Results

The levels of *Fusarium* toxins found in wheat grains, whole-wheat flour and breads prepared by three alternative procedures are summarized in Table I. Out of 8 monitored target mycotoxins only NIV, DON and its conjugates ADONs and DON-3-Glc were present at levels exceeded method detection limits. No Fus-X, HT-2, T-2 and ZON were found in examined samples. It should be noted, that conjugated DON was contained in experimental wheat at relatively high level, in this particular case the DON-3-Glc: DON ratio value was approximately 3 : 10, while in previous studies the ratio was reported to be 1 : 10 ref.[2].

Within the baking experiments decrease of both DON and DON-3-Glc occurred (up to 33 % and 49 %, respectively), ADONs and NIV decreased below the limits of detection. Although no significant differences in mycotoxins levels were obtained in bread loaves prepared under various experimental conditions, some indirect correlation between DON and DON-3-Glc concentrations could be seen. The longer was fermentation and proofing phase the lower was DON decrease, while decline DON-3-Glc was higher. These results indicate a potential breakdown of DON-3-Glc during bread-making yielding in free DON. At the same time other unknown transformations of these mycotoxins take place resulting in overall lower trichothecenes levels in bread in comparison with wheat taken into the process.

Table I
Levels of mycotoxins in wheat, flour and bread variations

Sample	Mycotoxins [μg kg^{-1}]			
	DON	DON-3-Glc	ADONs	NIV
Wheat	469	162	73	47
Flour	337	102	23	15
Bread-var.1	263	29	<5	<5
Bread-var.2	278	25	<5	<5
Bread-var.3	292	22	<5	<5

The data obtained in the second part of experiments concerned with beers are presented in Table II. The majority of beers contained detectable levels of DON, ADONs and DON-3-Glc. Occurrence of DON-3-Glc was shown in all of light beer samples. Interestingly, its mean levels exceeded DON levels in light as well as dark beers. Although bioavailability of DON-3-Glc has not been documented until now, guestion arises whether this “masked” DON does not increase the healt risk associated with trichothecenes dietary intake. The relative ratio of DON-3-Glc and DON in beers ranged from 0.8 to 1.3.

Table II
Levels of mycotoxins in beers

Beer	Mycotoxin [μg dm^{-3}]	Incidence [%]	Mean [μg dm^{-3}]	Range [μg dm^{-3}]
Light	DON	82	12.9	1.5–33.7
	DON–3–Glc	100	16.3	3.2–38.0
	ADONs	69	8.1	3.9–27.8
Dark	DON	84	9.3	1.0–29.5
	DON–3–Glc	92	11.0	2.5–40.2
	ADONs	71	8.8	5.1–19.8

Conclusions

The results obtained in this study can be summarized as follows:

(i) Some decrease of *Fusarium* mycotoxins including DON and its major conjugates ADONs and DON-3-Glc occurred during baking.

(ii) No significant impact of the tested technological times (proofing and fermentation) was observed.

(iii) Extensive contamination of beers obtained at Czech market demonstrated. The levels of DON, ADONs and DON-3-Glc in light and dark beers were comparable, the later compound was dominating.

This study was carried out within the scope of research projects NPV II, 2B08049 supported by the ministry of Education, Youth and Sports of the Czech Republic.

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P56 LIGNANS IN FLAXSEED – LC-MS/MS DETERMINATION

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Introduction

Lignans constitute a group of important biologically active plant phenols, structually characterized by the coupling of two phenylpropanoid units by a bond between β-positions in propane side chains. Lignans were identified in trees already in the 19th century. Currently, wide occurrence of lignans in various vascular plants has been well documented[1].

The interest in lignans and other phytoestrogens has grown in the recent years because of their putative beneficial health effects[2]. They have been suggested to induce a various effects, such as antioxidant, antitumor, estrogenic, and antiestrogenic activities, and the protection against coronary heart disease[3].

The reachest dietary source of lignans are flaxseeds, with glycosides of secoisolariciresinol (SECO) (Fig. 1.) and matairesinol (MAT) (Fig. 2.) as the major representatives of this group[2]. After ingestion, these lignans are deglycosylated and partly converted to the mammalian enterodiol and enterolactone by colonic bacteria. These forms are considered as the biologically active substances responsible for the benefical effects in humans[4].

In the presented study, a quantitative method (LC MS/MS) for determination of the plant lignans, secoisolariciresinol and matairesinol, in flaxseed has been developed. The study of variation in SECO and MAT content was based on examination of various cultivars of flaxseed.

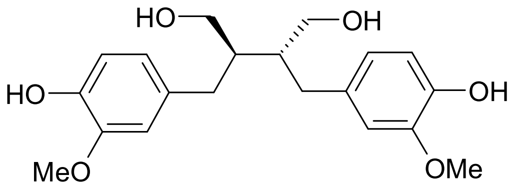

Fig. 1. **SECO**

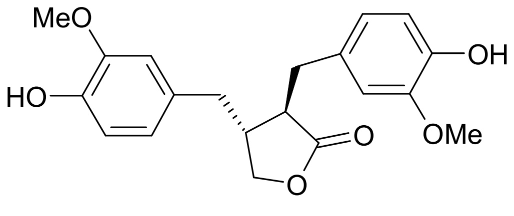

Fig. 2. **MAT**

Experimental

Chemicals

The lignan standards, secoisolariciresinol and mataresinol were purchased from Sigma Aldrich (Germany). The β-glucuronidase/sulfatase was purchased from Sigma-Aldrich (Germany).

Ethylacetate, cyclohexane, and acetic acid glacial were purchased from Sigma-Aldrich (Germany). Sodium hydroxide was purchased from Penta Chrudim (Czech republic). The deionised water was prepared using Milli-Q water system (Millipore, USA).

Samples

The flaxseeds were obtained from Agritec Plant Research s.r.o. (Šumperk, Czech republic). The levels of SECO and MAT were monitored in different cultivars. Cultivars AGT, Alaska, Astral, Atalante, Bajkal, Baladin, Flanders, Lola, Oural, Recital represented oil cultivars. Cultivar Venica represented fibre flax.

Sample Pretreament

Defated flaxseeds were incubated overnight with enzyme of Helix pomatia β glucuronidase/sulfatase in sodium acetate buffer at 37 °C. Subsequently the enzymatic hydrolysates were extracted with ethylacetate/cyclohexane. After removing the solvent, the residue was transfered into mobile phase.

Analysis

LC-MS/MS method was employed for examination of extracts. The system consisted of Alliance chromatography separation Module 2695 (Waters, USA) equipped with Discovery C 18 column (Supelco, Germany) (50 mm × 3.0 mm i.d., 5 µm), and Quattro Premier XE (Waters, UK) mass spectrometer detector employing an electrospray ionization source operating in negative mode.

Method Validation

Analytes were quantified by the standard addition method to compensate the influence of the co-isolated matrix on the effectivity of the ionisation process. Matrix standards could not be used due to unavailability of blank flaxseeds.

Repeated measurements were carried out to get performance characteristic of the method.

Results

Optimization of extraction and hydrolysis. The release of aglycones (SECO, MAT) from their glycosidic forms prior to analysis is necessary. Enzymatic hydrolysis was tested, and optimized. β glucuronidase/sulfatase (Helix pomatia) in sodium acetate buffer was used for hydrolysis of defated ground flaxseeds. Subsequent extraction of target analytes was carried out with ethylacetate/cyclohexane solvent mixture.

Chromatografic and detection conditions. The LC MS/MS method earlier described by Milder at al. (2004) was tested in the first stage. However, due to relatively high limits of detection, the composition of mobile phase was modified using acetic acid to enhance MS ionisation.

Performance characteristics. The quantification was carried out using the standard addition method. The crude flaxseed extracts were spiked with analytes to increase the analytical signal by a factor of 1.5 to 3.

The limits of detection (LOD) were determined by five repetitive analysis of standard solutions, the found values were for SECO 0.020 mg kg^{-1} and for MAT 0.025 mg kg^{-1}.

The repeatability was determined by six times analysis of flaxseed extracts and was expressed as relative standard deviation (RSD, %), the value for MAT was 3.2 % and for SECO 4.7 %.

Table I
Average levels of SECO and MAT in different cultivars of flaxseed

Cultivar	MAT [mg kg^{-1}]	SECO [mg kg^{-1}]
AGT	13	1157
Alaska	22	2317
Astral	14	1955
Atalante	14	1317
Bajkal	13	1172
Baladin	11	1197
Flanders	14	1193
Lola	12	1150
Oural	11	716
Recital	12	1030
Venica	16	682

Concentration of lignans in flaxseeds. The mean levels of lignans are shown in Table I. Their amount in various flaxseed samples ranged for SECO from 219 (Venica) to 2610 (Alaska) mg kg^{-1}, and for MAT from 5 (AGT 984) to 22 (Alaska) mg kg^{-1} with average levels 1031 mg kg^{-1} of SECO and 13 mg kg^{-1} of MAT. The levels of SECO were significatly lower in fibre flax cultivar Venica (220–860 mg kg^{-1}) compared to linseed oil cultivars (280–2600 mg kg^{-1}). The differences in MAT content were comparable in both types of linseeds.

Conclusions

LC-MS/MS method for determination of plant lignans, SECO and MAT, in flaxseed has been developed and validated. Flaxseeds were shown to be a rich dietary source of lignans. The dominating phytoestrogens representing this biologically active compounds were SECO and MAT. The highest lignans content was in cultivar Alaska.

This study was carried out within the projects NPVII 2B06087 and MSM 6046137305 supported by the Ministry of Education, Youth and Sports of the Czech Republic.

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P57 THE OCCURENCE OF PHTHALIC ACID ESTERS IN SELECTED FEEDSTUFFS

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Introduction

Phthalate acid esters (PAEs) are used in large quantities as softeners in many plastic products, paint, glue, putty, pharmaceutical products and cosmetics.[1–3] Significant migration into the environment is demonstrated during their production, manufacture, use and disposal.[4–5] Phthalates are not chemically bound to these products and are therefore continuously released into the air or through leaching into liquids.[6–11] Humans can be exposed to vinyl plasticizers through ingestion, inhalation, direct injection, or by skin contact. Exposures to phthalates range from milligrams to micrograms[12]. The occurrence of di-(2-ethylhexyl) phthalate (DEHP) ranks over 50 % of total PAEs. Butyl-benzyl phthalate (BBP) and di-*n*-butyl phthalate (DBP) are common occurrence[13].

Experimental

The aim of this investigation was to determine the occurance and content of phthalate acid esters (DBP and DEHP) in feedstuffs and oils.

Material

Samples of feedstuffs, premixes and feed additives for livestocks were collected by qualified expert from ÚKZUZ (Central Agricultural Control and Testing Institute) in years 2005 (n = 73) and 2006 (n = 59). These samples were collected from received feeds, dispatch stores, reception stores and multicomponent scales and stocked in polyethylene bags and glass bottles. Samples of oils A, B, C (n = 23) were collected during technological production processing in 2007. Oils A were stored in glass bottles, B and C in plastic barrels.

Methods

Proved methods were used for PAEs determination in feedstuffs[14]. Samples were stored in refrigerator after sampling and transporting.

Before starting the analyses, glass and laboratory materials were washed and rinsed with acetone. Samples were sufficiently homogenized, extracted by organic solvents mixture hexan:acetone (1 : 1). PAEs were separated from co-extracts by GPC (gel permeation chromatography) in a Bio-beads S-X3 filling. After GPC, the eluate was cleaned with hydrated sulphuric acid. PAEs were determined by HPLC and UV detection at 224 nm in a Cogent e-Column, C 18, using acetonitrile:water (9 : 1) as the mobile phase. The detection limit for PAEs in feeds is 0.03 mg kg^{-1}.

Table I
Concentrations of DBP, DEHP and ΣDBP+DEHP in feedstuffs in 2005

Sample	DBP [mg kg^{-1}]	DEHP [mg kg^{-1}]	ΣDBP+DEHP [mg kg^{-1}]
Wheat	0.91	3.15	4.06
Soybean oil	110.96	20.46	131.42
Natrium chloride	0.12	0.79	0.91
Vitamin K3	0.30	0.22	0.52
Vitamin D3	0.09	0.99	1.08
L-lysin	<0.03	<0.03	<0.03
Vegetable soybean oil	19.89	8.60	28.49
Alimet-methionine	38.36	<0.03	38.36
Animal fat	47.29	11.58	58.87
Animal fat	26.22	15.33	41.55
Folic acid	0.07	1.46	1.53
Corn	1.02	1.01	2.03

Results

Detected concentrations of DBP, DEHP and ΣDBP+DEHP in the first year of study (2005) are digested in Table I. The highest concentration of ΣDBP+DEHP was found in soybean oil (131.42 mg kg^{-1}) and animal fat (58.87 mg kg^{-1}). The lowest concentration was measured for L-lysin (<0.03 mg kg^{-1}). In 2006 (n = 59) levels of ΣDBP+DEHP ranged from 0.04 mg kg^{-1} (Wheat – start of processing) to 32.4 mg kg^{-1} (Rapeseed oil – end. plastic). A list of results in 2006 is given in Table II. PAEs in oils are listed in Table III. The highest concentrations of PAEs were measured in oils C (ΣDBP+DEHP = 61.55 mg kg^{-1}), whereas lowest levels were found in oils A (5.14 mg kg^{-1} ΣDBP+DEHP).

Table II
Concentrations of DBP, DEHP and ΣDBP+DEHP in feedstuffs in 2006

Sample	DBP [mg kg^{-1}]	DEHP [mg kg^{-1}]	ΣDBP+DEHP [mg kg^{-1}]
Vitamin D3	0.08	0.28	0.36
Vitamin K3	<0.03	0.19	0.19
Biotin	0.21	1.68	1.89
Nicotinic acid	0.04	2.98	3.02
L-lysin	0.05	1.71	1.76
Vitamin E	0.62	1.53	2.15
Wheat - start of processing	<0.03	0.04	0.04
Rapeseed oil – start	2.43	18.55	20.98
Rapeseed oil – end	4.68	17.19	21.87
Rapeseed oil – end plastic	26.63	5.77	32.4
Alimet	1.29	3.23	4.52
Mycocarb	0.05	0.04	0.09

Table III
Concentration of DBP, DEHP and ΣDBP+DEHP in oils

Sample	DBP [mg kg^{-1}]	DEHP [mg kg^{-1}]	ΣDBP+DEHP [mg kg^{-1}]
Oil A	1.11	5.71	6.82
Oil C	2.13	37.38	39.51

Conclusions

Samples of feedstuffs for livestock (complementary feeds, premixes, feed additives and raw materials) were collected in years 2005 and 2006, oils in 2007. Various levels of contamination were determined in feedstuffs. Feed additives, premixes and complementary feeds as well are poor in phthalates. Raw materials have higher amounts of PAEs, but the highest concentrations were measured in materials with high fat content. Because of phthalate´s lipophilic nature, they are released mainly into feedstuffs containing fat[15]. Phthalic acid esters determined in this investigation are similar to RASZYK et al. (1998)[16], where was DBP level in feed additives 0.207 mg kg^{-1} and DEHP 0.216 mg kg^{-1}. Oils before technological production processing (in plastic barrels) contained lower levels of PAEs than after processing, so this study confirmed, that PAEs can easily migrate from platics to fat.

This study was carried out within the scope of project NAZV 234/2202/MZ260041, supported by the Department of Agriculture, Res. & Dev., Czech Republic.

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P58 NEW APPROACH IN ANALYSIS OF FUSARIUM MYCOTOXINS AND THEIR “MASKED” FORMS: IMMUNOAFFINITY COLUMN CLEAN-UP

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Introduction

Mycotoxins are classified as toxic, low molecular weight secondary metabolites of microscopic filamentous fungi. Fusarium toxins are the most common contaminants in cereals. Recently several research study have been focused on masked deoxynivalenol and other mycotoxins of these group. The hyphothesis on existence of “masked” mycotoxins was postulated as soon as in mid-1980s. Mycotoxicosis cases were observed in farm animals although the laboratory examination of a feeding stuff did not indicate high levels of toxins. It was concluded that hydrolysis of precursor toxins in digestive tracts of animals occurs. Follow up research showed that mycotoxins can be partly metabolized by living plants as a result of detoxification process. “Masked” mycotoxins escape routine analysis for several reasons. These substances are more polar than the precursor toxins, they are difficult to extract with the common organic solvents or get lost in the clean-up procces. In addition, standards of these substances are not commercially available. Currently deoxynivalenol-3-glucoside (D3G) representing the “masked” form of the most common mycotoxin deoxynivalenol (DON) is available.

The most common method in mycotoxin analysis is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). To reduce interferences due to matrix coextracts various clean-up procedures are involved in sample processing step.

This study assessed the afficacy of several immunoaffinity columns (IACs) designed either for single fusarium toxins (DONPREP®, EASI EXTRACT HT-2̦T-2®, EASI EXTRACT ZON®) and DZT Multi Myco IACs for multimycotoxin analysis. Based on our recent research we investigated occurence of this phenomenon that documented cross reactivity of D3G in DON dedicated ELISA kits in the above IACs.

MycoSep® 226 AflaZoṇ columns clean-up based on adsorbtion chromatography usage were used for crude extract processing.

Experimental

Chemicals

Standards of analysed mycotoxins DON, D3G, HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenone (ZON) were purchased from Biopure (Austria).

Calibration standard solutions were prepared in the LC mobile phase methanol:water (50 : 50, v/v) by diluting of stock standard solutions in concentration range 5–1000 ng ml^{-1}. While standards in solvent were employed for calibration in case of purified samples, matrix-matched standards were used for unpurified extract.

Samples

Naturaly contaminated maize containing several fusarium toxins (see Table I) was used for our experiments.

Table I
Contents of trichothecene mycotoxins

Trichothecene	Mean $[\mu g\,kg^{-1}]$	s.d. $[\mu g\,kg^{-1}]$
deoxynivalenol	620	21
deoxynivalenol-3-glucoside	100	26
HT-2 toxin	27	18
T-2 toxin	52	15
zearalenone	462	23

Immunoaffinity Columns and MycoSep

Immunoaffinity columns DONPREP®, EASI EXTRACT HT-2T-2®, EASI EXTRACT ZON® and DZT Multi Myco IACs were purchased from R-Biopharm Rhône Ltd (Germany). DONPREP® columns contain a highly specific monoclonal antibodies purifying and concentrating DON from a sample extract. EASI EXTRACT HT-2̦T-2® columns are made for determination of HT-2 and T-2 and EASI EXTRACT ZON® are specified for ZON analysis. DZT Multi Myco IACs are designed as multi-mycotoxins columns. MycoSep® 226 AflaZoṇ columns (Romer Labs®, Austria) are designed for complex matrices with more interference such as gluten, meal feed and processed food.

Sample Preparation (Extraction and Clean-up)

For preparation of purified extract column application instructions recommended by producers were followed. Extraction processes and all subsequented steps – diluting, passing through column and eluting are summarized in Table II.

In addition, the acetonitrile:water (84 : 16, v/v) crude extract was prepared for LC-MS/MS analyses. This procedure is accredited to according ČSN EN ISO/IEC 17025:2005 for direct LC-MS/MS analysis and accuracy of generated data is regularly documented through proficiency tests (FAPAS®).

Analysis of Mycotoxins Using LC-MS/MS

High performance liquid chromatograph (HP1100 binary series LC system, Agilent Technologies, USA) coupled with tandem mass spetrometer (Finnigan LCQ Deca, USA) were employed. Chromatographic separation of

Tabulka II
Procedures employed for sample processing (according to producer recommendation)

Step of sample preparation	Immunoaffinity columns DONREP®	 Easi exctract HT-2 & T-2®	 Easi exctract ZON®	 DZT Multi MyCo	SPE columns MycoSep® 226
extraction solution	deionised water	methanol : water (90 : 10, v/v)	acetonitrile : water (75 : 25, v/v)	methanol : water (75 : 25, v/v)	acetonitrile : water (84 : 16, v/v)
dilution crude extract	no	phosphate buffered saline	phosphate buffered saline	phosphate buffered saline (PBS)	no
mLs of extract passed through comumn	2 ml extract	25 ml diluted extract	25 ml diluted extract	25 ml diluted exctract	8 ml of extract
washing	5 ml deionised water	20 ml deionised water	20 ml PBS	10 ml deionised water	no
elution	3 × 1.5 ml methanol	3 × 1.5 ml methanol	3 × 1.5 ml methanol	3 × 1.5 ml methanol	no

samples components was carried out in a reverse phase system using the column with polar endcapping (Synergi Hydro RP, 150 mm × 3mm × 4 μm).

MS-detector was operated in atmospheric pressure chemical ionization (APCI) mode, selective negative ions were aquired for DON, D3G and ZON, while for HT-2 and T-2 positive ions were monitored.

Results

The recoveries of fusarium toxins obtained within validation process employing spiked samples (200 μg kg^{-1}) for repeated analysis procedures (n = 3) characterized in Table II were summarized in Table III. The cross reactivity value for D3G is higher than recovery for DON in DONPREP®. Similarly D3G crossreacted in DZT Multi Myco IACs, nevertheless, it was fairly lower than aprox. 40 %. These DZT Multi Myco IACs provided good recoveries for HT-2 and T-2 even higher than in EASI EXTRACT HT-2T-2® IACs dedicated these two toxins. Similarly, higher recovery was achieved in DZT Multi Myco IACs than in EASI EXTRACT ZON® IACs.

The levels of fusarium toxins determined in analysis of contaminated maize corrected to recoveries are shown in Table IV. When taking the results in Table I obtained by direct LC-MS/MS as a reference (= 100 %), some more pronounced differences for results were seen. The most pronounced are higher concentration determined for HT-2 and T-2 by DZT Multi Myco IACs and ZON by EASI EXTRACT ZON® IACs. On the other hand, underestimation of D3G content accords when it is used IACs clean-up step. We can see good agreement of generated data for most of tested approaches.

Conclusions

The usage of immunoaffinity columns represent the challenging approach in selective pre-concentration of target

Table III
Recoveries of mycotoxins for various columns

Columns	Analyte	Recovery [%]
DONPREP®	DON	76
	D3G	103
EASI EXTR. HT-2T-2®	HT-2	90
	T-2	73
EASI EXTR. ZON®	ZON	66
DZT Multi MyCo	DON	79
	D3G	40
	HT-2	102
	T-2	106
	ZON	94
MycoSep® 226	DON	94
	D3G	40
	HT-2	92
	T-2	84
	ZON	98

Table IV
Data of different extraction and clean up process corrected to reference values noted in Table I

Columns	Analyte	%
DONPREP®	DON	86
	D3G	44
EASI EXTR. HT-2T-2®	HT-2	93
	T-2	125
EASI EXTR. ZON®	ZON	151
DZT Multi MyCo	DON	97
	D3G	35
	HT-2	152
	T-2	131
	ZON	101
MycoSep® 226	DON	98
	D3G	25
	HT-2	104
	T-2	110
	ZON	124

mycotoxins, moreover, isolation of same metabolite of parcial toxins is possible depending on cross reactivity of particular antibodies. Thanks to removing most of interferences with ionization process (resulting in signal suppression) and on this account, a lower detection limits can be obtained in LC-MS/MS. In addition, the improvement of method performance characteristics, the usage of standards in net solvent is possible for calibration. On the other hand, the cost of analysis is fairly increased by using immunoaffinity columns. Therefore, for analysis of sample containing relatively high levels of mycotoxins such as maize used in this study, the direct analysis of crude extract is better option. Moreover, more accurate quantification of D3G is obtained in this extract. As far as, usage of IACs is prefered to obtained high sensitivity of analysis then DZT Multi MyCo IACs are recommended for reliable pre-concentration of trichothecenes and ZON.

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P59 THE EFFECT OF CELL ADHESION ON POLLUTANT BIODEGRADATION

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Introduction

The unicellular microorganisms naturally form multicellular communities called biofilm. Bacterial biofilms are generally described as surface associated community consisting of microcolonies surrounded by a matrix of exopolymers (EPS) with varied composition. The characteristics of biofilm which are in focus of present research are the ability to withstand and degrade high concentration of various toxic substances.

Phenol and catechol are toxic and persistent pollutants of the environment. They enter the environment from a number of industrial sources, namely from the production of pesticides, herbicides and many others. The method of removal by bacterial population is one of the possible solutions[1].

For our experiments gram-positive *Rhodococcus erythropolis* was chosen as a bacterium with a broad degradation potential, owning to its highly versatile metabolism.

Experimental

Rhodococcus erythropolis CCM 2595 was cultivated in BSM[2] medium at 28 °C. Carbon source was added (phenol or catechol 0.7 g dm^{-3}) after sterilisation.

Phenol and catechol concentration in media were determined by HPLC: Watrex column 250 × 4 mm, Nucleosil 100 C18, acetonitrile/deionized water (40/60), 1.0 ml min^{-1}, UV detection 254 nm.

Changes in biomass concentration were measured by spectrophotometric method at 595 nm as a total protein concentration[3].

The cell surface hydrophobicity was assayed using a procedure according to Rosenberg[4] – BATH test. Polymer material surface hydrophobicity was measured by a contact angle measurement method[5].

For EPS composition analysis the biomass was removed from carrier by ultrasound and 2% EDTA addition. Saccharides were assayed according to Dubois[6], proteins according to Bradford[3].

Rate of adhesion during initial period of cultivation was determined by fluorescent microscopy (Microscope Nikon Eclipse E400). After staining of biofilm by SYTO 13 the part of colonized area was measured by the method of image analysis (LUCIA, Laboratory Imaging Ltd., CZ).

Results

Suspended Cells

Experiments were carried out in shaken flasks (120 rpm) in medium with phenol or catechol as a sole carbon source. The concentration of 0.7 dm l^{-3} of the pollutant was chosen as a stressed concentration according to previous experiments (data not shown). The changes of biomass concentration during cultivation were expressed as a total protein concentration. The results are presented in the Fig. 1. The biomass concentrations at the end of both cultivations were 13 mg dm^{-3}. The phenol (0.7 g dm^{-3}) was totally degraded after 49.5 h, catechol (0.7 g dm^{-3}) after 119.4 h.

Experiments have shown that catechol biodegradation is affected by day light. During cultivation catechol was decomposed to compounds which interacted with glass. The repeated use of glass vessels was impossible.

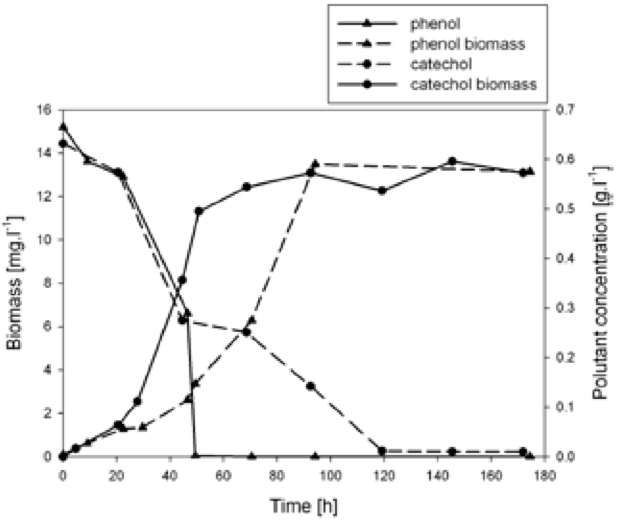

Fig. 1. ***Rhodococcus erythopolis* suspended cells growth and pollutant biodegradation. The cells were cultivated in shaken flasks in BSM medium at 28 °C. The phenol or catechol was used as sole carbon source (concentration 0.7 g dm^{-3}). The phenol/catechol and biomass (expressed as a total protein) concentration were monitored.**

Biofilm

The cell adhesion on solid materials is significantly affected by cell envelope composition and by hydrophobic/hydrophilic interactions between cells and carrier materials. The hydrophobicity and variance in EPS composition were studied.

Table I
Carrier materials hydrophobocity

Material hydrophobicity	Contact angle [°]
Glass (microscopic slide)	26.4 ± 6.6
Silicone	97.0 ± 3.6

The material hydrophobicity was determined by contact angle measurement. The results are summarised in the Table I. If value of contact angle is less than 90 ° the material is considered to be hydrophilic, if contact angle is greater than 90 ° the material is hydrophobic.

The cell hydrophobicity was determined by the BATH test. It was found that *Rhodococcus erythropolis* belongs to microorganisms with hydrophobic cell envelope character; the determined hydrophobicity is 89.0 ± 6.3 %.

The quantity of proteins and saccharides in EPS is shown in the Table II. Both proteins and saccharides amount is approximately two times higher in biofilm EPS from silicon carriers.

Table II
The concentration of saccharides and proteins in EPS

EPS composition		silicon	glass
Saccharides	$\left[\frac{mg_{EPS}}{g_{biomass}}\right]$	4.9	2.7
Proteins	$\left[\frac{\mu g_{EPS}}{g_{biomass}}\right]$	4.8	2.5

Rate of adhesion during initial period of cultivation was determined by the method of image analysis. Results have shown (Table III) that silicone is colonized more rapidly and better that glass. Small colonies were visible on silicone materials immediately after beginning of cultivation. Their area increased in time.

Table III
Portion of carrier occupied area by biofilm

Image analysis		1h	24h
glass	colonized area [%]	0.0	0.2
	number of objects in the measured area	0	46
silicone	colonized area [%]	12.5	47.9
	number of objects in measured area	1,394	1,103

The biofilm experiments were carried out in glass columns (Ø = 5 cm, volume 220 ml). The silicone and glass tubes (external diameter 0.5 cm, 1.0 cm length) were chosen as carriers. The biofilm growth was the first stage of the process. The cells were cultivated in columns in mineral medium (BSM) with phenol (concentration 0.3 g dm^{-3}, changed at regular intervals) for 3 weeks at 20 °C. After this period the stabilized biofilm was used for phenol degradation. The results are summarised in the Fig. 2. The biofilm concentration on glass (silicone) tubes was 4.1 (8.7) g dm^{-3}, respectively. The concentration of suspended cells in medium was lower in column with silicon tubes than in column with glass ones. The phenol was totally degraded in column with glass/silicone after 65 h/90 h, respectively.

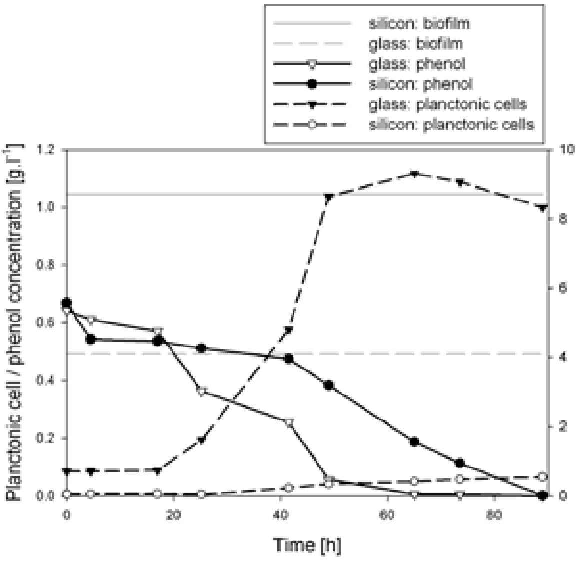

Fig. 2. **Phenol biodegradation by biofilm of *Rhodococcus erythopolis*. The cultivation was carried out in glass columns with two types of carrier (glass and silicone tubes, external diameter 0.5 cm, length 1 cm). The biomass was measured as total protein concentration at 595 nm**

The comparison of the biodegradation rates of the pollutants are summarised in the Table IV. Results indicate that catechol has a lower degradation rate than phenol. The highest biodegradation rate is reached in biofilm system with glass carriers.

Table IV
Comparison of biodegradation rate of the pollutants by suspended and sessile cells

Biodegradation rate $\left[\frac{mg_{pollutant}}{g_{protein} \cdot h \cdot l}\right]$			
	Phenol 0.7 g dm^{-3}		Catechol 0.7 g dm^{-3}
Suspended cells	3.89		1.61
Biofilm	glass 12.01	silicon 4.09	not tested in glass collums

Conclusions

It was found out that *Rhodococcus erythroposlis* cells better colonize silicone carriers to glass ones. Although the biomass concentration on glass carriers was two times lower the biodegradation rate was three times higher than in system with silicone. This indicates that only a part of biofilm is metabolically active. Results confirmed the hypothesis of higher biodegradation velocity of pollutants in biofilm system.

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P60 CATEGORIZATION OF OLIVE OILS

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Introduction

Olive oil is an important food component, which enjoys special and increasing popularity in many countries not only due to its delicate taste but also because of its nutrition value. Depending on regional conditions a variety of oils are produced in different quality.

Olive oil has several favourable health effects related to: reducing the content of adversely acting blood LDL cholesterol causing risk of cardiovascular diseases, decreasing the blood pressure, glucose content in blood, and increasing absorption of vitamins A, D, E, and K. The beneficial health effects of olive oil are connected to high contents of mono-unsaturated fatty acids and antioxidative substances.

Chemical analysis of olive oil is cumbersome since it consists of a complex mixture of chemical compounds and due to enormous matrix effect[1]. In this study, olive oil samples of different oil types were characterized by absorbances in their UV-VIS spectra and by sensorial assessment. Using a new chemometrical approach[2], based on the absorbances at the most informative wavelengths, neither the standard materials nor assignment of chemical compounds were necessary so that it is cheaper, requires less laboratory work but demands more computations.

Experimental

Olive Oil Samples and Their Characteristics

193 olive oil samples of Greek origin were studied belonging to five different oil types coded by M (31 samples), K (37 samples), E (13 samples), N (94 samples), and T (18 samples); these codes were used instead of commercial brand on demand. The sensorial assessment of the oils was made in a ten-point scale. Absorbances were measured at 2001 wavelengths in the range 200–700 nm. Acidity, the peroxide value, and traditional measurements K232 and K270 were used as further characteristics of the oil quality.

Instrumentation and Chemometrical Techniques

UV-Vis spectra of olive oil samples were measured by spectrophotometer Varian, Cary 50 Conc (Varian, Vic., Australia) and software Cary Win UV was used for data acquisition and processing. Absorption spectra of diluted (1 : 300, v/v) olive oil were measured in isooctane (spectroscopy grade); then they were digitized using a 0.25 nm step and saved to the PC.

For classification purposes new categorical variables *Sens*, *Variety* and *Location* were used denoting sensorial quality, oil type and geographical origin of the sample, resp. Linear discriminant analysis, quadratic discriminant analysis, logistic regression, the K-th nearest neighbour method and artificial neural networks were utilized as the classification techniques. The classification performance was evaluated for: *(i)* the *training set* used for computing the classification model, *(ii)* the *test set* created by the individual samples excluded from the training set by the "leave-one-out" principle[3].

Results

Categories of Olive Oils

According to sensorial characteristics, the collected samples were categorized into three classes using categorical variable *Sens*: the highest quality oils (6.5–9.0 points, denoted as "best"), the medium quality samples (3.5–6.4, "good"), and not acceptable quality (1.0–3.4, "worst").

Two further categorization principles were applied: *(i)* the type of olive oil using categorical variable *Variety* - five categories M, K, E, N, and T, *(ii)* the geographic locality, Peloponnese, Central Greece, and Crete, using the three-class categorical variable *Location*.

Classification of Olive Oils by Different Criteria

Linear discriminant analysis (LDA) and other classification techniques need an optimal reduction of the original number of variables eliminating unimportant ones. For this purpose the stepwise variable selection was used, by which 60 optimal wavelengths were selected in the case of the oil type categorization, 37 wavelengths were selected when sensorial quality was categorized, and 60 wavelengths when categorizing the oils by geographical origin.

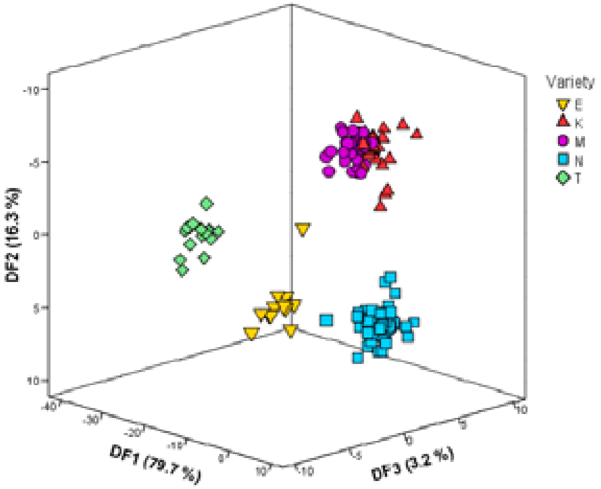

Fig. 1. **3D LDA plot of 5 different varieties of olive oils**

Display of the olive oils discrimination by the oil type (*Variety*) in 3D space of first three discriminant functions is shown in Fig. 1. Analogical 2D displays in Fig. 2. and Fig. 3. depict the oils classification by sensorial quality and geographic origin. A very good separation of the oil classes is evident in all three studied cases.

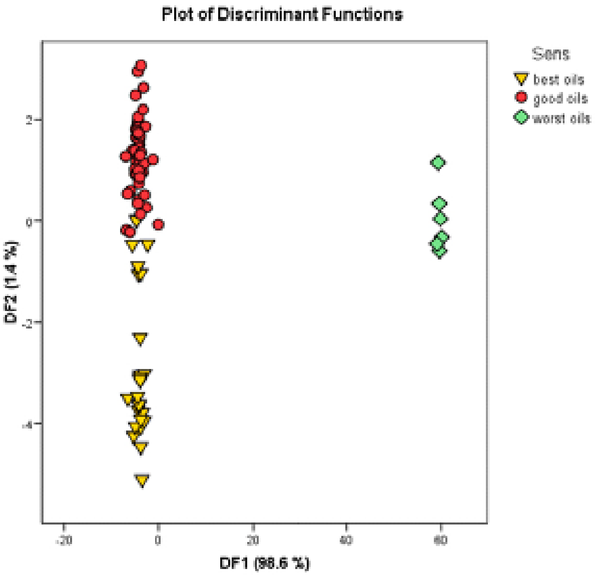

Fig. 2. **2D LDA plot of three olive oil classes differing by the sensorial quality**

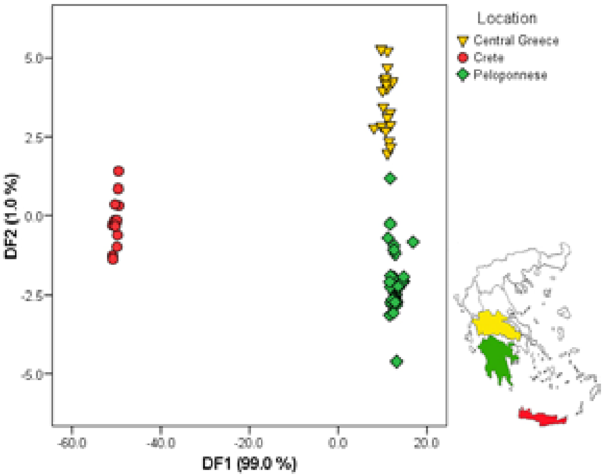

Fig. 3. **2D LDA plot of three olive oil classes differing by geographical origin**

Quadratic discriminant analysis (QDA), logistic regression (LR) and K-th nearest neighbour method (KNN) are further frequently used classification techniques[4]. They were used together with LDA and artificial neural networks (ANN)[5] for a complex classification of olive oils. A very good separation of the olive oil samples according to two selected criteria is shown in Table I and Table II.

Table I
Results of classification of 187 olive oils by variety

Method	Classification success	Model	Leave-1-out	Software
LDA	Number of correct	186	177	SPSS
	%	99.5	94.7	
QDA	Number of correct	187	131	SAS
	%	100.0	70.1	
KNN	Number of correct	186	185	SAS
	%	99.5	98.7	
LR	Number of correct	187	–	SPSS
	%	100.0	–	

The effectivity of classification using different classification techniques and software is here expressed by the ratio of the successfully categorized objects to all objects in per cents. The most important are the results of cross-validation achieved here by leave-one-out method. They show the effectivity of prediction new objects (olive oils) not involved in the training procedure; the best techniques are close to 100 %.

Table II
Results of classification of 93 olive oils by sensorial quality

Method	Classification success	Model	Leave-1-out	Software
LDA	Number of correct	87	81	SPSS
	%	95.6	89.0	
QDA	Number of correct	91	60	SAS
	%	100.0	65.9	
KNN	Number of correct	85	80	SAS
	%	93.4	87.9	
LR	Number of correct	91	–	SPSS
	%	100.0	–	
ANN	Number of correct	91	–	JMP
	%	100.0	–	

Classification of the oil samples by geographic origin was performed only by the LDA technique, which categorized 98.4 % of samples by leave-one-out technique in a correct way (consistent with Fig. 3.).

Conclusions

Classification of olive oils by variety, sensorial quality and geographic location was successfully performed by several chemometrical techniques using modern commercial software. The classification effectivity was in best cases close to 100 %. A speciality and novelty of the applied approach is processing of spectral data without the need to utilize standards representing the most important components of olive oils. Therefore a very complex quantitative multicomponent analysis is not necessary and the expenses for it and for the standard materials are saved.

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P61 THE PROTEOMIC ANALYSIS OF BARLEY ALBUMINS AND GLOBULINS

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Introduction

The proteins and glycoproteins play many important roles not only in nature, but also in technologies (*e.g.* food chemistry, medical technologies).

The characterization of glycoproteins is not an easy task and several techniques have to be used for the resolving of their total chemical composition. Today, methods including gel electrophoresis, LC, and MS together with the database searches have been increasingly used. MS has become a widely used method in protein analysis after the invention of MALDI and electrospray ionization techniques[1].

Proteomics is increasingly used to address questions of development, physiology and quality of crop plants[2,3]. Modern analytical techniques are used for the characterization of cereal seed composition in order to increase basic knowledge about these plants for economical and nutritional aspects. One of the most important crops is barley (*Hordeum Vulgare*), which is used mainly for malting and animal feed[4]. The production of (glyco)proteins in barley seed is of particular interest, because this approach can be utilized to produce barley lines with improved properties, such as malting quality due to expression of thermotolerant enzymes[5].

Experimental

Extraction of Proteins

The grains of malting barley (cultivar Jersey) were obtained from Research Institute of Brewing and Malting (Brno, Czech Republic). 250 mg of flour was mixed with 2 ml of extraction reagent (TRIS buffer – 0.1 M, pH 7.8 containing 0.5 M NaCl; 1 mM $MnCl_2$; 1 mM $CaCl_2$) and shaken 2 h. The suspension was centrifuged (20 min; 14,000 rpm). The supernatant containing the proteins was filtered using 0.45 μm microfilter (Millipore) and used for the following experiments.

Affinity Chromatography (AC)

A glycoprotein enriched fraction was obtained by Concanavalin *A* (Con *A*) lectin chromatography (Calbiochem). 4 ml of TRIS extract was applied on the column. Purified glycoproteins were released by 500 mM glucose.

1D-GE

The proteins were separated by 12.5 % SDS gels. SDS-PAGE separations were performed on 16 × 14 cm gels (OWL SEPARATION SYSTEMS).The visualization was carried out by Coomassie Brilliant Blue R-250 (CBB).

In-Gel Trypsin Digestion and Mass Spectrometry

The particular barley proteins and glycoproteins separated by 1-D GE were subjected to in-gel trypsin digestion[6]. Peptides, obtained by trypsin digest, were analyzed by MS. MS experiments were carried out on an *Applied Biosystems 4700 Proteomics Analyzer* mass spectrometer. α-Cyano-4-hydroxycinnamic acid (10 $mg\,m^{-1}$ 0.1% trifluoroacetic acid/acetonitrile (1 : 1, v/v)) was used as a matrix for MALDI-TOF MS analysis of peptides obtained by trypsin in-gel digestion. Nitrogen was used as the collision gas for MS/MS experiments. Protein identification was performed by searching the peptide masses and MS/MS sequence stretches against the sequence databases using the MASCOT or ProFound search engines.

Results

This work was focused on the systematic proteomic study of barley grains. Barley was selected as a model sample because it contains a complex mixture of proteins and glycoproteins and has a lot of analogies in other plant materials.

In general, it is not possible to identify the proteins without previous combination of MS with the separation techniques. Therefore especial attention was paid to the optimization of extractions and following separation methods. The different extraction reagents, selected on the basis of Osborn's protein classification, were tested and obtained protein patterns were compared. Fig. 1. showed noticeable differences between individual extract. The attention was focused

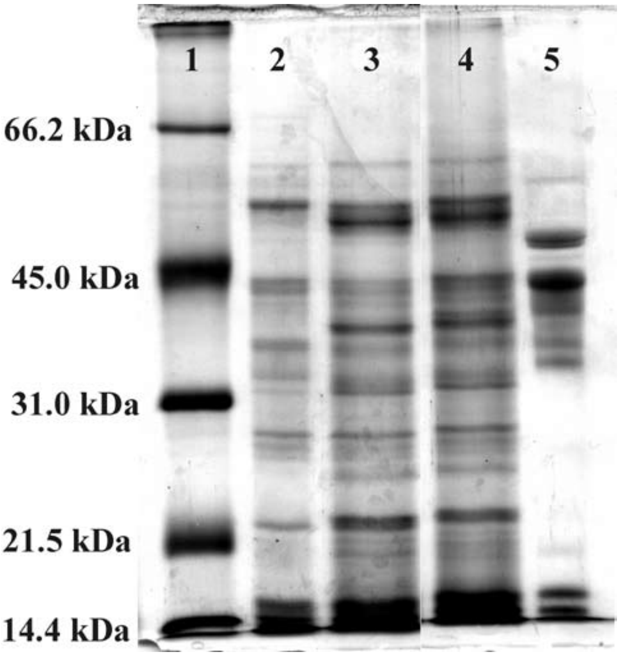

Fig. 1. **CBB stained 12.5 % 1-D SDS gel of barley proteins extracted by different extraction reagents. Lane 1 – molecular weight markers, Lane 2 – aqueous extract; Lane 3 – salt extract; Lane 4 – TRIS extract, Lane 5 – ethanol extract**

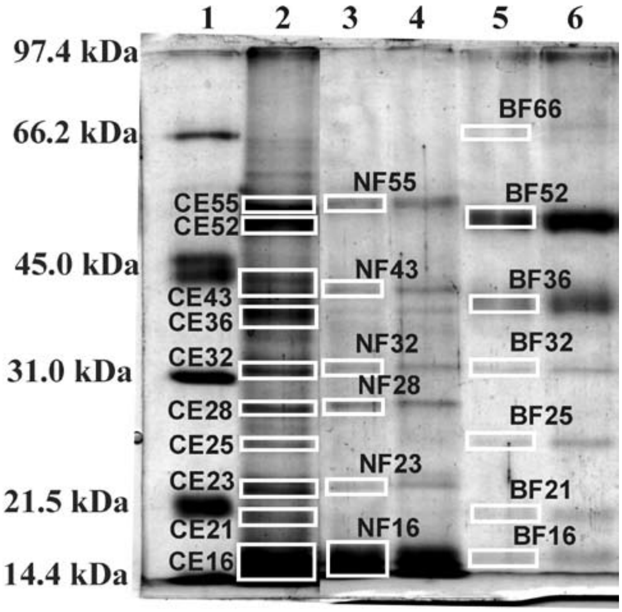

Fig. 2. **CBB stained 12.5 % 1-D SDS gel of TRIS extracts and its AC fractions. Lane 1 – molecular weight markers, Lane 2 – crude TRIS extract (CE); Lanes 3, 4 – Con A non-bound protein fractions of TRIS extract (NF); Lanes 5, 6 – Con A bound glycoprotein fraction of TRIS extract (BF)**

on albumin/globulin fraction extracted by TRIS buffer (see Experimental) that is important for food industry, because the proteins belonging to this fraction survive malting and brewing procedures and influence beer quality.

Lectin AC, one of the most effective glycoprotein isolation methods, was used for other simplification of albumins/globulins mixture and for the acquisition of fraction containing a high degree of barley glycoproteins. Since a common feature of plant seed glycoproteins is the presence oligosaccharide chains having an affinity for Con *A*, it was used to enrich the fraction of barley high-mannose and hybrid-types *N*-glycoproteins having affinity for this kind of lectin. The particular proteins (Con *A* non-bound fractions) and glycoproteins (Con *A* bound fraction) were subjected to SDS-PAGE. 1-D gel of AC fractions is shown in Fig. 2. Major bands were cut out (Fig. 2.) and exposed to the trypsin in-gel digestions. Extracted mixture of peptides was subjected to MS and MS/MS analyses. Subsequent database searching led to the identification of numerous groups of barley proteins with molecular masses ranges from about 70 to below 20 kDa. Their summary is shown in Table I.

Conclusions

These experiments confirmed that combination of AC, SDS-PAGE and MALDI-TOF MS is convenient for the characterization of barley (glyco)proteins. The developed procedures can be used for the solution of important industrial (*e.g.* brewing) problems.

Table I
Identified proteins from TRIS extract and its AC fractions

Indication of gel spots (extract)	Indication of gel spots (AC fractions)	Molecular mass (theor.) [kDa]	Molecular mass (exp.) [kDa]	Accession number	Protein name
–	BF66	72.5	~ 66	gi|421978	BEG1
CE55	NF55	57.9 59.6	~ 57	gi|38349539 gi|10953877	β-Amylase
CE55	BF52	57.7	~ 57	gi|804656	β-Glycosidase
CE 52	BF52	72.5	~ 55	gi|421978	BEG1
CE43	NF43	43.3	~ 43	gi|1310677 P06293	Protein Z serpin Protein Z
CE36	BF36	72.5	~ 36	gi|421978	BEG1
–	BF32	31.5 25.6	~ 31	gi|15824660 gi|72333	Con A
CE32	NF32	30.0	~ 32	gi|132577	rRNA N-glycosidase
CE28	NF28	28.5	~ 28	gi|116316	Endochitinase
CE25	BF25	72.5	~ 25	gi|421978	BEG1
CE23	NF23	20.0–22.4	~ 23	gi|439275 gi|4699834 gi|18920 gi|18916	Mixture of α-amylase/subtilisin inhibitors
–	BF21	72.5	~ 21	gi|421978	BEG1
CE16	NF16	16.1	~ 16	gi|439275	CMa-α-amylase inhibitor
CE16	NF16	17.9	~ 16	gi|452325	CMd-α-amylase inhibitor
–	NF16	16.7	~ 16	gi|6634471	CMe-α-amylase inhibitor
–	BF16	72.5	~ 16	gi|421978	BEG1

This work was supported by the project Centre for Study of Extract Compounds of Barley and Hop No. 1M0570, and Research Plan of IAC of the ASCR (AV0Z40310501).

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P62 COMPARATIVE STUDY OF THE PROTEIN COMPOSITION INDUCED BY MALTING BARLEY OF TWO VARIETIES

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Introduction

The selection of barley cultivar has great importance for the malting and brewing industries, as well as for breeders, because malting properties and resistance of barley plant to fungal or viral diseases is cultivar-dependent. Since protein content is one of the most important barley character related to malt qualities, proteomics is ideal tool for both differentiation of barley cultivars and description of malting course. Proteomic analysis usually includes some separation technique connecting with MS and the database searches. SDS-PAGE has been commonly used as a separation step before MS analysis of proteins. Moreover, it seems to be convenient tool for the study of qualitative differences between barley cultivar.[1–3]

The aim of this study was the characterization of protein changes and the identification of major proteins that undergo some modifications during malting process. For these experiments two barley cultivars were selected.

Experimental

Extraction of Proteins from Barley Grains

Barley grains in different malting degrees (cultivar "Jersey" and KM1910) were obtained from Research Institute of Brewing and Malting (Brno, Czech Republic). 50 mg of sample was mixed with 1 ml of deionized water and shaken 1 hr. The suspension was centrifuged (10 min; 13,000 rpm). The supernatant containing the proteins was lyophilized.

Protein Separation

Lyophilized extracts were resuspended in 150 µl sampling buffer (Laemmli Sample Buffer (Bio-Rad) with β-mercaptoethanol, 19 : 1) and denaturated (10 min, 95 °C). SDS-PAGE separations were performed on 15 % TRIS-HCl (Bio-Rad) or gradient (4–20 % TRIS-HCl, Bio-Rad) gels. The visualization was carried out by Coomassie Brilliant Blue (CBB) R-250, CBB G-250 and silver staining.

Protein Identification

The selected proteins separated by 1-D GE were digested in-gel by chymotrypsin[4]. Obtained peptides were analyzed by MALDI-TOF MS (*Applied Biosystems 4700 Proteomics Analyzer*). α-Cyano-4-hydroxycinnamic acid (CHCA; $10\ \mathrm{mg\,ml^{-1}}$ 0.1% trifluoroacetic acid/acetonitrile (1 : 1, v/v)) was used as a MALDI matrix. Air was used as the collision gas for all MS/MS experiments. Protein identification was performed by searching the peptide masses and MS/MS sequence stretches against the sequence databases using the MASCOT search engine (database NCBInr).

Results

Protein Changes During Malting

Water-soluble proteins were extracted from barley mature grains, grains after 1–5 days of malting and from green and ready malt. 1-D GE was performed to obtain a complex characterization of changes of protein profiles during malting. The gradient gel (4–20 %) was used to acquire the results covering the largest interval of molecular masses. Since the demand for a protein staining (sensitivity, compatibility with MS) is one of the most important tasks in GE, especial attention was paid to the selection of protein visualization. Two types of CBB (R-250 and G-250) and silver staining were compared (data not shown). The best results were obtained with CBB-G of which using the differences between protein patterns during malting were distinct (Fig. 1.).

A visual inspection of gradient gel confirmed significant modifications observed after three days of malting at minimal five protein bands at molecular masses about 53 kDa, 43 kDa, 26 kDa, and 23 kDa (see arrows in Fig. 1.). The major changes were noticed at the band with molecular mass about 43 kDa. Therefore it was cut out, digested by chymotrypsin and obtained mixture of peptides was subjected to MS and MS/MS analyses (Fig. 2. a, b). Database searching revealed its identity as *Protein Z* (gi/131091). This protein is important for the brewers, because due to its posttranslational modifications (glycations) influence the sensory and technological properties of beer (e.g. foam).

Protein Pattern for Different Barley Cultivars

Extracts from mature grains and malt of two different varieties, Jersey and KM, were separated via 15 % 1-D SDS-

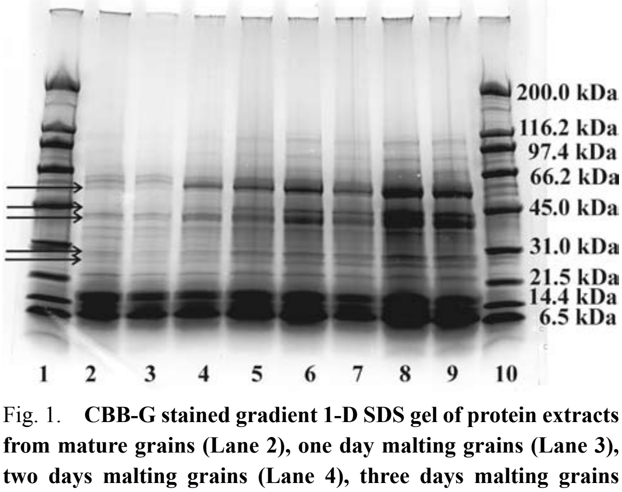

Fig. 1. **CBB-G stained gradient 1-D SDS gel of protein extracts from mature grains (Lane 2), one day malting grains (Lane 3), two days malting grains (Lane 4), three days malting grains (Lane 5), four days malting grains (Lane 6), five days malting grains (Lane 7), green malt (Lane 8) and ready malt (Line 9). Molecular weight markers are in Lanes 1 and 10**

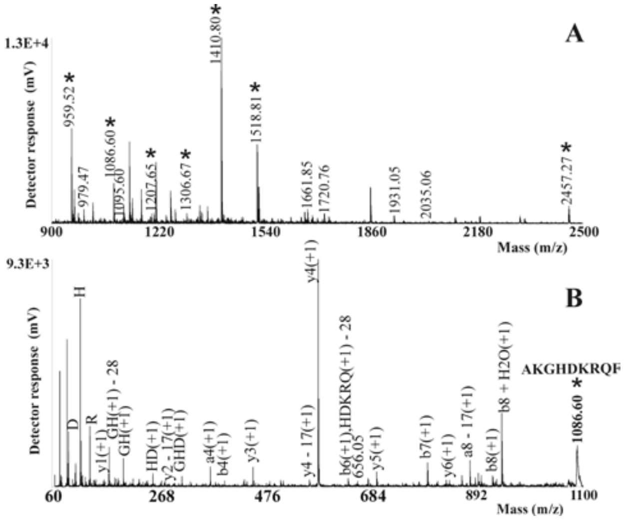

Fig. 2. **A) MALDI-TOF mass spectrum of peptide mixture from in-gel tryptic digest of a protein band 43 kDa. The asterisks (*) mark peptides coming from barley Z protein (confirmed by MS/MS). B) Representative MALDI-TOF fragmentation spectrum of $[M+H]^+$ ion peptide at m/z 1086**

PAGE in order to compare their protein profile. An example of differences in the protein pattern between cultivars is given in Fig. 3. The most relevant changes were observed between mature grains. As Fig. 3 showed, some of perceptible differences are in the protein concentrations (e.g. bands about 40 kDa), some bands were not present in KM cultivar, others in Jersey (see arrows in Fig. 3.).

Conclusions

Combination of 1D-GE and MALDI-TOF MS was successfully used for the revealing of characteristic changes in protein profile during malting process as well as the differences between two selected barley cultivars.

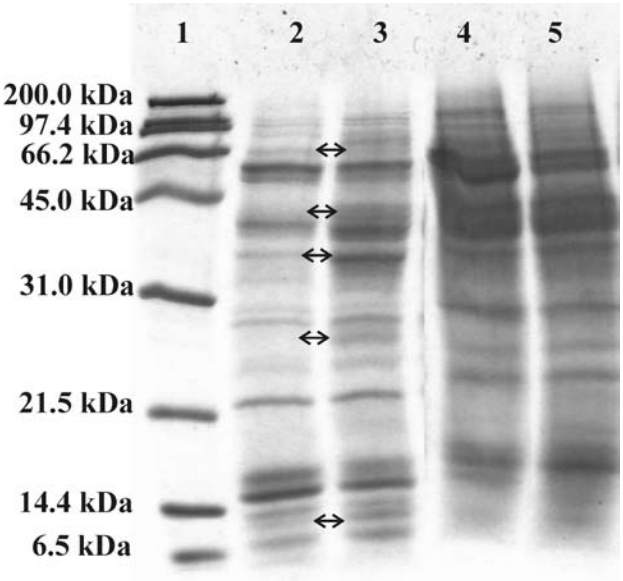

Fig. 3. **CBB-G stained 15 % 1-D SDS gel of protein extracts from mature grains (Lane 2 – variety Jersey; Lane 3 – variety KM), and ready malt (Lane 4 – variety Jersey; Lane 5 – variety KM). Molecular weight markers are in Lane 1**

This work was supported by the project Centre for Study of Extract Compounds of Barley and Hop No. 1M0570, and Research Plan of Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic (AV0Z40310501).

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P63 FATTY ACIDS LIKE MARKERS OF PROCESSED CHEESE CHANGES DURING STORAGE

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Introduction

Pasteurized processed cheese products are cheese-based food produced by comminuting, blending and melting one or more natural cheeses and optional ingredients into a smooth homogenous blend with the aid of heat, mechanical shear and usually emulsifying salts.

Optional ingredients which are determined by the product type include dairy ingredients, vegetables, meats, stabilizers, emulsifying salts, flavours, colours, preservatives and water[1,2]. Dairy ingredients means butter, cottage cheese, milk concentrates (e.g. skimmed milk powder, whey powder, caseinates).

Chemical composition of processed cheese depends on a processed cheese type and raw materials used[3].

Although the dairy products fall into the class of so called non-acid foodstuffs and suitable methods must be used to prolong their durability, the processed cheese was proposed as a part of Combat Ratio. The Combat Ratio serves for nutrition of soldiers in specific combat situations such as separation from a unit, battle, etc.[3]. There are special requirements for a minimum durability of individual components of Combat Ration which has been set as 24 months depending on an ambient temperature[4].

Practically the only method of achieving the 24 month durability of processed cheese is the thermo-sterilization. In the article the effect of a defined sterilization heating and storage conditions, such as temperature and time, on changes of fatty acids contents in sterilized processed cheese was investigated.

Experimental

Processed cheese (40 % dry matter, 45 % fat in dry matter) was manufactured following the traditional technology process in a selected dairy. The processing temperature was 91 °C, total time of heating was 5 minutes. Cheeses were than sterilized at 117 °C for 20 minutes and then cooled down to 10 °C in 2 hours.

Cheese samples were stored at 6 ± 2 °C, 23 ± 2 °C and 40 ± 2 °C during 24 month.

Extraction of Fat

The cheese sample (5.0 g) was heated with 15 ml of hydrochloric acid in boiling water bath until it dissolves and then still 20 minutes.

Ethanol (15 ml), diethyl ether and petrol ether (both 30 ml), were added into stirred mixture after cooling down. Closed flask was shaken and then allowed to separate the water and organic phases. The upper (organic) phase was displaced by the pipette to dried weighed flask. Next the water phase was reextracted with 15 ml of diethyl ether and 15 ml of petrol ether two times.

The rest of organic solvent was evaporated in 60 °C water bath (to dispose smell). Flask with fat sample was dried at 105 °C for one hour[1].

Methanol Esterification Method

The fat sample (5.0 g) was saponified with 50 ml methanolic solution of potassium hydroxide ($c = 0.5\ \mathrm{mol\,dm^{-3}}$) for 30 minutes in distilling flask with condenser and was esterified after neutralization by sulphuric acid on methyl orange for 30 minutes again.

After cooling methyl esters were shaken with 10 ml of heptane three times. The extract was dried by anhydrous sodium sulphate and filtered to a 50 ml volumetric flask again. Both heptane portions were rinsed with 20 ml of water twice. The extract was dried by anhydrous sodium sulphate and filtered to a 50 ml volumetric flask and filled up to the mark with heptane[5].

GC Analysis

Prepared heptane methyl esters solutions were injected to gas chromatograph using autosampler.

GC conditions: gas chromatograph TRACE GC (ThermoQuest Italia S. p. A., I) equipped with flame ionization detector, split/splitless injector and capillary column SPTM 2560 (100 m × 0.25 mm × 0.2 μm) with the temperature programme 60 °C held for 2 min, ramp 10 °C min^{-1} up to 220 °C, held for 20 min. The injector temperature was 250 °C and the detector temperature was 220 °C. The flow rate of the carrier gas N_2 was 1.2 ml min^{-1}.

Results

This work deals with the fatty acids included in processed cheese. The most abundant fatty acids such as lauric,

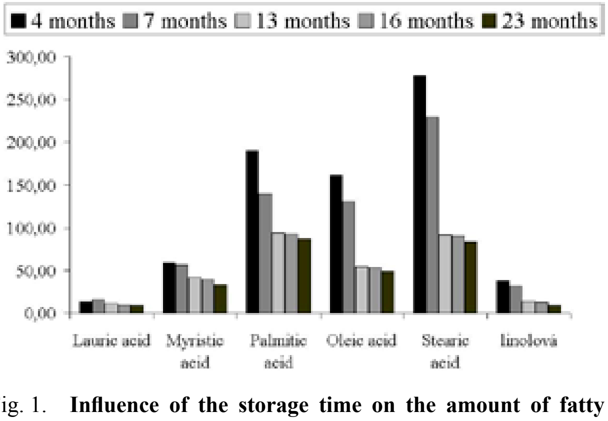

Fig. 1. **Influence of the storage time on the amount of fatty acids (cheeses stored at 6 ± 2 °C) (mg g^{-1}of cheese)**

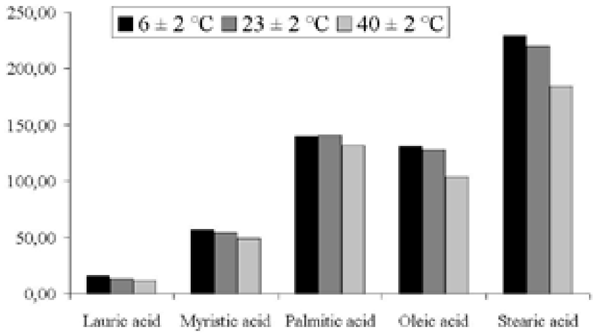

Fig. 2. **Differences between amounts of fatty acids in cheeses stored at different temperature (storage time 23 months) (mg g^{-1}of cheese)**

myristic, palmitic, stearic and oleic acid were elected like markers of changes during storage. The differences between amounts of these fatty acids depending on the storage temperature and time of storage were observed in this experiment.

In the Fig. 1 there are showed the differences between the processed cheeses stored at 6 ± 2 °C. By the increasing time of storage the amouths of elected fatty acids decrease. This can be explained by autooxidation process which can not be averted.

Since the first months the volume of fatty acids decreased with the temperature. In the Fig. 2. we can see differences between the cheeses stored for 23 months at different conditions. The temperature promotes the oxidative process.

Conclusions

The changes of fatty acids inclued in sterilized processed cheeses during storage were observed in these experiments. The cheeses were stored at different conditions for the 24 months.

We can conclude that the storage conditions significantly influence the content of individual fatty acids in cheeses. The contents of fatty acids in cheeses decreased as the consequence of longer time and higher temperature of storage. This fact can be probably caused by oxidation reactions of cheese fat.

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P64 DETERMINATION OF SELECTED ANTIOXIDANT ENZYMES IN BARLEY AND MALT

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Introduction

Presence of Reactive Oxygen Species (ROS) negatively affects the sensory stability of beer, thus, final quality of beer is related to its antioxidant activity. Antioxidant content in beer is substantially dependent on anti-oxidative activity of the primary raw materials – barley and malt. In barley antioxidants prevent the undesirable and generally negative changes of grain quality during the cropping and stocking. Generally, antioxidants can act against ROS by radical trapping, by inhibition of their formation or by decreasing of their effects. Complex antioxidant system acts as a well-coordinated complex of low molecular weight- and enzyme antioxidants. In barley antioxidant enzymes are represented mainly by superoxide dismutase, catalase and peroxidase.

Superoxide dismutase (SOD) is the main antioxidative enzyme in plant cells, where it stands for catalyse of dismutation of superoxide to dioxygen and hydrogen peroxide. This hydrogen peroxide is subsequently decomposited to dioxygen and water; catalases and peroxidases stand in this reaction as catalyzators.

In this work the influence of selected biological and chemical factors on superoxide dismutase and catalase activity in barley and malt were studied. As the part of the work was the optimalization of enzyme activity determination methods associated.

Methods

Samples of Barley and Malt

The enzyme activities were studied in two sets of 24 samples (6 barley varieties – Bojos, Jersey, Malz, Radegast, Sebastian and Tolar, cultivated on 4 localities – Branišovice, Hrubčice, Lednice and Krásné Údolí): the first set treated by standard spraying, the second was not treated. The malts originated from these samples were analysed using the same methods as for barley grain analysis.

Determination of Superoxide Dismutase

The modified method using the diagnostic kit RANSOD (Randox Laboratories, UK) was used for superoxide dismutase activity determination. This method employs xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride forming a formazan dye. The superoxide dismutase activity is then calculated by the degree of this reaction's inhibition.

Because the original method was developed for superoxide dismutase determination from human blood, the modification for SOD analysis in barley and malt consists in extract preparation from smashed and homogenized samples (Belcredi *at al.*, 2006): The sample is grinded and compounded in distilled water at 45 °C. The mixture is compounded for 15 minutes in the tub at the same temperature of water bath. After half-hour chilling the mixture is filtered. The filtrate is analysed for SOD activity according to the RANSOD kit manual.

Determination of Catalase

The most frequently used method for catalase activity determination (Bergmeyer, 1970) modified by Góth (1991) was used in this work. Góth's modification is based on fact, that the catalytic action of catalase could be stopped by addition of ammonium molybdate forming stable coloured complex with hydrogen peroxide.

Grinded sample is mixed with phosphate buffer at pH = 7.0 and than is this mixture compounded for one hour at the temperature of 5 °C. After subsequent centrifuging is supernatant filtered and used for analysis: hydrogen peroxide (at 12 mmol dm^{-3} concentration) is added to specimen and after 1.5 minutes is also added ammonium molybdate (at 16.2 mmol d^{m-3} concentration). The absorbance of formed yellow complex is measured at 375 nm. The concentration of remaining hydrogen peroxide is observed at calibrating curve. Calculated amount of used hydrogen peroxide (in mmol min^{-1}) agree with catalase activity (in U-units).

Results

SOD activity determination in chosen samples of barley and malts shows significant differences. The highest values of SOD activity were obtained in Bojos variety samples, the lowest in Radegast variety; concerning locality differences the highest values were founded in Branišovice.

Activity of catalase is corresponding with SOD activity – also these values were the highest namely for Bojos variety

Table I
SOD and catalase activity (U g^{-1} of dry matter) in barley and malt, depending on the localities

Locality	Treatment	SOD activity		Catalase activity	
		Barley	Malt	Barley	Malt
Branišovice	non-treated	120	143	114	166
	treated	128	149	115	148
Hrubčice	non-treated	108	129	116	118
	treated	116	118	116	120
Krásné Údolí	non-treated	112	109	78	92
	treated	97	106	71	85
Lednice	non-treated	90	119	86	105
	treated	86	117	90	138

Table II
SOD and catalase activity (U g^{-1} of dry matter) in barley and malt, depending on the varieties

Variety	SOD activity		Catalase activity	
	Barley	Malt	Barley	Malt
Bojos	128	133	114	140
Jersey	114	127	109	135
Malz	93	126	97	110
Radegast	92	113	107	123
Sebastian	110	127	113	115
Tolar	106	116	110	112

and field in Branišovice; the lowest values were observed in Malz variety. Used method for catalase activity determination shows itself to be easy and quick. Above referred method was slightly modified for this work's purposes: original concentration of ammonium molybdate (32.4 mmol dm^{-3}) recorded non-linear dependence on analyzed hydrogen peroxide depletion, therefore it had to be halved, and hence the absorbance of formed yellow complex is measured at 375 nm instead of original 405 nm.

This work has been supported by project MSM 6019369701.

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P65 FAT CONTENT AND ITS QUALITY IN RAPESEED (*BRASSICA NAPUS* L) DURING STORAGE

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Introduction

Fats and oils contain unsaturated fatty acids bound with triacyglycerols. Plant oils consist of polyunsaturated fatty acids which are susceptible for oxidation (Pokorný, 2001).

Colza oil is low in saturated fatty acids content (6–8 %), but is source of linolenic acid, oleic acid (60 %), and has favourable ratio of n-3 and n-6 fatty acids.

By the influence of higher temperatures the fat is decomposed. Chemical and physical changes of fat during frying cause an increase of fat viscosity, free fatty acids content, color changes, decrease of iodine value, and tendency to foaminess of fat (Venskutonis et al., 2000, Salková, 2002).

The aim of this work was evaluation of the influence of storage time on fat stability in rapeseed (oxidation changes of fat fraction).

Material and Methods

Rapessed was stored in silo. Samples (15 kg) were continuosly taken and analysis were performed - at the begining of storage (after harvest), during the storage and at the end of storage time. Rapeseed was stored in Poľnonákup Hont in Hontianske Nemce, as the mixture of variants. Analysis were performed at Department of Plant Processing and Storage, Faculty of Biotechnology and Food Sciences (SUA Nitra), then at Slovak Agricultural Research Centre in Nitra and at Food Research Institute, Biocenter Modra. Results are present from 3 years of observations (2003–2005).

Methods

Determined parameters of the rapeseed quality were the content of dry matter by Slovak Technical Standard (STS 560116), ash content (combustion at 900 °C), fat content (STS 560116), peroxide value (STS 580130), iodine value (by Hanuš), acid value (STS 580130), thiobarbituric value (distilling method), nitrogen compounds (by Kjeldahl). Fatty acids representation (chromatographically) was determined as follows: myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidonic, behenic and erucic.

Terms of samplings:

- year 2003: 1st July 10, 2003, 2nd October 12, 2003, 3rd January 15, 2004, 4th April 20, 2004
- year 2004: 1st August 23, 2004, 2nd October 28, 2004, 3rd January 24, 2005, 4th April 20, 2005
- year 2005: 1st August 10, 2005, 2nd November 15, 2005, 3rd February 15, 2006, 4th May 20, 2006

Results and Discussion

Mean dry matter content varied from 94.34 % (2005) to 94.81 % (2003). Higher content of dry matter is important point for storage stabilize. In term of rapeseed storage it is important to take into account that water is situated in the hydrophilic part of the seeds, in the direction to the skin. In the middle of the seed there is the fat.

Nutritional compunds were found to be stabile. Mean nitrogen compounds were determined more than 22.42 %. Mean ash content of the rapeseed was 3.83 %, ranged from 3,65 (2004)–3,93 % (2003).

Evaluation of Oxidative Changes in Lipid Fraction of Rapeseed

Mean fat content ranged from 43.16 (2003) to 45.52 % (2004). Linhart (2006) declared that colza oil was for its benefits on immune system evaluated more favourable than the rest of edible oils. Biodiesel production from rapeseeds makes this more preferable compared to another oils. Both advantages increased the price of rapeseed.

Peroxide value is optimal up to 10 mmol O_2 kg^{-1}. Our mean results showed 2.4 mol O_2 kg^{-1}. Acid value was more than 2 mg KOH g^{-1} of fat (2.28 mg KOH g^{-1} fat). According to the STS (46 2300-2) acidity of rapeseed during the storage got higher during the years 2004–2005. Higher content of free fatty acids can cause problems with oil refining.

Mean iodine value was 102.3, then this fat can be classified as semi-drying. Thiobarbituric value is desired to be low

Table I
Oxidative changes of lipid fraction of rapeseed during the storage

Rape seed	Fat [%]	Peroxide value [mmol O_2 kg^{-1}]	Acid value [mg KOH g^{-1}]	Thiobarbituric value [mg MDA kg^{-1}]
		2003		
1.	39.83	2.90	1.77	0.081
2.	44.83	3.71	1.75	0.088
3.	44.80	3.87	1.77	0.092
4.	43.16	3.85	1.79	0.096
Mean	43.16	3.58	1.77	0.089
		2004		
1.	45.20	1.82	2.02	0.058
2.	44.18	2.21	3.19	0.056
3.	46.50	3.08	3.09	0.066
4.	46.20	3.75	3.20	0.063
Mean	45.52	2.72	2.86	0.061
		2005		
1.	42.63	1.72	1.95	0.043
2.	43.60	1.95	1.95	0.051
3.	44.15	1.98	2.42	0.056
4.	44.26	2.24	2.52	0.064
Mean	43.66	1.97	2.22	0.053

during the storage, our mean value (0.0068 mg MDA kg^{-1}) showed the stability in oxidative changes (Table I).

The highest representation of fatty acids was found at oleic acid content (61.658 %), followed by linoleic acid (24.112 %) and linolenic (6.690 %). Mean content of erucic acid was found 0.399 %.

Conclusion

Storage conditions affect quality of raw materials. We can conclude that during observed years were not found any important oxidative changes in fat of rapeseed, seems due to optimal conditons during its storage.

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P66 ANALYSIS OF ACTIVE SUBSTANCES IN HONEY – A CONTRIBUTION TO HONEY AUTHENTICITY

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Introduction

Honey belongs to the oldest delicacies of natural origin. Moreover, honey contains many biologically active substances with positive effect on human health. This work was focused on study of antioxidant content, enzyme activities, protein and saccharide composition in 26 sorts of honey, which differed in geographical and botanical origins. Several qualitative as well as quantitative parameters were determined with the aim to contribute to identification of these compounds which could be considered as markers of the floral origin of honey.

Material and Methods

26 samples of honey, one sample of propolis and one sample of royal jelly were analyzed. Honey were harvested in year 2006 and 2007 and bought in retail chain, special shops and directly from bee-keeper. The study was focused on analysis of antioxidants. Followed groups of antioxidants were determined: flavonoids, catechins, carotenoids, vitamins E, C, A. Authenticity of quality were determined by hydroxymethylfurfural analysis.

Active Substances Analysis

Total phenolics were analyzed colometrically with Folin Ciocalteu reagent (750 nm). Total flavonoid content was analyzed colometrically with $NaNO_2 + AlCl_3$ (510 nm).Total antioxidant capacity was measured by Randox kit.

Individual flavonoids were analyzed by RP- HPLC/UV-VIS method. Using extrenal standards concentration of ((-)catechin, catechin gallate, chlorogenic acid, epicatechin, morin, quercetin, rutin was done. Samples (20 µl) were injected into the RP-18 column (Biospher PSI 200 C18, 7 µm, 150 mm × 4.6 mm). Mobile phases were methanol/water (55 : 45) for catechins and methanol/acetonitrile/water + 1% phosporic acid (20 : 30 : 50) for flavonoid analysis. The flow rate was maintained at 0.75 ml min^{-1} , analysis was performed at 30 °C. Carotenoids (beta-carotene, lycopene, luteine) were analyzed by RP-HPLC with spectrophotometric detection using Hypersil C18, 5 µm, 250 mm × 4.6 mm column, isocratic elution by methanol at the flow rate 1.1 ml min^{-1} and at 45 °C.

Identifiaction of individual flavonoids and catechins was performed by on-line LC/MS/ESI analysis (Mass spectrometer LCQ Advantage Max). Optimalization of mass spectrometry analysis in negative mode was done using chlorogenic acid. Samples of beer were mixed with 5 times higher amount of 2% HCl and extracted by SPE (Amid-2 column). Isocratic elution was performed by mobile phase acetonitrol:1% acetic acid 50 : 50 at flow rate 0.4 ml min^{-1} at 30 °C. Individual components were detected in full scan module.

Ascorbic acid was determined usin RP-HPLC on Hypersil APS-2, NH2, 5 µm, 150 mm × 4.6 mm column. Samples were stabilized by 2% HPO_3, 20 µl was injected. Mobile phase was natrium acetate/acetonitrile (95 : 5). Analysis was performed at flow rate 0.6 ml min^{-1} and 30 °C. Microtitration metod with 2,6-dichlorindofenol was used as comparative mthod too. The end point of titration was determined by pink colour.

5-hydroxymethylfurfural Assay

Concentration of 5-HMF was analyzed by RP- HPLC/UV-VIS method using external calibration. Honey extracts (20 µl) were injected into the RP-18 column (Kromasil C18, 7 µm, 150 × 4.6 mm). Isocratic elution was performed by mobile acetonitrile/water + 1% acetin acid (3:97). The flow rate was maintained at 1 ml min^{-1} , analysis was performed at 30 °C, UV detection was done at 284 nm.

Sensory Analysis

A group of 21 respondents were enrolled into orientation sensory study. They tested several fruit tea and evaluated basic sensory parameters. The droup of respondents was divided into two age-different groups:

- seniors: total 13, age 68.5 ± 7.16; 10 F/3 M,
- juniors: total 8, age 27.13 ± 3.63; 6 F/2 M.

Additionally, consumer questionnaire was completed by 35 student respondents. Preferences and consumption of dried fruit and cereal bars were evaluated.

Results

Average values of total antioxidant capacity ranged (12.75–137.49) mmol 100 g^{-1}. Average values of total phenolic ranged (8.51–61.34) mg 100 g^{-1} and average values of total flavonoids ranged (0.75–6.04) mg 100 g^{-1}. Honey samples contained (41.83–585.10) µg 100 g^{-1} of rutin, (9.30–313.40) µg 100 g^{-1} of myricetin, (6.5–171.90) µg 100 g^{-1} of luteolin, (3.19–436.37) µg 100 g^{-1} of quercetin, (2.10–242.66) µg 100 g^{-1} of apigenin, (0.15–105.12) µg 100 g^{-1} of caempferol and (0.07–17.52) mg 100 g^{-1} of naringenin. From group of catechins there were measured (5.98–310) µg 100 g^{-1} of catechin, (17.77–486.29) µg 100 g^{-1} of epicatechin, (0.18–64.90) µg 100 g^{-1} of catechin gallate and (0.59–140.56) µg 100 g^{-1}of epicatechin gallate. From lipophilic compounds the most abundant in honey samples was tocopherol, its value ranged (29.20–8531.17) µg 100 g^{-1}.

Table I
Total antioxidant activity of several kinds of honey

Honey kind	TAS [mmol 100 g^{-1}]
linden	45.01 ± 0.51
floral (whisked) 2007	26.86 ± 0.38
floral (whisked) 2006	51.34 ± 0.61
sunflower	55.76 ± 0.58
rape (natural)	16.51 ± 0.31
rape (pasted)	40.23 ± 0.50
meadow (floral) (supermarket 1)	22.31 ± 0.35
forest (floral mixed) (supermarket 1)	37.84 ± 0.49
meadow (floral) (supermarket 2)	16.20 ± 0.30
forest (supermarket 2)	44.61 ± 0.48
thyme and herbal	55.04 ± 0.58
acacia	16.73 ± 0.32
royal jelly in honey	3,118.93 ± 11.02
meadow (meadow flora)	21.99 ± 0.34
eucalyptus	62.14 ± 0.67
meadow	47.09 ±0.44
acacia	33.28 ± 0.45
orange flower honey	12.75 ± 0.29
cereal	58.63 ± 0.55
raspberry leaves	40.20 ± 0.42
Floral – bee-keeper	146.68 ± 1.28
honydew	137.49 ± 1.16
Royal yelly	198.62 ± 1.33
propolis	57,355.93 ± 300.42

Ascorbate content ranged (0.65–4.65) mg 100 g^{-1}. Content of hydroxymethyl furfural ranged (0.26–4.06) mg 100 g^{-1}.

By LC/MS method luteolin, naringenin, protocatechin acid, coffee acid and p-cumaric acid in honeydew were detected and, furthermore, kyanidin and pinocembrin in floral honey were found.

Mono-floral honey contained biologically active compounds the most, imperceptibly less contained multi-floral honey and honeydew honey. Acacia honey contained the lowest amount of biologically active compounds.

Table II
5-HMF concentration in several kinds of honey

Honey kind	5-HMF [mg 100 g^{-1}]
linden	0.24 ± 0.00
floral (whisked) 2007	0.52 ± 0.01
floral (whisked) 2006	1.46 ± 0.00
sunflower	3.09 ± 0.00
rape (natural)	0.23 ± 0.01
rape (pasted)	0.38 ± 0.00
meadow (floral) (supermarket 1)	0.26 ± 0.00
forest (floral mixed) (supermarket 1)	0.20 ± 0.00
meadow (floral) (supermarket 2)	1.79 ± 0.01
forest (supermarket 2)	0.88 ± 0.00
thyme and herbal	0.89 ± 0.02
acacia	4.06 ± 0.00
royal jelly in honey	0.76 ± 0.01
meadow (meadow flora)	0.56 ± 0.00
eucalyptus	1.64 ± 0.01
meadow	1.13 ± 0.00
acacia	1.63 ± 0.00
orange flower honey	0.73 ± 0.00
cereal	2.09 ± 0.01
raspberry leaves	0.66 ± 0.00
Floral – bee-keeper	0.38 ± 0.00
honydew	0.50 ± 0.02
Royal yelly	0.41 ± 0.00
propolis	0.54 ± 0.01

Conclusions

According to our analytically obtained data there is no possible to say which kind of honey (meadow or forest) exhibited more beneficial health effect. It seems that honey quality is more significantly influenced by processing and storage conditions than by honey origin alone.

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P67 ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITY OF DRIED FRUIT, DRUIT TEAS AND CEREAL FRUIT PRODUCTS

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Introduction

Cereal products form important par of human nutrition. They contain high amount of saccharides, proteins, fiber, vitamins, minerals and other nutrients. There are many studies about benefitial healt effect of cereals in prevention of cardivascular diseases, cancer and hyperlipidaemia. At this time cereals are recommended mainly in the form of complex natural saccharides digested for a long time. Many cereals are mixed with other components as cocoa, nuts, seeds, hioney, chocolade, and also dried fruit. Addition of several kinds of dried fruit could enhance beneficial effect of cereal product.

In this work some biologically active substances in dried fruit, fruit teas and cereal products were analyzed.

Material and Methods

8 kinds of fruit teas (apple, pear, black currant, lemone, cherry etc.) were erolled into this study. 14 kinds of dried fruits were analyzed (apple, pear, plum, berries, tropical kinds etc). 9 kinds of cereal müsli bars with addition of dried fruit (e.g. berries, bear, apple, pear, plum, orange etc.) were analyzed too.

Antioxidant Analysis

Total phenolics were analyzed colometrically with Folin Ciocalteu reagent (750 nm). Total flavonoid content was analyzed colometrically with $NaNO_2 + AlCl_3$ (510 nm).Total antioxidant capacity was measured by Randox kit.

Individual flavonoids were analyzed by RP-HPLC/UV-VIS method. Using extrenal standards concentration of ((–)catechin, catechin gallate, chlorogenic acid, epicatechin, morin, quercetin, rutin was done. Samples (20 µl) were injected into the RP-18 column (Biospher PSI 200 C18, 7 µm, 150 mm × 4.6 mm). Mobile phases were methanol/water (55 : 45) for catechins and methanol/acetonitrile/water + 1% phosporic acid (20 : 30 : 50) for flavonoid analysis. The flow rate was maintained at 0.75 ml min^{-1} , analysis was performed at 30 °C. Carotenoids (beta-carotene, lycopene, luteine) were analyzed by RP-HPLC with spectrophotometric detection using Hypersil C18, 5 µm, 250 mm × 4.6 mm column, isocratic elution by methanol at the flow rate 1.1 ml min^{-1} and at 45 °C.

Identifiaction of individual flavonoids and catechins was performed by on-line LC/MS/ESI analysis (Mass spectrometer LCQ Advantage Max). Optimalization of mass spectrometry analysis in negative mode was done using chlorogenic acid. Samples of beer were mixed with 5 times higher amount of 2% HCl and extracted by SPE (Amid-2 column). Isocratic elution was performed by mobile phase acetonitrol:1% acetic acid 50 : 50 at flow rate 0.4 ml min^{-1} at 30 °C. Individual components were detected in full scan module.

Ascorbic acid was determined usin RP-HPLC on Hypersil APS-2, NH2, 5 µm, 150 mm × 4.6 mm column. Samples were stabilized by 2% HPO_3, 20 µl was injected. Mobile phase was natrium acetate/acetonitrile (95 : 5). Analysis was performed at flow rate 0.6 ml min^{-1} and 30 °C. Microtitration metod with 2,6-dichlorindofenol was used as comparative mthod too. The end point of titration was determined by pink colour.

Antimutagenity Assay

Test strain *Saccharomyces cerevisiae* D7 was used for antimutagenic effect trstiny. The frequency of spontaneous convertants at the tryptophan locus (trp5-12/trp5-27) and revertants at the isoleucine locus (ilv-1-92/ilv1-92) was tested. In each assay positive and negative controls were included. For positive controls 4-nitroquinoline-1-oxide was used as standard mutagen. Experiments were carried out by 1-day treatment of the growing cells with tested samles. After treatment the cells were washed with with phosphate buffer and plated on a solid medium. After 5-days cultivation at 28 °C Ile-revertants and Trp-convertants colonies were counted. Five consecutive experiments were performed.

Sensory Analysis

A group of 21 respondents were enrolled into orientation sensory study. They tested several fruit tea and evaluated basic sensory parameters. The droup of respondents was divided into two age-different groups:

- seniors: total 13, age 68.5 ± 7.16; 10 F/3 M,
- juniors: total 8, age 27.13 ± 3.63; 6 F/2 M.

Additionally, consumer questionnaire was completed by 35 student respondents. Preferences and consumption of dried fruit and cereal bars were evaluated.

Results

Analysis of dried fruits showed high level of vitamins and phenolics mainly in berries. High level of carotenoids was observed in dried apricots and plums. High antioxidant activity was found mainly in dried apples, cranberries and blueberries (Table I).

The highest content of total phenolics, total flavonoids, total antioxidant capacity, procyanidin B2 and chlorogenic acid was measured in plums and blueberry bars (Table II). The highest level of reduced saccharides was evaluated in raspberry and cereal bars.

Higher antixidant content was found in herbal teas than in fruit teas. Comparing bag teas with loose leaf teas higher antioxidant activity was shown in loose leaf teas. In all tea samples catechins – catechin, epicatechin, epicatechin gallate and other flavonoids – rutin, morin, quercetin, kaempferol, myricetin and luteolin were determined. In most of teas high level of catechin and rutin was detected. Higher vitamine C level was found in most of fruit teas and in rose hip tea.

High antimutagenic activity exhibited dried cranberries and blueberries. Most of tested dried fruits with high antimutagenic effect exhibited also high antioxidant activity as well as high content of some antioxidants. No direct correlation was found among these parameters. High antimutagenic activity showed most of fruit teas, mainly pear tea.

Table I
Total antioxidant activity of dried fruit

Dried fruit	TAS [mmol dm^{-3}]
apple	123.194 ± 1.280
pear	55.020 ± 0.440
apricot	37.380 ± 0.480
plum	56.640 ± 0.980
cherry	54.998 ± 3.840
blueberry	92.396 ± 3.570
cranberry	87.996 ± 4.280
strawberry	59.874 ± 1.350
raisin	82.862 ± 1.280
date	40.456 ± 1.257
fig	28.342 ± 1.100
pineapple	46.198 ± 2.560
mango	1.1568 ± 0.230
papaya	17.9304 ± 0.985

Table II
Total antioxidant activity of cereal bars with dried fruit

Fruit cereal bar	TAS [mmol dm^{-3}]
plum	8.15 ± 1.28
apple	4.02 ± 0.64
apricot	5.26 ± 0.75
raspberry	4.96 ± 0.52
tropical	2.85 ± 0.48
blueberry	8.76 ± 1.76
pear	6.24 ± 1.02
mixed	6.45 ± 1.14
orange	6.02 ± 0.96

During long-term storage of fruit teas (1 year, 20 °C, darkness) a significant decrease of all analyzed antioxidant parameters was followed.

Conclusions

In this work selected kinds of dried fruit, fruit teas and cereal fruit bars were compared. The hioghest values of active substances were found in dried berries, apples and apricote. Similarly, the highest amount of antioxidant substances was found in cereal bars with berries. Cereal part of bars exhibited also important contribution to total antioxidat activity. In fruit teas similar content of active substances was found because of identical basic composition of most teas (apple, rose hip, ibis).

It can be concluded that cereal products, dried fruit ant their mixtures could be important foods recommended for modern human nutrition

P68 CHARACTERIZATION OF "CZECH BEER" – A PILOT STUDY

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Introduction

The healthful and nutritive properties of beer have been recognized by the medical profession for thousands of years. Clinical and statistical evidence and laboratory studies have shown that active substances in beer could influence immune system, block cancer formation, protect against coronary disease and even prolong life. Total benefitial effect of beer and malt samples is a result of many individual contributions of natural substances present in such complicated biological material.

Within the last few years, development of modern instrumental analytical methods has gained increasing importance in authenticity control of food and food ingredients. The characterization of beer samples has a lot of interest because their composition can affect the taste and stability of beer and consumer health. Many substances could contribute to final taste of beer. To the most important probably belong proteins and phenolic substances.

Polypeptides and proteins that influence, direct or indirectly, beer foam quality are protein Z, LTP1 and hordein/glutelin fragments, which originated from malt and have a direct influence on beer foam quality. Other proteins, like malt hordeins and albumins and wheat are, to some degree, also important for beer quality. Protein hydrophobicity is pointed out as a key parameter to enhance foam quality. Electrophoretic, chromatographic and immunological analytical methods are currently used to study polypeptides and proteins present in barley, malt, wort, beer, and foam. Best results are obtained when combinations of these methods are applied.

The aim of this pilot study is to compare composition and to find specific and/or characteristic components in several kinds of beer, malt and other brewing materials.

Material and Methods

13 samples of several types of malt (2 × Czech malt, 2 × German malt, 2 × carapils, 2 × caramel, 3 × dark-caramel, 2 × wheat) were gained from Malting Institute in Brno. 10 kinds of analyzed beer samples were obtained from retail chain, additional 6 samples of Czech beer were gained from breweries.

Antioxidant Analysis

Total phenolics were analyzed colometrically with Folin Ciocalteu reagent (750 nm). Total flavonoid content was analyzed colometrically with $NaNO_2 + AlCl_3$ (510 nm).Total antioxidant capacity was measured by Randox kit.

Individual flavonoids were analyzed by RP-HPLC/UV-VIS method. Using extrenal standards concentration of ((-)catechin, catechin gallate, chlorogenic acid, epicatechin, morin, quercetin, rutin was done. Samples (20 µl) were injected into the RP-18 column (Biospher PSI 200 C18, 7 µm, 150 mm × 4.6 mm). Mobile phases were methanol/water (55 : 45) for catechins and methanol/acetonitrile/water + 1% phosporic acid (20 : 30 : 50) for flavonoid analysis. The flow rate was maintained at 0.75 ml min^{-1} , analysis was performed at 30 °C. Carotenoids (beta-carotene, lycopene, luteine) were analyzed by RP-HPLC with spectrophotometric detection using Hypersil C18, 5 µm, 250 mm × 4.6 mm column, isocratic elution by methanol at the flow rate 1.1 ml min^{-1} and at 45 °C.

Identifiaction of individual flavonoids and catechins was performed by on-line LC/MS/ESI analysis (Mass spectrometer LCQ Advantage Max). Optimalization of mass spectrometry analysis in negative mode was done using chlorogenic acid. Samples of beer were mixed with 5 times higher amount of 2% HCl and extracted by SPE (Amid-2 column). Isocratic elution was performed by mobile phase acetonitrol:1% acetic acid 50 : 50 at flow rate 0.4 ml min^{-1} at 30 °C. Individual components were detected in full scan module.

1D Gel Electrophoresis

1D PAGE-SDS electrophoresis of proteins was carried out by common procedure using 15% and 17.5% polyacrylamide gels. Proteins were staining by Coomassie Blue and by silver staining. For comparison, microfluidic technique using 1D Experion system (BioRad) and P260 chips was used for yeast protein analysis too.

2D Gel Electrophoresis and LC-MS/MS

2D electrophoresis of proteins was optimized in cooperation with Laboratory of Functional Genomics and Proteomics, Faculty of Science, Masaryk University of Brno. 2D gels were obtained using protein preparatives isolated from lyophilized cells. After optimization of separation conditions proteomes from lyophilized beer samples were analyzed. Quantitative analysis was done using BioRad Laboratories 2D software. Identification of some spots was done using LC-MS/MS.

Results

The content of total phenolics in malt was about 1.4 × higher than in wort and hop wort and 1.5–2.5 × higher than in beer samples. Phenolic content decreased in sequence dark caramel – caramel – Czech – German – wheat malt. The main flavonoid detected in most of malt and wort samples was (–) catechin followed by rutin and quercetin. Czech malt contained about 14 μg g^{-1} of (–)catechin, 2 μg g^{-1} of rutin and 1.4 μg g^{-1} of caempherol, while wort contained about 5x higher (–)catechin level. The ration of individual flavonoids changed with malting temperature. Levels of flavonoids changed similarly as phenolic content. TAS levels were several times higher in dark types of malt than in wheat or Czech malt.

Barley and malt proteins are relatively well-documented and their analysis belongs to commercially used tests for barley variety authenticity. Beer proteome has been not studied in detail. In this work main protein fractions (40 kDa, lower than 8–10 kDa, peptides – see Fig. 1. and Fig. 2.) were determined in lyophillized beer samples. Pilot 2D electrophoretic analysis of some beer samples was done mainly for optimization of analysis conditions.

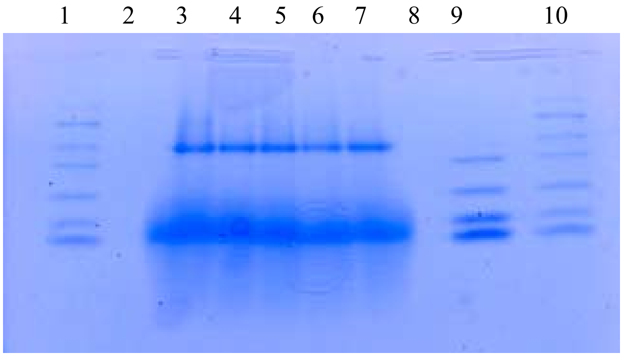

Fig. 1. **1D PAGE-SDS analysis of beer proteins. 1 – standard 6 (97.4, 67.0, 45.0, 29.0, 21.0, 12.5, 6.5 kDa; 3-7 – proteome from lyofilized beer; 9 – standard 5 (29.0, 21.0, 12.5, 6.5 kDa); 10 – standard 6**

Conclusions

The aim of presented pilot study was to use modern techniques to qualitative and quantitative analysis of characteristic components of Czech beer. One of the main goals is separation and analysis of specific proteins and peptide fragments and changes of their content during beer processing. Based on analysis of barley variety purity using standard 1D PAGE-SDS detailed characteristics of proteins by microfluidic techniques, 2D-electrophoresis, LC/ESI-MS, LC/MS/MS was tested. Further aim was qualitative and quantitative analysis of individual phenolics in Czech beer by chromatographic techniques (RP-HPLC, LC/MS). All analyses will be performed in several kinds of Czech beer in comparison with beers processed by different technology. All results will be compared to obtain a set of parameters suitable to final characterization of Czech beer. This set could be used in food control to authenticity assesment of Czech beer.

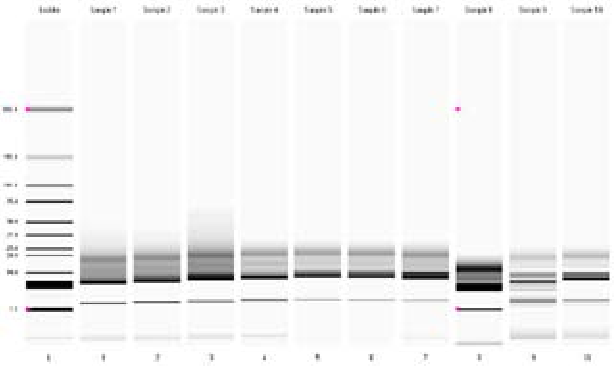

Fig. 2. **Microfluidic 1D electrophoresis of beer proteins**

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P69 TOCOPHEROL AND FATTY ACIDS CONTENTS OF SELECTED ROMANIAN CEREAL GRAINS

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Introduction

Cereals grains are one of the main sources of tocopherols (Ts) for humans[1]. These compounds are recognized for their inhibition of lipid oxidation in biological systems and each isomer shows , to a different extent, vitamin E activity in the order α – T>β – T>γ – T>δ – T(ref.[2]). The interest in the relationship between Ts and fatty acids (FA) is outlined by the fact that fats are carriers for Ts and they have at the same time a prooxidant effect since they are susceptible to oxidation[3].

The objective of this study is to determine the content of tocopherols (α– , β+γ and δ–) and FA composition in 19 elected Romanian cereal grain varieties (wheat, maize and oat).

Experimental

Cereal Samples

Cereal grain samples harvested in 2006 and 2007 were obtained from Agricultural Research Institute Turda. The samples included eight wheat, eight maize and three oat varieties (var.). Samples from two replicates were chosen for analysis. All samples were ground in a laboratory mill. Ground samples were stored at –20 °C until extracted.

Methods

Total lipids (TL) were determined according to a routine procedure 4.

The tocopherols were analysed using a Shimadzu VP Series liquid chromatograph equipped with a degasser and a fluorescence detector FR-10 AXL with excitation wavelength of 290 nm and emission wavelength of 325 nm. Chromatographic separation was performed on a Alltima RP C-18 column (250 mm × 4,6 mm, 5 μm). The column was used at 40 °C. The mobile phase was a mixture of acetonitrile and methanol (50 : 50 , v/v) and eluted at a flow rate of 1.0 ml min^{-1}.

Sample treatment included saponification of 0.5 g cereal flour in a Pyrex glass tube with 0.5 ml 50% KOH and a mixture of 5 ml + 2 ml water in the presence of 0.1 g ascorbic acid. The tube was transferred to a boiling water bath for 30 min. Then 6 ml of ethanol 50% was added to the cooled tube. Unsaponified lipids were extracted by using three portions (each 10 ml) of n-hexane: diethyl ether (7 : 3, v/v). After separation of the phases, the organic layers were collected in a separatory funnel. Organic extracts were washed three times with water and then evaporated in a vacuum rotary evaporator at 35 °C. The dried residue was extracted with 2 ml methanol + 2 ml acetonitrile by mixing on a vortex mixer for 2 min. The tocopherol contents were calculated from the peak areas using standard curves of tocopherols (α – T, β – T, γ – T and δ – T) obtained from Merck and Sigma.

FA were analyzed by gas-liquid chromatography (GLC) with flame ionization detection (FID). The sample (1 μl) was injected into gas-chromatograph, a Shimadzu GC-17 A series equipped with a 30 m Alltech AT-WAX coated with polyethylene glycol (0.25 mm I.D., 0.25 μm film thickness).The oven temperature was programmed as follows : 70 °C for 2 min, then raised to 150 °C at 10 °C, held at 150 °C for a further 3 min, then raised to 235 °C at 4 °C min^{-1} . The final oven temperature was maintained for 5 min. The injector and detector temperature was 260 °C. The carrier gas was helium at a pressure of 147 kPa.

FA were converted to methyl esters by reaction with boron trifluoride/methanol at 80 °C for 2 h in a Pyrex glass tube. Esters were extracted twice with 1 ml n-hexane, the extracts were combined, neutralized with sodium carbonate and dried with anhydrous sodium sulfate and filtered. Finally the filtrate was concentrated under a stream of nitrogen.

Table I
The content of tocopherols in different cereal grains

Cereal	Tocopherols [mg 100 g^{-1}] range α	β+γ	δ
Wheat	0.77	0.23	–
	0.64–0.98	0.16–0.31	–
Maize	0.26	0.96	0.08
	0.16–0.34	0.75–1.21	0.05–0.12
Oat	0.31	0.03	–
	0.27–0.37	0.02–0.04	–

Results

The means and ranges of tocopherols in different cereal grains are shown in Table I.

α-tocopherol was the main isomer found among analysed tocopherols in wheat and oat var. The content of α-T in wheat var. was similar to that reported by other authors[1,5]. Maize had the highest β+γ tocopherol content. Small quan-

Table II
Total lipids and fatty acid profiles of selected cereals

TL and fattyacids [g 100 g^{-1}] food	Wheat	Maize	Oat
TL	1.6	5.2	5.8
SFAs[1]	0.17	0.55	0.74
MUFAs[2]	0.18	1.32	1.80
PUFAs[3]	–	–	–
Total cis	0.67	2.66	1.75
n-6 (as 18 : 2)	0.64	2.59	1.70
n-3	0.03	0.07	0.05

[1] Saturated fatty acids
[2] Monounsaturated fatty acids
[3] Polyunsaturated fatty acids

tities of δ–T was found in maize whereas in wheat and oat it was not found.

Table II presents the mean values for the analysis of TL and fatty acid profile of selected cereals. Lipids are only a minor component of cereals, with the amount varying from a lipid content of 1–3 % in wheat , to 5–9 % in maize and 5–10 % in oats[6].

Total unsaturated fatty acids (UFAs) ranged from 57–86 % of total fatty acids with linolenic and oleic acid predominant for wheat var. Total UFAs ranged from 72–87 % respectively 62–83 % for maize and respectively oat var. and the same predominant fatty acids. The content of linoleic acid was significantly higher in maize var. than in wheat and oat var.

The lower content of α–T in maize var. does not correlate with the standpoint that higher PUFA content is accompanied by higher content of α–T (ref.[7]).

Conclusions

This study focused on the quantification of tocopherols and fatty acids in 19 selected Romanian cereal grain varieties. The results of this study should be helpful in updating the national database for food composition and for assessment of the nutritional intake of the population.

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P70 MONITORING OF BARLEY STARCH AMYLOLYSIS BY GRAVITATIONAL FIELD FLOW FRACTIONATION AND MALDI-TOF/TOF MS

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Introduction

In barley grain, starch occurs in form of granules with bimodal size distribution. The oval large starch granules (A) have diameters in the range from 10 to 40 µm and they prevail in weight. On the other hand, the spherical small starch granules (B) have diameters from 1 to 10 µm and they prevail in number[1]. Enzymatic starch granule hydrolysis is one of the most important reactions occurring during malting and mashing.

In this study, the capacity of gravitational field flow fractionation (GFFF) to monitor the amylolysis of starch granules was investigated. In previous works[2-6] GFFF was successfully used for study of size distribution of starch granules in different barley varieties. Degradation of starch particles from wheat was monitored by sedimentation FFF[7]. Lower saccharides released during amylolysis can be studied by MALDI-MS.

Experimental

Kernels of barley variety Jersey were used for isolation of starch granules. Kernels were graded and the fractions over 2.5 mm were used. The isolation procedure is described in detail elsewhere[8]. It combines classical approaches (incl. crushing of barley kernels by a roll crusher, steeping in 0.02M HCl, repeated rubbing and filtering through sieve 0.08 mm) and present knowledge (treatment with ß-glucanase and cellulase).

Starch granules were hydrolyzed by 2 units of α-amylase mg^{-1} starch (Sigma-Aldrich) added to 2 ml of 3% (w/v) starch suspension at 35 °C. Aliquots were removed in time interval during hydrolysis and centrifuged. Supernatant was mixed with 2,5-dihydroxybenzoic acid (DHB) and measured by 4700 Proteomics Analyzer (Applied Biosystems, USA) MALDI-TOF/TOF mass spectrometer (equipped with Nd/YAG laser; 355 nm).

Starch granules were resuspended in 10^{-3} % sodium dodecyl sulphate (SDS) (Fluka, Germany), sonicated and analyzed by GFFF. Apparatus for GFFF is described in[6]. The channel dimensions were 360 × 20 × 0.150 mm. The highpressure pump HPP 4001 (Laboratory Instruments, Prague, Czech Republic) was used to introduce the carrier liquid into the channel via an inlet capillary situated at the channel head. UV/VIS Spectra 100 (Spectra Physics, San Jose, USA) operated at 470 nm was used as a detector. The samples were injected at the stopped flow by using a Hamilton microsyringe. Just after injection, a loading flow rate of 0.2 ml min^{-1} was applied for 10 s. Then a relaxation time (stopped-flow period) was 1.5 min. After this time period, a linear flow rate of 0.8 ml min^{-1} was applied.

Results

Experimental conditions of GFFF separation were chosen in order to elute starch particles in focusing or lift-hyperlayer elution mode[6]. In this elution mode, starch granules do not interact with the channel bottom; therefore particle-wall interactions are negligible.

Fig. 1. shows a representative GFFF elution fractogram obtained for native starch of barley variety Jersey. Three major peaks can be seen: the first corresponding to unretained species (void volume), the second corresponding to the large A starch granules and the third peak corresponding to the small B starch granules.

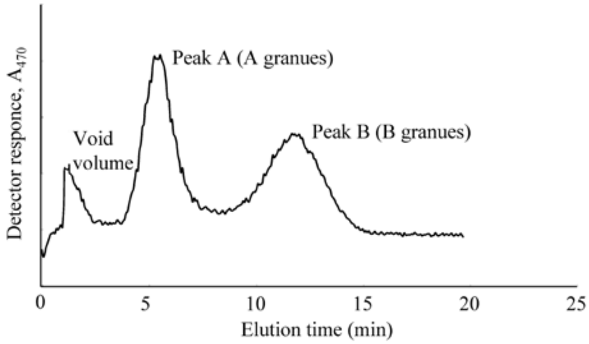

Fig. 1. **Representative fractogram of native starch from barley variety Jersey after GFFF elution**

The α-amylase type VIII-A from barley (Sigma) was chosen for starch granule attack. In the case of bimodal starch population, the amylolysis mechanisms are more complex than those for small monomodal starches. Amylases start enzymatic digestion on specific points of the large A starch granules. Degradation then continues towards the centre of the granule by the formation of channels leading to particle

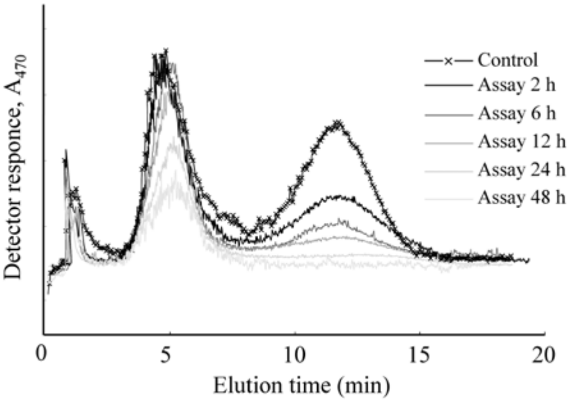

Fig. 2. **GFFF monitoring of barley starch enzymatic hydrolysis. Starch was isolated from barley variety Jersey**

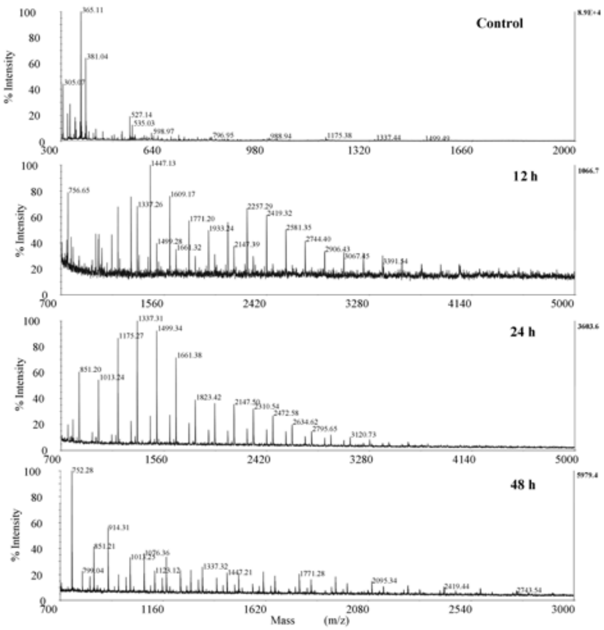

Fig. 3. **MALDI-TOF mass spectra of saccharides released during amylolysis. DHB was used as a matrix**

disruption and fragmentation after certain time[9]. Contrary, B granules are gradually digested from the whole surface.

Fig. 2. shows fractograms of native barley starch incubated with amylase. Changes in GFFF fractogram profiles were observed during amylolysis. The rapid decrease of the intensity of B peak corresponds to the greater susceptibility of B granules to amylolysis[9]. The time depended decrease in peak A signal after certain incubation period is also well-marked. On the other hand, the fractograms of the control starch sample showed minimal changes through experiment. The results of experiments well reflects concept of pitting degradation of large A starch granules and pheripheral hydrolysis of small B granules.

Lower sugars released during amylolysis were studied by MALDI-MS. The spectra of the control sample and digestion mixture during amylolysis are shown in Fig. 3. Increasing intensities of peaks related to sugars correspond to released saccharides during amylolysis. With progress of hydrolysis several series of + 162 Da peaks can be seen in mass spectra (see the last spectrum in Fig. 3.).

Conclusions

The results of incubation of native starch granules isolated from Jersey barley variety with amylases confirmed that GFFF appeared to be a good tool for monitoring of degradation progress of native barley starch. MALDI-MS of lower sugars released during amylolysis showed its potential for study of products of starch hydrolysis.

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P72 THERMOGRAVIMETRIC ANALYSIS OF LIQUID PYROLYSIS PRODUCTS OF WOODY BIOMASS

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Introduction

Thermal analysis (TA) represents a set of methods, used to study the changes of investigated material by measuring some physical properties as a function of temperature.

Nowadays, several thermoanalytical methods are used, differing in monitored physical property (mass, volume, length, magnetic susceptibility, temperature difference between sample and reference sample), etc.

In thermogravimetric analysis (TGA), mass changes of sample are determined as a function of temperature.

Data obtained from TG measurements are used to investigate thermal stability of substances, determination of relative volatility and determination of other physical and chemical parameters. First derivative of TGA curve according to temperature is denoted as DTGA or DrTGA and represents the rate of reaction.

TGA is used very frequently to study thermal behavior of different kinds of biomass (in inert or oxidative atmospheres), mainly agricultural crops or their residues[1], woody wastes[2], some fossil fuels – coal and blend fuels (biomass/coal)[3,4].

Pyrolysis of plant or woody materials generates solids, liquids and gaseous fuels or products. Their composition depends on the pyrolysis process conditions.[5–8]

Thermal behaviour of liquid and solid pyrolysis products – bio-oil and wood coal – usable as fuels or for energetic purposes – can be studied by means of thermoanalytical methods. Experimental part of this work is focus on TGA, which was used for study of thermal behaviour and thermal stability of fractions of bio-oil in inert atmosphere.

Experimental

Materials

Bio-oil fractions originating at industrial pyrolysis of beech wood – pyrolysis condensate (PC), pyro-oil (PO), and wood tar (WT) were suplied from SLZ Chémia, Slovakia.

Methods and Instruments

Thermogravimetric analysis (TGA) was performed on a Mettler Thermoanalyzer 2, in inert gas atmosphere (nitrogen).

Conditions: flow rate: 60 ml min^{-1}, temperature program was 10 °C min^{-1} from 25 to 600 °C, analysis time 60 min, and sample amount was ~ 20 mg.

Results

As can be seen from TGA results (Fig. 1.) of pyrolysis condensate (PC), temperature rise up to ~ 120 ºC resulted in more than 90 % weight loss. PC is formed of a mixture of compounds which distillated with the highest rate at 97 ºC.

This fraction of bio-oil according to TGA curve contains mainly volatile organic compound (VOC) and water. VOC is defined as a compound which has boiling point equal or less than 250 °C at pressure 101.3 kPa[9].

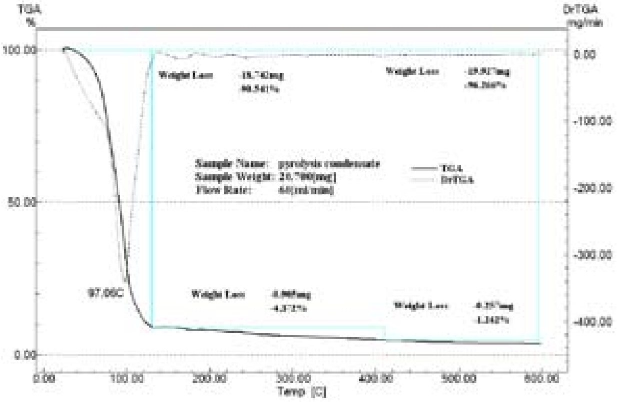

Fig. 1. **TG analysis of pyrolysis condensate**

The results of TGA analysis of pyro-oil (PO) document (Fig. 2.) that PO still contains VOC (6 %), but their amount is much lower than that present in the fraction of PC. Increasing the temperature to ~ 410 ºC leads to ~ 93 % weight loss and so the most part of PO distilled off or is thermally decomposed.

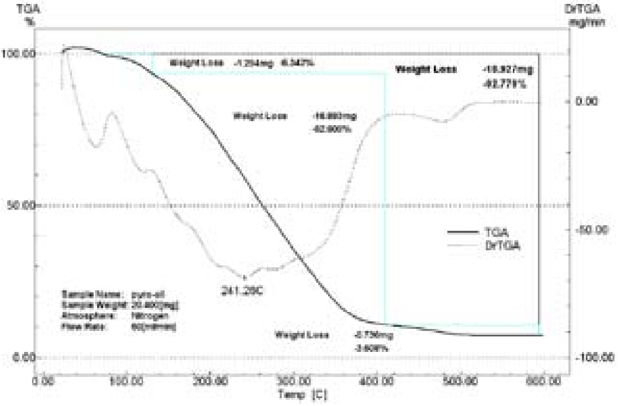

Fig. 2. **TG analysis of pyro-oil**

The results of TGA analysis of wood tar (Fig. 3.) led to a conclusion that reaching temperature ~ 400 ºC, 75 % of wood tar fraction (WT) underwent decomposition.

This fraction contains also thermally stable compounds as evidenced by residual more than 20 % amount at 600 ºC.

The individual phases of the thermal decomposition or degradation – namely I. dehydratation and volatilisation, II. active pyrolysis and III. passive pyrolysis[10] were analysed.

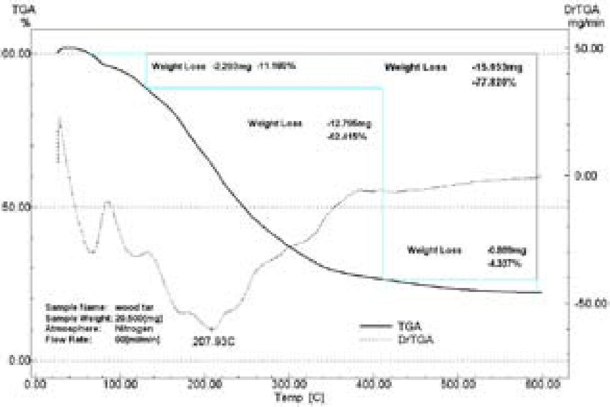

Fig. 3. **TG analysis of wood tar**

Fig. 4. shows comparison of TGA curves of fractions of bio-oil. It is obvious from this comparison that the least stable fracion is PC, followed by PO and ending with WT.

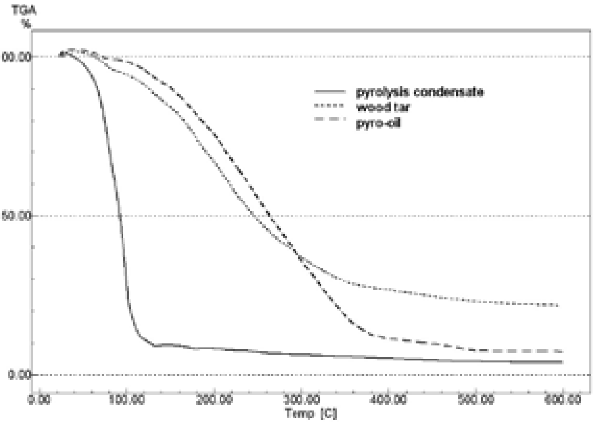

Fig. 4. **Comparison of TG analysis of bio-oil fractions at the same conditions**

Temperatures and weight losses for individual stages are listed in Table I.

Table II indicates total weight losses for individual fraction of bio-oil and corresponding decomposition temperatures at which the maximum weight loss per minute occurs.

Data in tables for individual stages differ because every fraction contains other compounds and in different quantitative amounts.

Table I
Decomposition stages of bio-oil fractions and weight loss

Stage	I.		II.		III.	
T [°C]	25–130		130–410		410–600	
Sample	[mg]	[%]	[mg]	[%]	[mg]	[%]
PC	18.80	90.8	0.91	4.4	0.26	1.3
PO	1.29	6.3	16.89	82.8	0.74	3.6
WT	2.29	11.2	12.80	62.4	0.89	4.3

PC contains mainly water, VOC and hydrocarbons with short chains. PO contains hydrocarbons with longer chains, benzene and phenolic structures. WT is composed mainly of condensed aromatic substances, benzene and phenolic structures.

Table II
Total weight loss of bio-oil fractions and decomposition temperature at max. weight loss

Sample	Weight [mg]	Loss [mg]	Loss [%]	t_{max} [°C]
PC	20.7	19.963	96.4	97.1
PO	20.4	18.927	92.8	241.3
WT	20.5	15.953	77.8	207.9

Conclusions

In this work, we investigated three liquid fractions of bio-oil generated in process of woody biomass pyrolysis beechen wood.

TGA measurements indicated different composition of individal fraction of bio-oil.

Pyrolysis condensate contains substantially volatile compounds (VOC) and water than pyro-oil and wood tar. Pyro-oil contains more VOC than wood tar, which is confirmed by total weight losses of these fractions. Pyrolysis condensate has total weight loss 96.4 %, pyro-oil 92.8 % and wood tar 77.8. So the residue of wood tar is highest at 600 °C.

At all fractions it is possible to deduce from TGA curves that if temperature increases gradually, then distillation of volatile compounds and their exothermal decomposition occur.

Distillation and decomposition process proceed in different temperature intervals and weight losses at these intervals are different for individual fractions, which means that each fraction contains different substances with different ratio.

Based on TGA results it can be concluded that the least thermal stability has fraction of pyrolysis condensate, followed by pyro-oil and, finally, the most stable was wood tar.

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P73 ANALYTICAL MEASUREMENTS AND PHISICOCHEMICAL PARAMETERS DETERMINATION IN STRAWBERRIES AND RASPBERRIES ENHANCE PRODUCTION

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Introduction

Strawberry is one of the most delicious fruits of the world, which is a rich source of vitamins and minerals[1].The strawberry harvest in Huelva was included in the main group of agricultural activities in which Spain have an important position in the framework of the European Union. Huelva offers adequate climatic characteristics and soil composition as well as water disponibility for irrigation that is exceptional in the national territory. Contemporany history of cultivating strawberry de Huelva started in 60's in Moguer city.

In recent years, several day-neutral varieties have been introduced and different agro-techniques were standardized at various research stations[2]. In addition, strawberry has become the most favourite fruit crop among the growers, especially near towns and cities, which has resulted a phenomenal increase in its area and production. In Huelva, many varieties of strawberry and raspberry are grown, but (*Fragaria vesca* var. *Camarosa* and *Rubus idaeus* var. *Glen Lyon)* has become the most popular dessert variety due to its high production potential and because it is an attractive red medium-sized fruit of better quality.

The safeguard of quality characteristics from harvest up to consumption is an essential requirement for the fruit sector. Quality control and authentication of fruits and derivatives is a key aspect for consumers and producers. Food authentication is the process by which a food is verified as complying with its label description. Labeling and compositional regulations, which may differ from country to country, have a fundamental place in determining which scientific tests are appropriate for a particular issue[3] Thus the availability of sound analytical methods which can ensure the authenticity of foods plays a fundamental role in the operation of modern society. In this sense, several analytical parameters have to be taken into account.

In this work, we have analysed several samples of strawberries and raspberries (*Fragaria vesca* var. *Camarosa* and *Rubus idaeus* var. *Glen Lyon*) in relation with phisicochemical parameters (elements such as Ca, Mg, K, Na, B, Cu, Fe, Ni and Zn; anions: phosphates, nitrates, sulphates, carbonates; solids in suspension, pH, redox potential). The experiment was carried out during six months in which several agrochemical experiments have been developed such as the use of new plastics for the harvest and the pollination with bumblebees.

Experimental

Samples

Samples of soils and leaves were analysed in this study. The samples of leaves were taken during the months of major production of both plants and the samples of soil at the beginning and at the end of the harvest.

Preparation of the Sample

(i) Samples of leaves: The samples were dried in an oven at 100 °C. After that they were extracted by using a microwave oven: 0.2 g of sample was weighted and 4 ml of HNO_3 (65%) were added. The program was from room temperature to 170 °C at 20 °C min^{-1}.

(ii) Samples of soil: the same parameters that for the leaves were analyzed, but different extractant solutions were used[4,5]:

- Phosphorus: Extraction with sodium bicarbonate at pH 8.5
- Manganese and iron: Extraction with ammonium acetate pH 4.8
- Sodium, potassium, calcium and magnesium: Extraction with ammonium acetate at pH 7
- Organic matter: was determined by a redox process using potassium dichromate and sulphuric acid, to measure the excess of dichromate with a salt-ferrous

Measured Elements and Methods of Analysis:

- ^{63}Cu and ^{66}Zn: measured with an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Model 4500 (Agilent Technologies, Tokyo, Japan)
- Orthophosphates: measured with the molybdenum blue method[4].
- Mn, Fe, Ca, Mg: they were measured with Atomic Absorption Spectroscopy (AAS).
- K and Na: Atomic Emission Spectroscopy (AES).
- Determination of the soil texture: it was measured by using the Mastersizer 2000, a laser diffraction instrument.

ICP-MS Parameters:

- RF power: 1,370 W
- Plasma gas flow-rate: 15 $dm^3 min^{-1}$
- Auxiliary gas flow-rate: 1.00 $dm^3 min^{-1}$
- Nebulizer gas flow-rate: 1.15 $dm^3 min^{-1}$
- Sampling depth: 6.5
- Sampler and skimmer cones: Ni
- Time per isotope: 0.3 s per isotope
- Isotopes monitorized: ^{63}Cu and ^{66} Zn

Results

The results of analyses of soil throughout the study are collected in the Table I.

- At the beginning of the study (February).
- At the end of the harvest, in June.

Table I
Results of analyses of soil throughout the study

	Strawberry		Raspberry	
	February	June	February	June
Carbon [%]	0.24	0.14	0.26	0.20
Oxidable organic matter [%]	0.42	0.24	0.44	0.34
Organic matter total [%]	0.54	0.31	0.57	0.45
N [%]	<D.L	<0.3	<D.L	<0.4
H [%]	<D.L.	<0.7	<D.L.	<0.8
P [mg kg^{-1}]	158.94	89.73	144.63	185.2
Extraction NH_4-Ac pH 7				
K [mg kg^{-1}]	62.55	35.64	86.24	95.37
Na [mg kg^{-1}]	14.5	N.D.	19.19	N.D.
Ca [mg kg^{-1}]	167.61	520	188.6	640
Mg [mg kg^{-1}]	30.91	33.1	39.67	62.09
Extraction NH_4-Ac pH 4.8				
Mn [mg kg^{-1}]	8.47	8.50	7.09	8
Fe [mg kg^{-1}]	14.78	6.91	10.54	8.41
Principio del formulario				
Soil texture[4]	Loamy sand		Loamy sand	
Final del formulario				
Clay	5 %		15 % Final del formulario	
Silt	17 %		37 %	
Sand	73 %		37 %	
Graves				
Final del formulario	5 %		11 %	

D.L. – Detection Limit; N.D. – Not determined

The results of the analyses of leaves throughout the study are represented in the Figs. 1., 2. and 3.

Once we have obtained the results of the measured concentrations of elements in leaves and soil, it is required to get conclusions of the analysis comparing the obtained values with the common known concentrations for each specie[6,7]. It is important that the levels of the elements in the plants are the required ones in each stage of the growing.

As it is shown in Fig. 1., the levels of copper and zinc are between required levels in both species. In this sense, we can observe that the level of zinc is major at the beginning of the harvest. Similar conclusions can be drawn for the concentration of lements in Figs. 2. and 3.

As it is shown in Fig. 2., in the leaves from strawberries the levels an

As it is shown in Table I, the levels of organic matter are low that is a characteristic of sand soils. In addition, the levels go down through the harvest. Although levels are low, the results of production has been good in both cases.

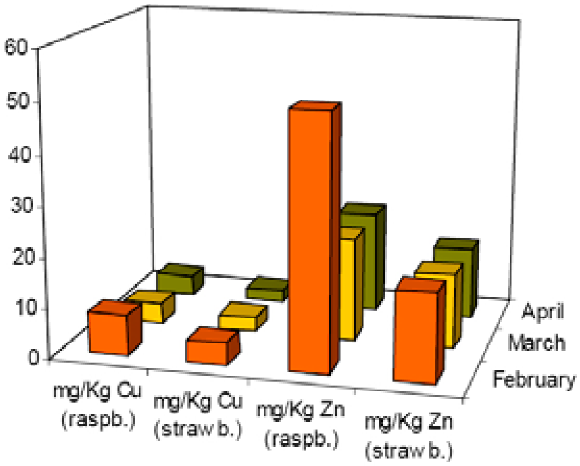

Fig. 1. **Evolution of copper and zinc in leaves of strawberries and raspberries**

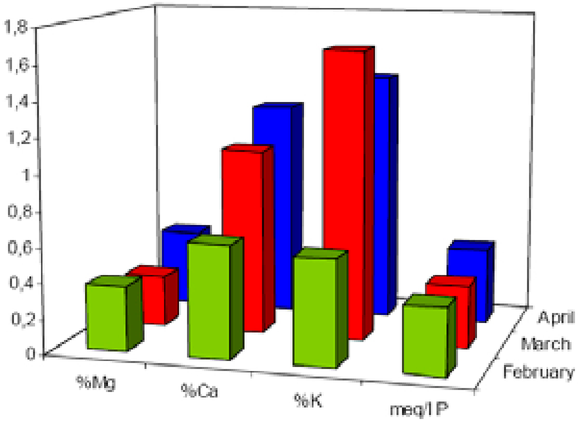

Fig. 2. **Evolution of magnesium, calcium, potassium and phosphorus in leaves of strawberries**

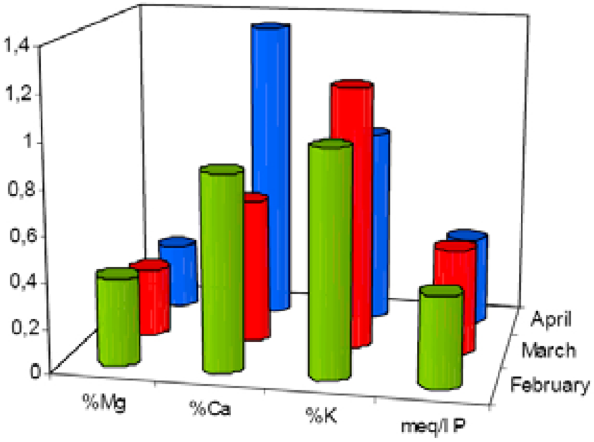

Fig. 3. **Evolution of magnesium, calcium, potassium and phosphorus in leaves of raspberries**

The samples were taken monthly, and they covered all stages of development of the crop: vegetative growth, flowering and fruiting.

These measured parameters were used to know the evolution of these two varieties and to observe the requirements of the plants. Future works will be focused in a discussion of a parelell study carried out to observe the evolution of the fruit, ºBrix, potential deformations, and other parameters such as pollination using bumble bees as well as the experimentation with new biodegradable plastic.

Conclusions

- An analytic methodology for the determination of a great number of analytical parameters of agronomic interest has been developed in this study.
- The obtained analytic data are of great utility to evaluate the experiences of the use of biodegradable padding plastics in the cultivation of raspberry.
- The use of pollination by bumblebees increases the production and improves the quality of the fruit.

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P74 MULTIVARIATE METHODS IN FOOD ANALYSIS

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Introduction

Chemometrical processing of the results of instrumental analytical measurements takes advantage of modern statistical methods and advanced software and creates new possibilities for solution of problems in various practical fields of application, e.g. in assessing quality of raw and processed food[1,2], or in advancement of diagnosis in laboratory medicine, which we have studied in recent years[3,4].

Wine belongs to the commodities, which are very frequent objects of falsification[1]. Therefore it is necessary to develop procedures which make possible wine classification and authentication[5,6], i.e. verification of the selected sample with regard to the wine variety, producer/locality as well as the year of production.

Pumpkin seed oils enjoy special and increasing popularity mainly due to their characteristic taste. The oil is contained in the seeds, consists of approx. 70 % unsaturated fatty acids and contain a number of important compounds like triterpenoides, carotenoides, tocopheroles and phytosteroles[2]. The oil quality also depends on the geographical origin, seasonal variations and climatic influences.

This study was focused on the classification of white varietal wines based on the results of chemical analyses. Another goal was spectral characterization of different sorts of pumpkin seed oils accompanied by the sensory evaluation, which after chemometrical data processing facilitated detecting the properties most informative about the oil quality.

Experimental

Wine Samples

Altogether 46 samples of Slovak varietal wines, Welsch Riesling, Grüner Veltliner and Chardonnay, were analyzed during two years using 18 traditionally analysed variables like SO_2, total acids, citric acid, malic acid, tartaric acid, lactic acid, sugars by Shoorle, glucose, fructose, polyphenols, density, etc. The wines were produced by two producers in Bratislava and Hlohovec. Sensorial analysis of all examined wine samples was also provided.

Pumpkin Oil Samples

36 commercially available pumpkin oil samples of Steyrian origin were studied. The samples were examined by sensorial analysis and spectroscopically using 38 variables representing the maximum fluorescence signal.

Sensorial Analysis

Sensorial analysis of wines was made by a group of experts who assessed in a twenty-point scale colour, bouquet, taste, and the total points. Two or three wine categories were made by sensorial quality according to the median or the lower and upper terciles of total points. Smell, taste and visual character of oils were concerned when rating the sensory quality of oils. The collected oil were categorized into two basic classes: fully satisfactory ("good") *vs*. not fully satisfactory ("bad").

Statistical Analysis

Statistical treatment of the obtained data was performed using program packages SPSS (SPSS Inc., Chicago, U.S.A.) and STATGRAPHICS Plus 5.0 (Manugistics, Inc., Rockville, U.S.A.).

Results

Wine

Principal Component Analysis (PCA)

Fig. 1. depicts the PCA representation of the samples of three wine varieties and two vintages where some natural grouping of the studied wines is visible. It is worth to note that the observed natural wine clusters are not created by the wine varieties but correspond mainly to the vintage categories: the 1999 samples are below –1.0 on the PC1 axis, the 2,000 samples are above + 1.0.

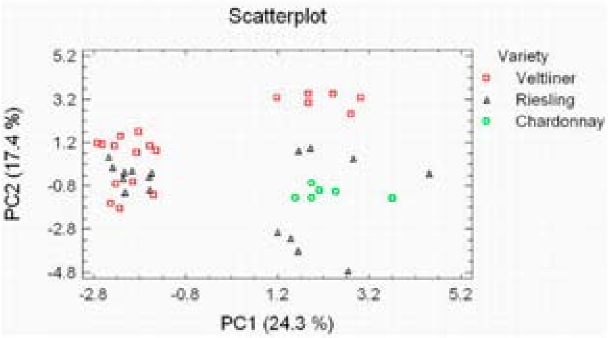

Fig. 1. **PCA scatterplot of the studied wine samples**

Discriminant Analysis (LDA)

LDA is a supervised learning method, in which the classification model is constructed using the the training data set. Then the developed model is used to classify the test samples data set. Three ways of classification were used: by variety, year of vintage, total sensorial quality. In addition, partial sensorial characteristics were used: colour, taste and bouquet of wine. The LDA results using different criteria are summarized in the last two columns of Table I. Success of classification for the set of 46 wine samples was close to 100 %. Fig. 2. exemplifies the successful classification by variety.

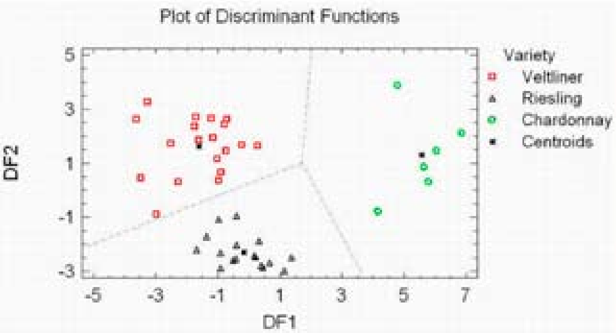

Fig. 2. **LDA plot of three wine varieties**

The stepwise discriminate analysis was applied to the complete set of variables in order to select the variables most important regarding the classification criterion. The classification performance was evaluated for the best group of variables, the number of which is given in brackets.

When the leave-one-out validation technique was applied for classification of wine by *sensorial quality* ("good" or "bad") a 78 % and 87 % success were obtained for all and five best variables, resp.

Table I
Criteria for wine classification and success in classification when all or best variables were used

Criterion	Number of classes	Classification success in % All*	Best*
Variety	3	100.00	95.65 (3)
Vintage	2	100.00	100.00 (2)
Quality	2	93.48	93.48 (5)
Quality	3	86.96	78.26 (9)
Colour	2	91.30	89.13 (7)
Colour	3	76.09	78.26 (6)
Bouquet	2	97.83	93.48 (8)
Bouquet	3	93.48	84.78 (10)
Taste	2	91.30	91.30 (10)
Taste	3	86.96	91.30 (7)
Producer	2	100.00	100.00 (4)

* "All" refers to 18 originally used variables; "Best" refers to the optimally selected variables with their number in brackets

Pumpkin Oils

Principal Component Analysis

The data set of pumpkin oils characterized by 38 variables (maximal intensity of fluorescence using exitation wavelengths 280–650 nm) was used for this study. The inspection of the PCA scatterplot (not shown) has revealed that two oil samples as outliers. The remaining oils are located in two natural clusters at negative values of PC1 and positive values of PC2, resp. In the loadings plot (Fig. 3.), all excitation wavelengths are divided into three groups. A reasonable assignment of these groups is a task of our current study.

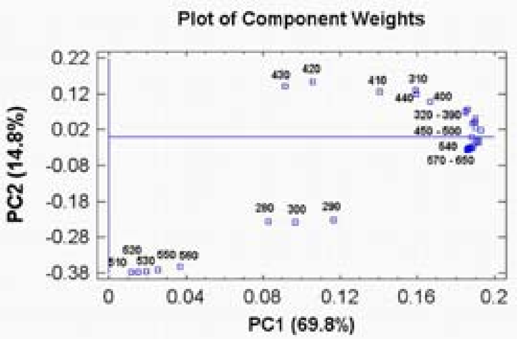

Fig. 3. **PCA loadings plot showing the interposition of the used wavelengths for fluorescence measurements.**

Discriminant Analysis

Fig. 4. represents the LDA graphical output, which shows that very good quality oils samples are located in a cluster at positive values of the first discriminant function (DF1) whilst the lower quality oils form a cluster at negative DF1 values. The separation of two sorts of oils differing by the sensorial quality is remarkable. The classification performance was 100 % for *cross-validation* using the leave-one-out procedure.

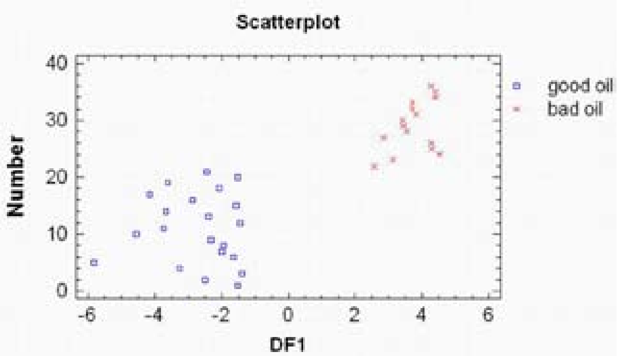

Fig. 4. **LDA plot of the oil sample number vs. the sole discriminant function DF1**

Conclusions

White varietal wines were succesfully classified according to several classification criteria: by variety, vintage, producer as well as partial sensorial descriptors. A very good quantitative separation of the wine samples according to all chosen criteria was obtained by discriminant analysis.

Stepwise variable selection enabled to find an optimal reduced set of variables. The established and validated discriminant models are fully applicable for the category prediction of the unclassified wine samples.

Fluorescence analysis can be successfully applied for classification of commercial pumpkin oils according to their sensorial quality. The investigation of the species causing the most important fluorescent signals reflecting the oil quality is the object of our further study.

This work was supported by grants APVV-0057-06 and VEGA 1/3584/06.

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P75 PHYTOCHEMICAL STUDY OF FLAVONOIDES FROM *MELAMPYRUM CRISTATUM* L. SPECIES

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Introduction

The Melampyrum genus is not studied in Romania that is why there had been chosen the study of *Melampyrum cristatum* L. species with the help of HPLC couplet with MS. A modification to this method achieved by a group of young researchers from Cluj was brought change of the mobile phase, from a mobile was brought phase with elements that are not volatile, there are arrived at a volatile one, the element from the chromatography column can be inserted in mass spectrometry being obtained supplementary information about the elements because the spectrum is registering the molecular mass and mass spectrum from the elements found.[1–4]

Experimental part

Method of Analysis

In a Sox let device the plant powder is treated, leaves respectively flowers from *Melampyrum cristatum* L., to remove the chlorophyll. There were taken work 10 g leaves, flowers, cut up sieve IV with 460 ml clorophorm, the extraction was carried out for 48 hours at 60 °C, until the solvent becomes completely colorless. The powder is subjected to another extraction with 140 ml methanol; the solution resulted being the basic one from which the qualitative determinations will be made.

For the analysis of the polyphenol compounds from vegetable extracts the high performance liquid chromatography coupled with mass spectrometry (LC/MS) was used.

Devices

HPLC coupled with mass spectrometer; binary pump; autosampler; thermostat HP 1100 Series; detector UV HP 1100 Series; mass spectrometer Agilent Ion Trap 1100 VL.

MS working conditions: source of ions- ESI (electrospray); ionization manner-negative ; nebulizer-nitrogen , pressure 60 PSI ; drying gas-nitrogen , flow rate 12 $dm^3 min^{-1}$, temperature 300 °C , capillary potential: +3000 V; method of analysis-monitoring specific ions (poly phenol carboxylic acids) or AUTO MS (flavonoides and their aglycons)

HPLC working conditions: analytical column Zorbax SB-C18 100 mm × 3.0 mm i.d., 3.5 µm (Waters); Precolumn Zorbax SB-C18; the mobile phase: mixture methanol: acetic acid solution 0.1% (v/v) elution in gradient (start 5% methanol, up to 35 min 42% methanol, up to 45 min 5% methanol – re-equilibration); flow rate: 1 $ml\,min^{-1}$, temperature: 48 °C; detection: UV 330 nm up tu 17 minutes, 370 nm up to 38 minutes; volums of injection: 5 µl.

Polyphenol Analysis by UV Detection

Every category of elements were detected at the wavelength corresponding to maximum of absorbtion of UV spectrum. So, the polyphenolcarboxilic acids were detected at the wave-length from 330 nm, but the flavonoides and their aglycons at 370 nm.

Processing of Samples to be Analysed

There were analyzed in parallel two samples of each vegetable extract, one as such, but another one hydrolyzed. The reason for which hydrolysis achieved is the fact that, in general, some floavonoide aglycons or some polyphenolicarboxilic acids are not free, but on bound (glycosides, esters, etc).

So, carrying out on acid hydrolysis will lead to freeing there compounds from the bound form and it would bring more information about the chemical composition of the studied product.

The samples that are analyzed as such are diluted 1 : 10 before the injection. The hydrolysis was performed following the procedure: one part of the extract is diluted with one part of HCl solution and it is maintained on water bath at 80 °C for 40 min. It is brought at 10 ml (measuring flask) and it is injected in the chromatographic system.

Thin layer Chromatography was Performed in the Following Conditions

Solution to analyzed: extract from *Melampyrum cristatum* flowers and leaves 25 µl applied.

Standard solution: methanol solution from rutozid 1.22 $mg\,ml^{-1}$ (Roth), hiperozid 1.1 $mg\,ml^{-1}$ (Merck), clorogenic acid 1 $mg\,ml^{-1}$ (Fluka) and cafeic acid 1 $mg\,ml^{-1}$ (Roth) – 10 µl applied; stationary phase: Kieselgel 60F254 (Merck); mobil phase: ethylacetate (Merck) – ethylmethylketone (Merck) – formic acid (Merck) – water (50 : 30 : 10 : 10, vol.); migration distance 10 cm: 12 cm; migration time: 60 minute.

The application of solution in layers was carried out in bands of 1 cm at 1.5 cm distance from the lower border of the plate.

Reveal of plate it has realized with iron clorure 10 % in ethanol.

The chromatogram analysis is realized in UV la 254 nm, after the revelation in visible light spectrum.

TLC Coupled with Photo Densitometry

The chromatographic plate was scanned with a Shimadzu CS9000 photo densitometer after pulverization with iron clorure. The photo densitometer parameters are: way of functioning in reflexion, wolfram lamp, scanning method: zig zag, λ = 550 nm.

Results and Discussions

Workings in the described condition there are presented the flavonoid concentrations in leaves, respectively in flowers from *Melampyrum cristatum L.*

After the photodensitometric evaluation for the *Melampyrum cristatum* leaves, the sample which is more concentrated in flavonoides, the densitogram from Fig. 4 was obtained. The height of the drops in densitogram, the low flavonoide concentration.

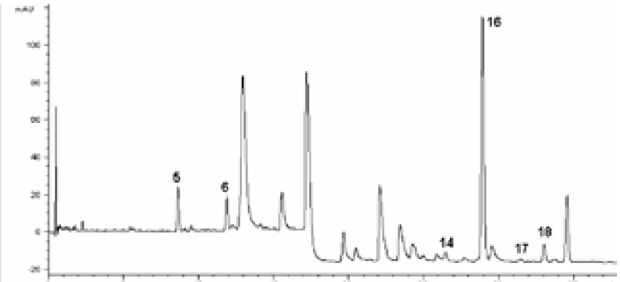

Fig. 2. **Mass spectrum of methanolic hydrolysed extract from *Melampyrum cristatum L.* leaves**

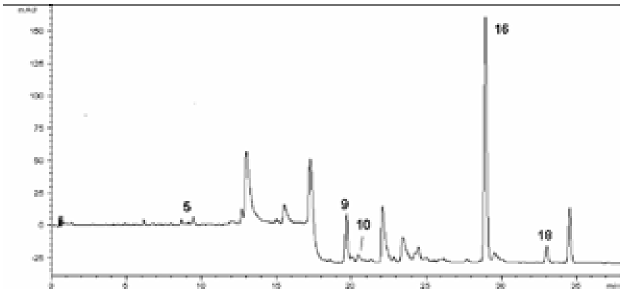

Fig. 1. **Mass spectrum of methanolic unhydrolysed extract from *Melampyrum cristatum L.* leaves**

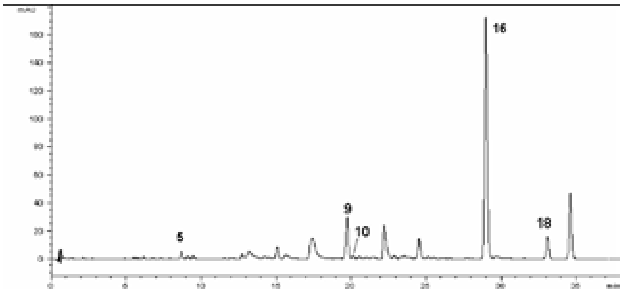

Fig. 3. **Mass spectrum of methanolic unhydrolysed extract from *Melampyrum cristatum L.* flowers**

Table I
Concentration of methanolic unhydrolyzed extract from *Melampyrum cristatum L.* leaves

Compound	Nr.	Identification UV	Confirmation MS	Concentration in extract [$\mu g\,ml^{-1}$]	The quantity in vegetable drug [$mg\,g^{-1}$]
Isoquercitrin	9	yes	yes	60.838	0.30419
Rutozid	10	yes	yes	3.715	0.01858
Luteolina	16	yes	yes	153.390	0.76695
Apigenina	18	yes	yes	15.195	0.07598

Table II
Concentration of methanolic hydrolysed extract from *Melampyrum cristatum L.* leaves

Compound	Nr.	Identification UV	Confirmation MS	Concentration in extract [$\mu g\,ml^{-1}$]	The quantity in vegetable drug [$mg\,g^{-1}$]
Quercetol	14	yes	yes	3.380	0.01690
Luteolina	16	yes	yes	131.092	0.65546
Kaempferol	17	yes	yes	1.439	0.00720
Apigenina	18	yes	yes	11.235	0.05618

Table III
Concentration of methanolic unhydrolysed extract from *Melampyrum cristatum L.* flowers

Compound	Nr.	Identification UV	Confirmation MS	Concentration in extract [$\mu g\,ml^{-1}$]	The quantity in vegetable drug [$mg\,g^{-1}$]
Isoquercitrin	9	yes	yes	46.930	0.23465
Rutozid	10	yes	yes	2.876	0.01438
Luteolina	16	yes	yes	140.680	0.70340
Apigenina	18	yes	yes	18.190	0.09095

Table IV
Concentration of methanolic hydrolysed extract from *Melampyrum cristatum L.* flowers

Compound	Nr.	Identification UV	Confirmation MS	Concentration in extract [μg ml^{-1}]	The quantity in vegetable drug [mg g^{-1}]
Quercetol	14	yes	yes	2.659	0.01330
Luteolina	16	yes	yes	11.122	0.05561
Kaempferol	17	yes	yes	1.642	0.00821
Apigenina	18	yes	yes	13.479	0.06740

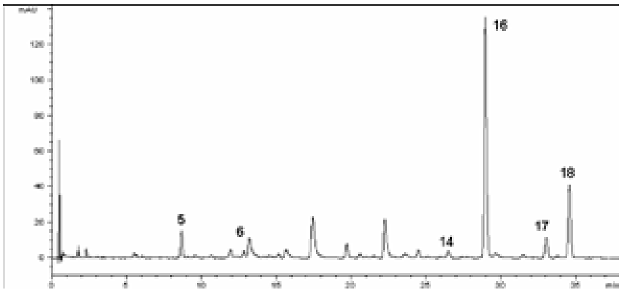

Fig. 4. **Mass spectrum of methanolic hydrolysed extract from *Melampyrum cristatum L.* flowers**

Table. V
The apigenin and luteolin concentration obtained by TLC

Test	Apigenina		Luteolina	
	Area	c [mg ml^{-1}]	Area	c [mg ml^{-1}]
M.cristatum flowers	21,474.6	0.82	102,253.9	0.74
M.cristatum leaves	18,528.3	0.71	107,944.8	0.78

Table VI
The concentration found in rutozid and hiperozid by *Melampyrum cristatum L.*flowers and leaves

	Melampyrum cristatum leaves	flowers
Rutozid %	1.850 %	1.750 %
Hiperozid %	0.43125 %	0.71875 %

The quantitative analyses are made after the method from *Cynarae folium* monography and *Crategus folium* cum flore monography by FR IX and FR X.

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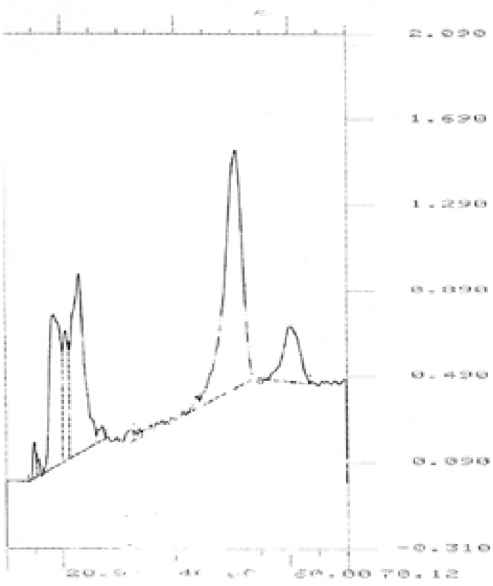

Fig. 5. **Densitogram by *Melampyrum cristatum* flowers**

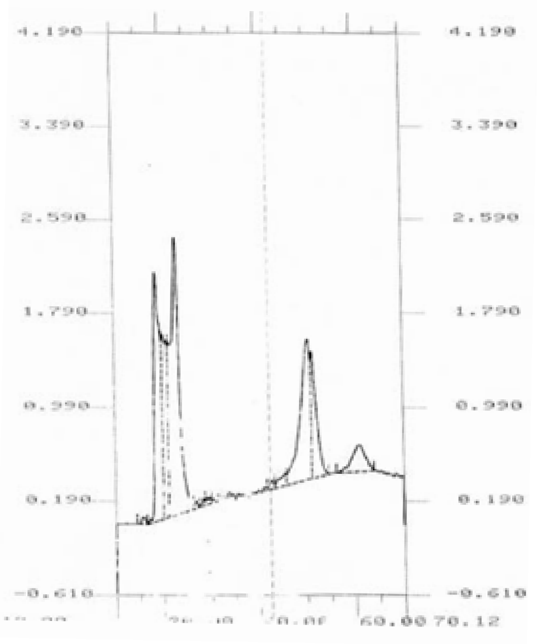

Fig. 6. **Densitogram by *Melampyrum cristatum* leaves**

P76 THE INFLUENCE OF CHOICE FACTORS ON FERMENTATION OF RED WINE

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Introduction

Winemaking can be summarized as the biotransformation of must into wine, which performed principally by *Saccharomyces cerevisiae* strains during the primary alcoholic fermentation. A secondary fermentation, the so-called malolatic fermentation is biodeacidifacation that is often encouraged, since it improves wine stability and quality. Malolatic fermentation (MLF) usually occurs either spontaneously or after inoculation with selected bacteria after fermentation. MLF, the enzymatic decarboxylation of L-malic acid to L-lactic acid, is an important secondary fermentation process carried out by lactic acid bacteria (LAB) during the vinification of red must, and certain white and sparkling wine styles. In addition to deacidification, MLF can increase microbiological stability and enhance the flavour and aroma of wine. The study was focused on the composition of microflora during the extraction of flavour components from grape solids and during fermentation as well. In this way the two technology processes for the production of red wine from Velké Pavlovice region were monitored.

Experimental

Three different media were applied for the cultivation of microorganisms; first for monitoring of total volume of microorganisms, second for yeasts and third for lactic acid bacteria. The indirect method was used for the determination of the amount of viable cells. This method consists of the enumeration of visible macroscopic colonies grown up on agar plates. When the cells grew up, the forms of colonies were analyzed visually and the morphology of microorganisms was microscopically detected.

Cultivation media:

- *Plate Count Agar*. Suitable for the determination of total number of microorganisms in foodstuffs.
- *Malt Agar with the addition of antibiotics*. Into the cultivation medium streptomycine 80 µg/l and propionic acid 0, 25 ml/l was added. Suitable for the isolation and identification of yeasts and fungi.
- *Tomato Juice Medium Base (for Lactobacillus from wine)*.Suitable for the isolation and identification of lactic acid bacteria occurring in wine.

Results

Two technological procedures in wine manufacturing, which differ in technological steps shown in the Table I, were compared.

Table I

Technological procedure	Preliminary fermentation			Alcoholic fermentation		
Application	SO_2	CE	CY	CB	Mode of heating	SO_2
Wine factory No. 1	–	×	×	–	regulated	×
Wine factory No. 2	×	×	×	×	non regulated	×

CE – Commercial Enzymes
CY – Commercial Yeasts
CB – Commercial Bacteria

Since the start of wine manufacturing, till the last addition of SO_2, the total number of yeasts in wine factory No. 1 was monitored. Number of cells in dependence on time is shown in Fig. 1. It is known[1], that the yeasts *Saccharomyces cerevisiae* do not start the fermentation. The yeasts starting the fermentation are *Hanseniaspora* (*Kloeckera*), *Candida Metschnikowia*. From Fig. 1. follows that the number of starting yeasts and yeast microorganisms reached the value $1.2 \times 10^6 \pm 1.3 \times 10^5$ cells in 1 ml. Other yeasts than *Saccharomyces* growing at the start of the fermentation, utilize aminoacids and vitamins for their growth and reduce the growth of *Saccharomyces cerevisiae*. These play an important role in the second half of the fermentation time[1]. From the Fig. 1. it is evident that even though the commercial culture of *Saccharomyces cerevisiae* was inoculated into the mash during the day of crushing, the increase of total number of yeasts occurred 4 days after the inoculation. The whole period of preliminary fermentation lasted 8 days and the highest value of total number of yeasts was achieved in a day of pressing. After that the decrease was observed. This effect can be attributed to the autolysis of yeasts after the alcoholic fermentation[1]. The decrease is also caused by the inhibition of starting population of bacteria *Oenococcus oeni* and malolactic fermentation, as shown in Fig. 2. After the alcoholic fermentation the autolysis of yeasts occurs, followed by the liberation of nutrients important for the growth of bacteria and the growth of present yeasts, as well. The decrease is also caused by the inhibition of starting population of bacteria *Oenococcus oeni* and malolactic fermentation, as shown

Fig. 1. **Number of yeasts – Wine factory No. 1**

in Fig. 2. After the alcoholic fermentation the autolysis of yeasts occurs, followed by the liberation of nutrients important for the growth of bacteria and the growth of present yeasts, as well.

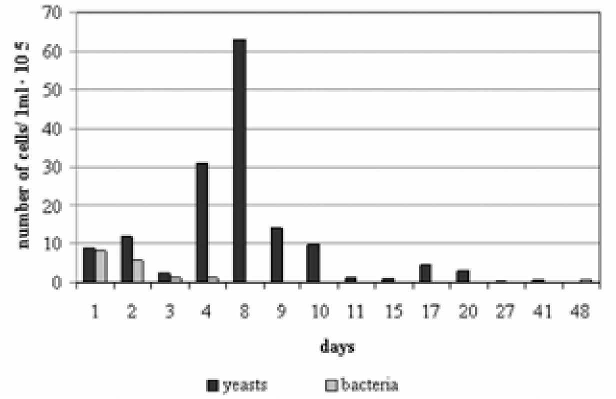

Fig. 2. **The comparison of the number of yeasts and lactic bacteria wine factory No. 1**

From the comparison of the number of yeasts in both wine factories (Fig. 3.) follows that after the pressing the total number of yeasts in wine factory No. 2 was lower. This difference is probably a result of the application of small amount of SO^2 in factory No. 2 by the start of the preliminary fermentation of the juice. The application of SO_2 was carried out to eliminate the development of undesirable microflora. The difference can also result from the difference in heating of the must in tank.

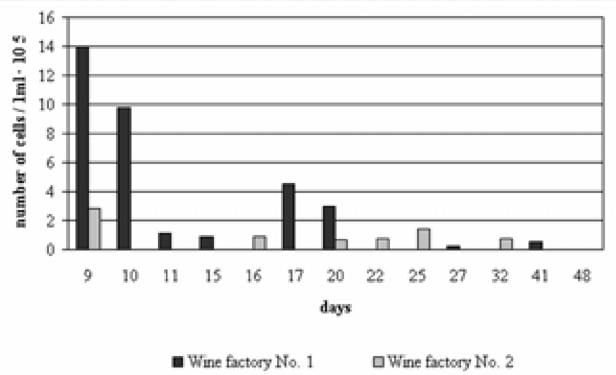

Fig. 3. **The comparison of the number of yeasts in wine factories No. 1 and No. 2**

After the alcoholic fermentation the start of malolactic fermentation is supposed. In wine factory No. 1 the amount of bacteria of malolactic fermentation was studied (Fig. 4.). In the case of wine factory No. 2 the commercial culture of bacteria *Oenococcus oeni* was applied. The heating of the must was realized using the heating rod. This procedure implies that the local overheating can occur, which destroys the microflora. In wine factory No. 2 by the application of malolactic bacteria no bacteria of lactic fermentation were detected.

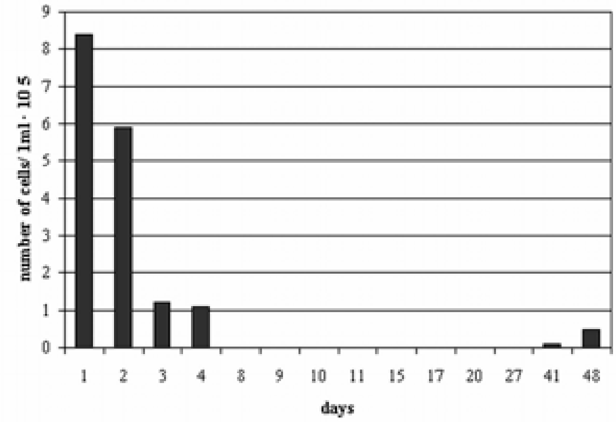

Fig. 4. **Number of lactic bacteria – wine factory No. 1**

During the study of the fermentation process the attention was also focused on the fungi in red wine manufacturing. From the results based on the visual evaluation of the increase on Petri dishes can be supposed that the fungi *Penicillium* and *Aspergillus* dominate. These can produce the different micotoxins. Fig. 5. presents the mixed culture of microorganisms, which grew from the grape juice in the day of crushing. The important result is the presence of the fungi *Botrytis*.

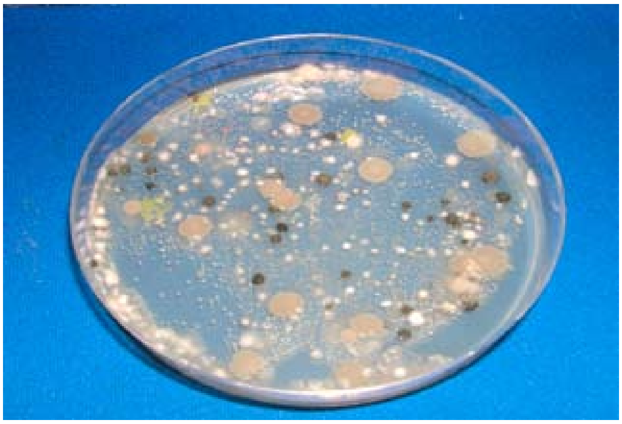

Fig. 5. **The mixed culture of microorganisms, which grew from the grape juice in the day of crushing**

Fig. 6. is an example of the growing blue-green colony of yeasts with the white edge, which appeared on plates with the grown population of the yeasts. This figure is also an example of the fungi, which during its development colonized the population of the yeasts

In Fig. 7. the blue-green fungi with the white edge can be seen again. In difference to Fig. 6. this fungi during its development did not colonize the site, where the population of yeasts was grown. These effect is in accordance with the observation[1] that the yeasts *Metschnikowia pulcherrima, Pichia*, *Candida*, *Cryptococcus* and some *Saccharomyces* and *Zygosaccharomyces* have strong antifungal aktivity

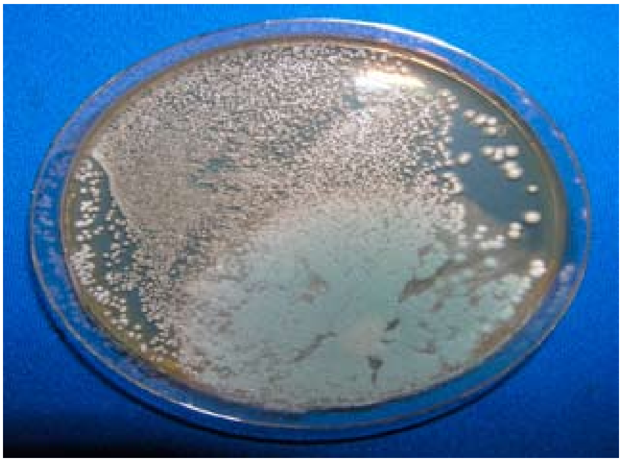

Fig. 6. **The fungi, which during its development colonized the population of the yeasts**

In the framework of the study it was confirmed that the wine is the product of many diverse interactions between yeasts, fungi and bacteria. This microflora can be positively and negatively influenced by the different technological interventions

Fig. 7. **The blue-green fungi with the white edge**

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P77 OPTIMALIZATION OF CULTIVATION MEDIA AND HYDROXYLASE ENZYME PRODUCTION BY *AUREOBASIDIUM PULLULANS*

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Introduction

In many countries, wheat straw is an abundant lignocellulosic by-product from farming, consisting of cellulose (35–40 % wt.) and hemicellulose (25–30 % wt.) in close association with lignin (10–15 % wt.)[1].

Lignin is a natural, complex, heterogenous, phenylpropanoid polymer comprising 25–30 % of plant biomass. Primarily three enzymes, i.e., lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase[2,3,4] have been held responsible for lignin degradation.

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic centre and usually called multicopper oxidases.

Laccases catalyze the reduction of oxygen to water accompanied by the oxidation of a substrate, typically a p-dihydroxy phenol or another phenolic compound. It is difficult to define laccase by its reducing substrate due to its very broad substrate range.

Although laccase was also called diphenol oxidase, monophenols like 2,6-dimethoxyphenol or guaiacol are often better substrates than diphenols, e.g. catechol or hydroquinone. Syringaldazine[N,N0-bis(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)] and ABTS are often considered to be a unique laccase substrates[5].

Laccase activity has been demonstrated in many fungal species, but laccase production has never been demonstrated in lower fungi. There are many records of laccase production by ascomycetes. Yeasts are a physiologically specific group of both ascomycetes and basidiomycetes. This basidiomycete yeast produces a true laccase capable of oxidation of phenols and aminophenols and unable to oxidize tyrosine[6]. The production of laccase was not demonstrated in ascomycetous yeasts.

In this study, the method suitable for production laccase as lignin-degrading enzymes by Aureobasidium pullulans was optimalized. Wheat straw was the only carbon source and only the most essential nutrients were added.

Experimental Microorganism

The fungal strain *Aureobasidium pullulans* F 8189 used in the present study was obtained from the culture collection of the Culture Collection of Yeasts (CCY), SAV, Institute of Chemistry. The tested culture was maintained at 26 °C on malt slant agar for 10 days.

Cellulolytic Substrate

Wheat straw from local sources (0.3–0.5 mm particle size) was used as natural substrate. This substrates was sterilized in autoclave at 120 °C for 20 minutes.

Optimalization of Culture Conditions

Growth of *A. pullulans* was studied in three cultivation media – glucose medium (GM), glucose medium with wheat straw (GSM) and basal medium with wheat straw (BSM) during solid-state (SSF) and submerged (SF) fermentation.

The glucose medium contained in grams per 1,000 g: KNO_3, 2; K_2HPO_4, 1; $MgSO_4$, 0.5; glucose, 50. The basal medium has the same composition as glucose medium but without glucose. The pH of the both media was adjusted to 5.6. These media were then autoclaved for 30 min at 121 °C and poured into Petri dishes with diameter of 9 cm and in 100 ml Erlenmayer flasks. Cultivation media with wheat straw also contained sterilized wheat straw (1 g wheat straw 100 ml^{-1} of medium).

Petri dishes and Erlenmayer flasks were inoculated by three ways – with fungal spores obtained from 4 days grown cultures on malt agar, with three mycelial discs (each 1 × 1 cm) obtained from 4 days fungal culture grown on glucose agar and inoculation by loops. Growth of *A.pullulans* was observed at 26 and 32 °C.

Enzyme Assay

Laccase (EC 1.10.3.2) activity was determined by the oxidation of 2,2‘-azino-bis(3-ethylthiazoline-6-sulfonate) (ABTS) according to Buswell et.al.[7] The reaction mixture for the standard assays contained 100 µl ABTS solution (1mM), 300 µl sodium acetate buffer pH 5.0 (0.1M) and 600 µl enzyme extract. Oxidation was followed via the increase in absorbance at 420 nm ($\varepsilon_{420} = 36{,}000\ M^{-1}\,cm^{-1}$). All enzyme assays were performed in triplicate.

Protein concentration was determined by Lowry et al. method[8] with bovine serum albumin as standard.

Results

A comparison of growth pattern of *A.pullulans* in various cultivation conditions as medium composition, temperature or method of inoculation was made and the results are presented in Table I. As Table I demonstrates, *A. pullulans* were grown in different type of solid media. In the course of liquid cultivations less growth was observed regardless of temperature. The best growth rate was obtained in GSM or BSM medium in comparison to GM during solid-state fermentation.

The effect of method of inoculation varied from inoculation with fungal spores (maximum growth) to inoculation by loops (minimum growth).

For testing of production of hydroxylase were chosen microorganism Aureobasidium pullulans by methods of Azure-B agar and ABTS agar[9]. In an attemp to compare the production laccase during solid-state and submerged

Table I
Growth rate of *A. pullulans* on different cultivations media – glucose medium (GM), glucose medium with wheat straw (GSM) and basal medium with wheat straw (BSM)

Incubation time [days]	Type of inoculation	Solid state fermentation		
		GM 26/32 °C	GSM 26/32 °C	BSM 26/32 °C
3	Loop	0/+	0/0	0/+
	Fungal spores	+/+	+/+	0/+
	Mycelial discs	0/0	0/0	0/0
5	Loop	0/+	0/0	0/+
	Fungal spores	+/+	+/+	+/+
	Mycelial discs	+/0	+/0	+/0
10	Loop	0/+	++/0	++/+
	Fungal spores	++/+	++/0	++/+
	Mycelial discs	+/+	+/0	++/+
14	Loop	0/+	++/+	+/+
	Fungal spores	++/+	++/+	++/+
	Mycelial discs	+/+	+/+	++/++

0 = no growth, + = growth, ++ = extremelly growth

fermentation, *A. pullulans* was studied in various solid and liguid media. No laccase activity could be detected in any solid media. In contrast to solid-state fermentation, this strain proceeded the maximum activity in liquid GM, but low level of laccase was detected in BSM. Laccase production reached its maxima on 8 and 15 day for GM and 7 day for GSM and BSM. (Fig. 1.).

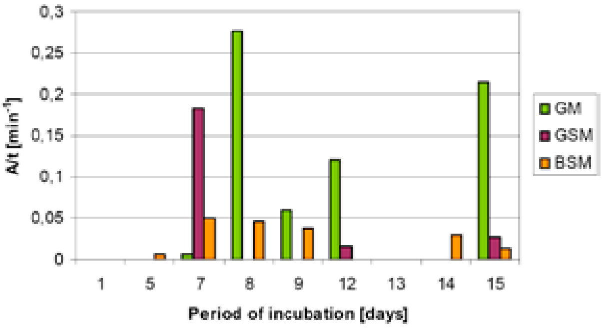

Fig. 1. **Comparison of the production of laccase by *A. pullulans* grown on three different culture media during submerged cultivation**

Conclusions

Our results indicated, that the conditions optimal for *A. pullulans* growth were determined for glucose medium with wheat straw, temperature 26 °C, inoculation by fungal spores and solid state fermentation. The results reported here also showed that optimal growth of *A. pullulans* and production of laccase were not be detected for the same cultivation conditions.

During the optimalization of the culture medium hydroxylase activity reached higher level in the presence of yeast *Aureobasidium pulllulans* in the glucose medium without wheat straw during submerged cultivation. Relatively higher laccase production on GM showed twin peaks during their incubation.

Reapperance of enzyme activity during later stage of wheat straw degradation might be attributed to fungal autolysis resulting in the release of cell membrane bound on intracellular enzymes in the medium[10].

This work has been supported by Ministry of Education, Youth and Sports under project MSM 021630501.

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P78 INFLUENCE OF MODIFIED BIOCOMPOSITES ON PRODUCTION OF EXTRACELLULAR POLYSACCHARIDES BY IMMOBILIZED *AUREOBASIDIUM PULLULANS*

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Introduction

Pullulan is an extracellular water-soluble polysacharid produced by yeast-like strain *Aureobasidium pullulans*. It is a linear homopolysaccharide usually described as an a-(1–6) lincagen polymer, consisting mainly of maltotriose units[1]. The regular alternation of alpha-1,4 and alpha-1,6 bonds results in two distinctive properties, such as structure flexibility, enhanced water-solubility and excellent film- and fiber forming properties[2]. Thanks these characteristic pullulan can be used in low-calorie food aidtives, cosmetic emulsions, oxygen-impermeable film for packaging, adhesives, and thickening and extending agents. Recently, pullulan has been getting renewed attention as an excellent material for pharmaceutical and biomedical application[3].

Fermentation can be affected by different types of carbon and nitrogen source resulted in varying pullulan yields during the culture growth process, since the philamentous forms or chlamydospores are less productive than the yeast or pigment-free blastospores[4].

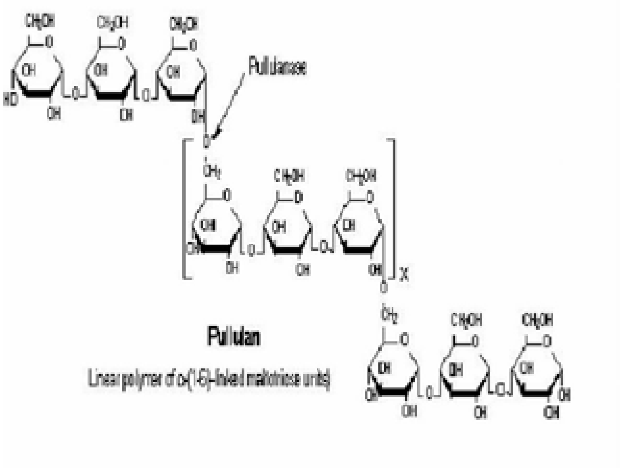

Fig. 1. **Chemical structure of polysaccharide pullulan**

In this work, several media with different type of carbon and/or nitrogen source were used for *A. pullulans* cultivation. PUR foams modified by 10 % of carboxymethyl cellulose, 2-hydroxyethyl cellulose, acetylated starch and acetyl celulose, respectively were used as immobilization agents. A. pullulans cells were cultivated in Erlenmeyer flasks at 28° C for 120 to 480 hours. First, all materials were tested as potential carbon/nitrogen source. Further, microorganisms were cultivated for 4–6 weeks in presence of PUR under permanent shaking. Samples were taken in 24-hour intervals, quantitative changes of biomass and pullulan content were determined gravimetrically and/or spectrophotometrically. Additionally, surface microscopy of all degraded polyurethanes was tested.

Material and methods

Microorganism and Culture Conditions

Strain of *A. pullulans*, CCM F-148 was purchased from Czech Collection of Microorganisms. The culture was stored on medium with malt extract and yeast malt agar, respectively, at 4° C. For biodegradation experiments 100 ml of inoculum was prepared in 500 ml Erlenmayer flask. Cultivation was performed at 28 °C and 150 rpm for 24 hours. Inoculum as well as production medium contained ($g\,dm^{-3}$): yeast extract 7, potassium phosphate 5, ammonium sulphate 5, magnesium sulphate 0.34, glucose 40 was used as carbon source, distilled water. Initial pH was 6.5 before autoclaving. All flasks were incubated in an incubator shaker operating at 28–30 °C and 150 rpm for 120 hours.

Materials

General chemicals: polyether polyol, tolylene diisocyanate 80/20 (TDI), tin and amine based catalysts, surfactant and water. Biodegradable fillers: acetylated starch (AS), acetylcellulose (AC) Mn = 30,000 Da and 2-hydroxyethylcellulose (HEC) Mn = 90,000 Da. The foams were prepared by a three-step reaction process. The chemical composition of the pulverized BIO-PU foams was proved by an infrared spectroscopy on the Nicolet Impact 400D Fourier Transform InfraRed (FTIR) spectrometer using the KBr technique.

Measuremend of Dry Cell Weight and Pullulan Production

Samples for analyses (10 ml) were taken from each flask in regular 24-hour intervals. Total biomass (summ of mycelial and yeast cells) was determined after centrifugation of the culture sample at 12,000 × g for 20 min and washing the sediment with distilted water gravimetrically (drying at 105 °C for 2 hours).

To analysis of pullulan production, supernatant was mixed with 2 volumes of absolute ethanol for 20 min. Precipited polysaccharide was separated by centrifugation or filtration and dried at 80 °C. Pullulan precipitate was purified twice by hot water and by ethanol. Crude pullulan yield was measured gravimetrically.

Pure polysaccharide content was determined by analysis of products of its enzymatic hydrolysis (effect of pullulanase resulted in maltotriose). The content of maltotriose was determined by Somogyi-Nelson method[5], calibration with maltotriose was performed.

Results

Fig. 2. shows biomass and pullulan production in presence of modified PUR foams. It seems that the highest pullulan production was observed in control medium (without PUR), so, PUR presence exhibited negative effect on pullan production.

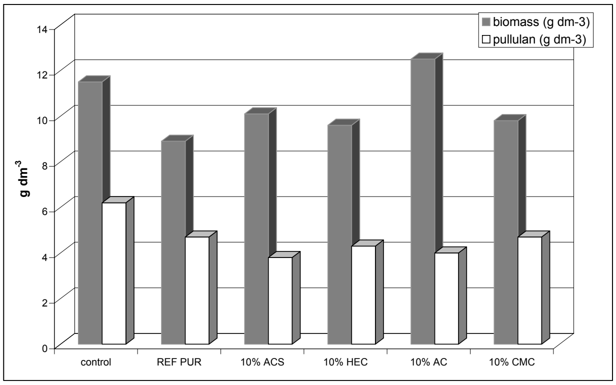

Fig. 2. **Biomass and pullulan production by *A. pullulans* grown in presence of modified PUR foams**

Conclusions

Yeast strain Aureobasidium pullulans can grow in presence of modified polyurethane foams. Degradation degree of BIO-PUR corresponded with growth of yeast culture. The highest degree of degradation was found in PUR modified by 40 % carboxymethyl cellulose; degree of degradation decreased with CMC concentration.

According to our results of *A. pullulans* grown in PUR presence it seems that most of modified polyurethane foams exhibited slight negative effect on pullullan (4.3 $g\,dm^{-3}$ control, 4.9 $g\,dm^{-3}$ 1% ACS-PUR, 3.2 $g\,dm^{-3}$ REF-PUR) as well as biomass production (11.5 $g\,dm^{-3}$ control, 7.8 $g\,dm^{-3}$ 1% ACS-PUR, 8.9 $g\,dm^{-3}$ REF-PUR). Acetyl cellulose was the only modification agent, which was able to enhance pullulan (6.5 $g\,dm^{-3}$) as well as biomass (12.5 $g\,dm^{-3}$) production.

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P79 FATTY ACIDS DISTRIBUTION IN THE LIPID FRACTIONS OF *CALENDULA OFFICINALIS* L. SEEDS OIL

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Introduction

Plant oils are important renewable resources used as food; feed or as industrial feedstocks[1]. The *Calendula* seeds oil is currently considered a potential oilseed crop with industrial or other functionalities. It is characterized by a high content of the unusual conjugated octadecatrienoic acid – calendic acid (18 : 3c 8t, 10t, 12c) and some other conjugated isomers, which give special chemical and physical properties[2]. There is an increasing interest for conjugated fatty acids since some of them were proved to have anticancer and lipid-lowering effects[3,4]. Calendic acids have inhibitory effect on human colon cancer cells, decrease body fat content and have hepatoprotective effect[5,6,7]. Here we present the fatty acids distribution in the lipid fractions of *Calendula* seeds and the fatty acids variation during seeds maturation.

Experimental

Extraction and Lipid Fractionation

Total lipids were extracted using Folch method[8]. Neutral lipids were separated by TLC with a solvent mixture of hexane: ethyl ether: acetic acid (95 : 15 : 1). Polar lipids were scratched, extracted and separated according to Heape method[9].

Fatty Acids Analysis

The total lipid extract and the lipid fractions were transesterified with BF3/methanol. The methyl esters of fatty acids (FAME) were dissolved in hexane and injected for GC analysis. A Shimadzu GC 17A with FID detector and a Crompack Silica 25 MXO capillary column (25 m × 0.25 mm i.d., film thickness 0.25 µm) was used. The temperature program was: 5 min at 70°C, 4 °C/min to 235 °C (hold 5 min). The injector temperature was 260 °C and the detector temperature – 260˚C. The carrier gas was helium.

Sterols Analysis

A part of total lipid extract was saponified by refluxing with 1M KOH ethanol/water (8 : 2, v/v) solution for 1 h. The unsaponifiables, containing total sterols, were then extracted first with petroleum ether and diethyl ether. The ether phases were combined, washed and evaporated to dryness.

The sterols were derivatized trimethyl silyl ether (TMS) derivatives and separated on fused silica capillary column coated with 5% phenyl/95% dimethylpolysiloxane (30 m × 0.25 mm i.d., film thickness 0.25 µm; Rtx-5; Restek Corporation, Bellefonte, PA, USA) and using the same GC system mentioned above. The temperature program was: 5 min at 200 °C, 10 °C/min to 300 °C (hold 20 min).

FAME and sterols peaks were identified by comparison of their retention times with those of commercially available standards (Sigma). All extractions and GC-FID runs were performed in triplicate and mean values were calculated.

Results

Calendula seeds were analyzed in different stages during their maturation, seeds collected: immediately after flower drops (0), one week after (1) and two weeks after flower drops. (2). The fatty acids composition is presented in Fig. 1. The calendic acid represents – 8.62 % at first stage, it increased at 26.6 % one week after and reached the maximum content of 53 % in the mature seeds. The increasing of 18 : 3c content occurs in the same time with the fast decreasing of linoleic acid and a slow decrease of oleic acid, while stearic and linolenic acid remain almost to the same values during seeds maturation.

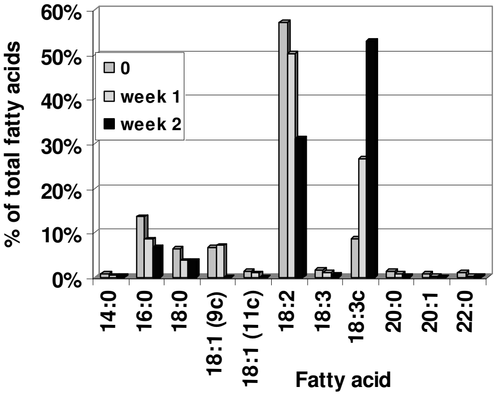

Fig. 1. **Fatty acids variation during seeds maturation**

Triacylglycerols (TAG) contain the highest amount of conjugated fatty acids (33 %), while in diacylglycerols (DAG) and monoacylglycerols (MAG) contain less than 10 % (data not shown). The polar lipid (PL) fraction is highly unsaturated, with more than 65 % of linoleic acid. Conjugated acids are present in polar lipids fraction fact which can

Table I
Fatty acids distribution in lipid fractions

Fatty acid	TAGSE	PL	
Miristic 14 : 0	0.75	1.58	0.34
Palmitic 16 : 0	6.41	21.25	12.88
Stearic 18 : 0	2.47	2.65	2.84
Oleic 18 : 1 (9c)	7.33	10.02	7.21
Linoleic 18 : 2 (9c, 12c)	47.2	47.8	65.56
α-linolenic 18 : 3 (9c, 12c, 15c)	1.17	3.15	3.13
Conjugated acids 18 : 3c	33.33	11.5	6.56
Arachidic 20 : 0	0.65	2.25	0.31
Gadoleic 20 : 1 (9c)	0.49	0.65	0.30

Table II
Sterols composition in *Calendula* seeds

Component	Retention time [min]	[mg 100 g^{-1}] seeds
5-α-cholestane-3-β-ol (Internal standard)	40.23	–
Cholesterol	39.63	4.23
Campesterol	45.07	9.21
Campestanol	45.60	0.42
Stigmasterol	46.50	17.6
Δ-7-Campesterol	48.57	1.42
β-Sitosterol	49.78	38.9
(Sitostanol + β-amyrin)	50.63	0.97
Δ-5-Avenasterol	51.05	3.72
Δ-7-sitosterol	53.95	13.98
Δ-7-avenasterol	55.13	2.91
Citrostadienol	61.33	1.56
Total sterol content		94.92

be explained by the implication of phosphatidylcholine and some of his derivatives in the desaturation and transacylation processes. The esterified sterols (SE) contain the highest amount of palmitic acid, but also more than 11 % conjugated fatty acids (Table I). β-Sitosterol is the major phytosterol of seeds, representing 41 % of total sterols, followed by stigmasterol with 18 %, Δ7 – sitosterol – 15 % and campesterol – 10 % (Table II)

Conclusions

The fatty acids composition of *Calendula* seeds is strongly influenced by the degree of seeds maturation. Conjugated fatty acids reached maximum content in mature seeds. Conjugated fatty acids are concentrated in triacylglycerols and they are present in lower amount in other lipid fractions. Beside the industrial application, *Calendula* seeds oil can be considered as a nutraceutical due to his high content (over 80 %) of biologically active polyunsaturated fatty acids and phytosterols.

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P80 LIPID PEROXIDATION PRODUCTS IN PLASMA OF PATIENTS WITH CHRONIC PANCREATITIS

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Introduction

The chronic inflammation of human pancreas (chronic pancreatitis) is known to be associated with an increased generation of reactive oxygen species damaging pancreatic tissue[1,2]. One of the primary targets of these radicals are unsaturated fatty acids. The lipid peroxidation leads to formation of mutagenic compounds such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Therefore, the main aim of the study was to determine MDA and 4-HNE plasma levels in patients with chronic pancreatitis using HPLC-DAD.

Experimental

Materials

EDTA tubes 7.5 ml (Sarstedt) were used for blood collection. Water G CHROMASOLV® (for gradient elution), methanol G CHROMASOLV® (for gradient elution, ACS), acetonitrile CHROMASOLV® (for HPLC, gradient grade) and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich Ltd. Solid Phase Extraction (DSC-C18; Discovery®, 1 ml Tube, 50 mg) tubes were obtained from Supelco. EDTA disodium salt (EDTA-Na_2) and trichloroacetic acid (TCA) were from Fluka – Biochemika. 2,4-dinitrophenylhydrazine (DNPH) and potassium dihydrogen-phosphate (KH_2PO_4) were purchased from Lachema and 35% hydrochloric acid (HCl) from Penta. All reagents and chemicals were analytical grade of highest purity. All organic solvents were HPLC grade.

Plasma Collection

A total of 105 patients (range 20 to 60 years; 69 males and 36 females) with chronic pancreatitis was included in the study. Blood samples were collected by venipuncture into EDTA tubes twice from each patient. The second blood collection was performed six months after the first round. Whole blood was immediately centrifuged (3,500 rpm, 22 °C for 10 min) and plasma was removed. Aliquots of plasma in test tubes (1.5 ml, with 1.96 mM BHT) were snap frozen in liquid nitrogen and then stored at –80 °C. The same procedure for plasma collection was used for the healthy male and female volunteers (range 20 to 60 years, 10 males and 17 females).

Plasma Preparation

Hydrolysis of protein bound MDA and 4-HNE was achieved by incubating 500 µl plasma with 250 µl 0.25 N hydrochloric acid in 60 °C water bath for 40 min. Then, protein was precipitated with 250 µl 15% trichloracetic acid, and the mixture was centrifuged (16,000 G, 4 °C for 10 min). The supernatant was further used for HPLC analysis.

HPLC Analysis

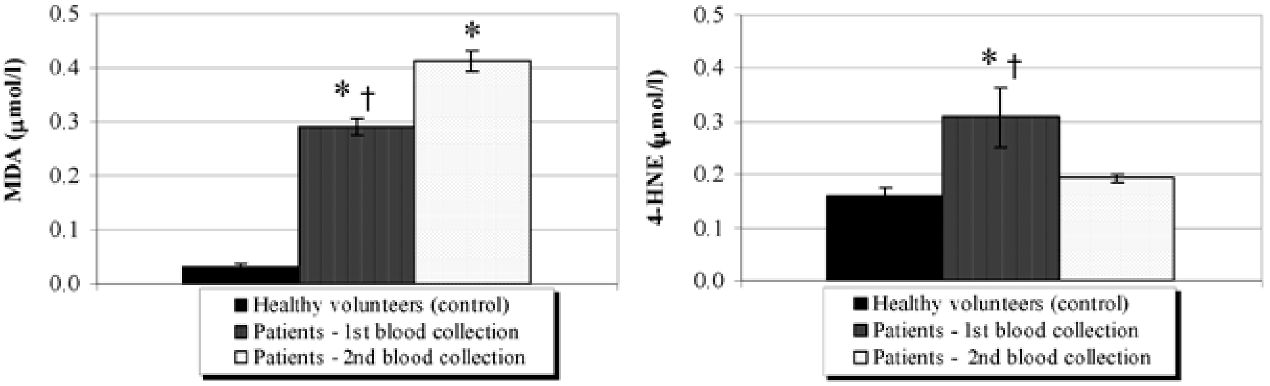
For derivatization, 100 µl or 200 µl of DNPH reagent (5 mM solution in 2 M HCl) was added to 600 µl of supernatant for MDA-DNPH products or to 1,000 µl of supernatant for 4-HNE-DNPH products. This reaction mixture was incubated for 60 min at room temperature protected from light. 4-HNE-DNPH adducts were extracted with SPE tubes – DSC-C18 which were conditioned with 1 ml of methanol, then with 1 ml 25 mM KH_2PO_4 for adjustment acidic pH 3 of the sample matrix. Concentrated 4-HNE-DNPH adducts were eluted with 300 µl of acetonitrile. The adducts of 4-HNE-DNPH and MDA-DNPH after reaction with DNPH were directly injected onto Cogent™ Bidentate C18 column (4.2 µm, 4.6 × 150 mm I.D.) with pre-column MetaGuard Polaris-C18 (5 µm, 4.6 mm). Chromatography was performed using Agilent 1100 series and DNPH derivates of aldehydes (MDA-DNPH and 4-HNE-DNPH) were detected with Agilent 1100 photo-diode detector at 310 nm (MDA) or 350 nm (4-HNE) at flow-rate 1 ml min^{-1} with an isocratic elution acetonitrile-water-acetic acid (40 : 60 : 0.2, v/v/v) for MDA-DNPH detection and with linear gradient of acetonitrile-water-acetic acid (50 : 50 : 0.2, v/v/v) to acetonitrile-water-acetic acid (80 : 20 : 0.2) in 20 min (for 4-HNE-DNPH determination). The amounts of MDA and 4-HNE were quantified by performing peak area analysis using external calibration curve. The plasma concentrations of MDA and 4-HNE were expressed as µmol dm^{-3}.

Statistical Analysis

Data are reported as mean ± S.E.M. The data were statistically analyzed by Student´s *t*-test. A *P* value of less than 0.05 was considered significant.

Results

Fig. 1.(a) and Fig. 1.(b) demonstrate the concentration of MDA and 4-HNE in plasma of patients with chronic pancreatitis in the 1st and 2nd blood collection compared with healthy volunteers (control). MDA concentrations (mean 0.29 ± S.E.M. 0.01 µmol dm^{-3}) in plasma from the 1st blood collection were significantly higher in comparison with healthy controls. Moreover, MDA levels (mean 0.41 ± S.E.M. 0.02 µmol dm^{-3}) in the plasma from the 2nd blood collection were significantly higher not only in comparison with healthy controls but also in comparison with MDA levels in plasma from the 1st blood collection. Similarly, the concentrations of 4-HNE were significantly increased in plasma from the 1st blood collection (mean 0.31 ± S.E.M. 0.05 µmol dm^{-3}) in comparison with healthy controls.



(a) **(b)**

Fig. 1. **Effect of the chronic inflammation of human pancreas on lipid peroxidation expressed as MDA concentration (a) and 4-HNE concentration (b); values are the mean ± S.E.M. * Symbol shows significant difference compared to the control, † symbol shows significant difference between the 1st and 2nd blood collection**

However, the levels of 4-HNE in the 2nd blood collection (mean 0.19 ± S.E.M. 0.01 μmol dm^{-3}) were only slightly elevated in comparison with healthy controls. The concentration trend of 4-HNE is not correlated with MDA between the 1st and 2nd blood collection.

Conclusions

In the present study, we demonstrated that the chronic inflammation in pancreas is accompanied with an increased concentration of secondary products of lipid peroxidation as MDA and 4-HNE in blood plasma. This observation is in agreement with published studies of other authors[3,4].

In contrast with 4-HNE, the concentration of MDA in plasma increased gradually during the followed time period. It can be suggested that MDA rather then 4-HNE could be a good biomarker[5,6,7] for monitoring of oxidative stress in patients with chronic pancreatitis and a progress of the disease.

To confirm this suggestion, these patients will be examined periodically in the consequent two years.

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P81 THE EFFECT OF LIPOPHILICITY ON ANTIFUNGAL ACTIVITY OF SOME 2-AMINO AND 2-METHYLBENZIMIDAZOLE DERIVATIVES

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Introduction

Benzimidazole and its derivatives have a long history as antimicrobial agents. Several thousands of benzimidazole analogs have been synthesized and screened for pharmacological activity. They are of wide interest because of their diverse biological activity and clinical applications. These heterocycle systems have different activities as they can act as bacteriostats or bactericides, as well as fungicides[1–5] and they are present in numerous antiparasitic, antitumoral and antiviral drugs.[6–7] Also, some of them exhibit appreciable antiprotozoal activity[8]. It was confirmed to have a moderate in vitro anti-HIV activity[9]. The success with these group of molecules stimulated the search for new biologically active derivatives. Understanding the role of chemical structure on biological activity is very important. Predictions of biological and physicochemical properties of molecules based on their structure are the fundamental and most interesting objectives of chemistry.

The conception that there exists a close relationship between bulk properties of compounds and the molecular structure of those compounds is quite rooted in chemistry. This idea allows one to provide a clear connection between the macroscopic and the microscopic properties of matter, and thus has been firmly established as one of the central foundations of chemistry. Therefore, it is the basic tenet of chemistry to attempt to identify these assumed relationships between molecular structure and physico-chemical properties and then to quantify them.

A large number of research studies are nedded to analyze the pharmacophore present in these compounds using the Three Dimensional QSAR (quantitative structure-activity relationship) methods.[10–12] The physicochemical properties predicted from structure are helpful in the search for new molecules of similar or increased biological activity. QSAR studies enable the investigators to establish reliable quantitative structure-activity relationships, to derive a QSAR model and predict the activity of novel molecules prior to their synthesis.[13–16]. These studies reduce the trial- and error element in the design of compounds by establishing mathematical relationships between physical, chemical, biological, or environmental activities of interest and measurable or computable parameters such as physicochemical, electronic, topological, or stereochemistry. 3D-QSAR methodology has been succesfully used to generate models for various chemotherapeutic agents.

Progress in the use of QSAR methods has shown the importance of the hydrophobic or lipophilic nature of biologically active molecules. The lipophilicity modifies the penetration of bioactive molecules through the apolar cell membranes. This property is usually characterized by partition coefficient (log$P$), which is essentially determined from distribution studies of the compound between an immiscible polar and non-polar solvent pair. To measure log$P$ by the conventional “shake-flask” technique[17] is difficult and time resuming. It is complicated to determine log$P$ for substances that are not very soluble in water or can not be detected by conventional techniques. Instead of measuring the log$P$ values by equilibrium methods, partition chromatographic data can be determined.

One of the most frequently used methods for lipophilicity measuring is reversed-phase thin-layer chromatography (RP TLC)[18–22]. Lipophilicity can be expressed in terms of many different descriptors (log$P$, π, f, logk_w, R_M, R_M^0) obtained experimentally or calculated. Most frequently used experimental parameters are retention constants, R_M^0 (RP TLC) and logk_w (RP HPLC), whilst the calculated factor is log$P$. Samples of pure compounds are not always available, so it is important to develop QSAR methods that can efficiently predict biological activity by using theoretical descriptors computed from the chemical structure. log$P$ is also used in many environmental studies to determine the environmental fate of chemicals. By knowing exact values for this parameter, it is possible to predict the inhibitory activity of a drug.

In this context, the aim of the present study was to investigate the activity of different substituted 2-amino and 2-methylbenzimidazoles against yeast *Saccharomyces cerevisiae* and study the quantitative effect of lipophilicity on antifungal activity. The main objective was to establish a quantitative lipophilicity-inhibitory activity relationships and derive a high-quality model which would link the lipophilicity of these compounds with their inhibitory activity.

Experimental

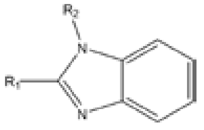
The structures of the benzimidazoles tested in this study are presented in Table I. All the compounds, except (1) and (8) were synthesized by a general procedure described by Vlaović[23]. 2-aminobenzimidazole (1) and 2-methylbenzimidazole (8) were of analytical reagent grade, commercially available.

Antifungal Activity

All the benzimidazole derivatives were tested for their *in vitro* growth inhibition activity against yeast *Saccharomyces cerevisiae* (ATCC 24860). For the evaluation of the antifungal activities of the samples, agar disc diffusion method was used as described by NCCLS[24].

The strains were grown on Sabouraud Dextrose slants for 24 hours at 25 °C and checked for purity. After incubation the cells were washed from the surface of agar and suspended in a sterile phisyological solution. The number of cells in 1 ml of suspension for inoculation, measured by Mc Farland

Table I
The structures of the compounds studied



Compound	R_1	R_2
1	NH_2	H
2	NH_2	$C_6H_5{-}CH_2$
3	NH_2	$4{-}CH_3{-}C_6H_4{-}CH_2$
4	NH_2	$4{-}Cl{-}C_6H_4{-}CH_2$
5	NH_2	$C_6H_5{-}CO$
6	NH_2	$4{-}CH_3{-}C_6H_4{-}CO$
7	NH_2	$4{-}Cl{-}C_6H_4{-}CO$
8	CH_3	H
9	CH_3	$C_6H_5{-}CH_2$
10	CH_3	$4{-}CH_3{-}C_6H_4{-}CH_2$
11	CH_3	$4{-}Cl{-}C_6H_4{-}CH_2$
12	CH_3	$C_6H_5{-}CO$
13	CH_3	$4{-}CH_3{-}C_6H_4{-}CO$
14	CH_3	$4{-}Cl{-}C_6H_4{-}CO$

nefelometer, was 1×10^7 CFU cm^{-3}. The 1 ml of this suspensions was homogenized with 9 ml of melted (45 °C) Sabouraud Dextrose Agar and poured into Petri dishes. On the surface of the agar the 6 mm diameter sterile paper discs (Hi Media, Mumbai, India) were put and impregnated with 10^{-3} ml of samples. The plates were incubated for 24–47 hours at 25 °C, and the diameter of the resulting inhibition zone (including the disc) was measured (in mm). The evaluation of antifungal activities of samples was carried out in three repetitions.

Minimum inhibitory concentration (MIC) was determined by the agar dilution method according to guidelines established by the NCCLS standard M7-A5[25]. The MIC of tested benzimidazoles is defined as the lowest concentration of the compound at which no growth of the strain is observed in time and under specified experimental conditions. Stock solutions of the compounds were prepared in dimethylformamide (DMF). Further dilutions were performed with distilled water. The inoculated plates were then incubated at 35 °C for 16–20 h. A control (using DMF without any test compound) was included for each organism. It was determined that the solvent had no activity against any of the test microorganisms. The negative logarithms of molar MICs ($\log 1/c_{MIC}$) were determined and used for further calculations.

Molecular Modeling and log *P* Calculations

Molecular modeling studies were performed by using CS Chem-Office Software version 7.0 (Cambridge software) running on a P-III processor[26]. All molecules were constructed by using Chem Draw Ultra 7.0 and saved as the template structure. For every compound, the template structure was suitably changed considering its structural features, copied to Chem 3D 7.0 to create a 3-D model and, finally, the model was clened up and subjected to energy minimization using molecular mechanics (MM2). The minimization was executed until the root mean square (RMS) gradient value reached a value smaller than 0.1 kcal mol^{-1}·A. The Austin Model-1 (AM-1) method was used for re-optimization until the RMS gradient attained a value smaller than 0.0001 kcal mol^{-1}·A using MOPAC. The lowest energy structure was used for each molecule to calculate lipophilicity parameters (Table II).

Statistical Methods

The complete regression analysis was carried out by PASS 2005, GESS 2006, NCSS Statistical Softwares[27].

Table II
Data of the lipophilicity parameters

Compound	$\log P$
1	0.99
2	2.96
3	3.44
4	3.52
5	2.84
6	3.32
7	3.39
8	1.48
9	3.45
10	3.94
11	4.01
12	3.33
13	3.81
14	3.89

Results

The results of antifungal studies of benzimidazoles tested against *Saccharomyces cerevisiae* are summarized in Table III. As indicated, all the compounds show antifungal activities against the tested yeast. Consequently, compounds with high $\log 1/c_{MIC}$ are the best antifungals. The MICs were compared with Ketoconazole and Amphotericin which were screened under similar conditions as reference drugs.

In order to identify the effect of lipophilicity on the inhibitory activity, QSAR studies of title compounds were performed.

A set of benzimidazoles consisting of 14 molecules was used for multilinear regression model generation. The reference drugs were not included in model generation as they belong to a different structural series. An attempt has been made to find structural requirement for inhibition of *Saccharomyces cerevisiae* using QSAR Hansch approach on benzimidazole derivatives. To obtain the quantitative effects of the lipophilicity parameter of benzimidazole derivatives on their antifungal activity, QSAR analysis with $\log P$ was operated.

Table III
Antifungal screening summary

Compound	$\log 1/c_{MIC}$ exp.	$\log 1/c_{MIC}$ predict.	Residuals
1	3.726	3.772	–0.046
2	4.854	4.946	–0.092
3	4.579	4.498	0.081
4	4.615	4.632	–0.017
5	4.880	4.986	–0.106
6	4.604	4.657	–0.053
7	4.638	4.568	0.07
8	4.325	4.231	0.094
9	4.551	4.483	0.068
10	3.277	3.435	–0.158
11	3.313	3.229	0.084
12	4.577	4.645	–0.068
13	3.602	3.778	–0.176
14	3.637	3.573	0.064
Ketoconazole	4.628	–	–
Amphotericin	4.869	–	–

Usually, lipophilicity parameters are linearly related to pharmacological activity (MICs), but in the more general case this relationship is not linear[28]. Therefore, it was made a complete regression analysis resorting to linear, quadratic and cubic relationships. The statistical quality of the resulting models is determined by correlation coefficient (r), standard error of estimation (s), and probability factor related to F-ratio (F). Good quality of mathematical models was obtained in cases of quadratic and cubic relationships, as depicted in Eqs.(1) and (2). It is noteworthy that both equations were derived using entire data set of compounds (n = 14) and no outliers were identified. The F-value obtained in Eqs.(1) and (2) is found statistically significant at 99 % level since all the calculated F values are higher as compared to tabulated values. It is apparent from the data that fitting equations improve when resorting to second order polynomial.

$$\log 1/c_{MIC} = -0.748 \log P^2 + 3.654 \log P + 0.712 \tag{1}$$
$$r = 0.962;\ s = 0.171;\ F = 69.32$$

$$\log 1/c_{MIC} = -0.201 \log P^3 + 0.863 \log P^2 - 0.263 \log P + 3.382 \tag{2}$$
$$r = 0.979;\ s = 0.134;\ F = 77.56$$

For the estimation of the quality with regard to predictive ability of the best model (2), the cross-validation statistical technique has been applied. The simplest and most general cross-validation procedure is the leave-one-out technique (LOO technique). This method uses cross-validated fewer parameters: PRESS (predicted residual sum of squares), SSY (total sum of squares deviation), r^2_{CV} and r^2_{adj} (Table IV). PRESS is an important cross-validation parameter as it is a good approximation of the real predictive error of the models. Its value being less than SSY points out that the model predicts better than chance and can be considered statistically significant. The present models have PRESS << SSY. From the PRESS and SSY, r^2_{CV} can be easily calculated:

$$r^2_{CV} = 1 - \text{PRESS/SSY} \tag{3}$$

But, the only way to estimate the true predictive power of a model is to test their ability to predict accurately the biological activities of compounds. In order to verificate the predictive power of the developed model, predicted $\log 1/c_{MIC}$ values of benzimidazoles investigated were calculated by using Eq.(2) and compared with the experimental values.

Table IV
Cross-validation parameters

Eq.(2)	
PRESS	1.317
SSY	4.370
PRESS/SSY	0.301
r^2_{CV}	0.699
r^2_{adj}	0.946

Based on the magnitude of residue, there is a close agreement between the observed and calculated inhibitory activity (Table III). Further the plot of linear regression predicted $\log 1/c_{MIC}$ values against the observed $\log 1/c_{MIC}$ values also favor the model expressed by Eq.(2) (Fig. 1.).

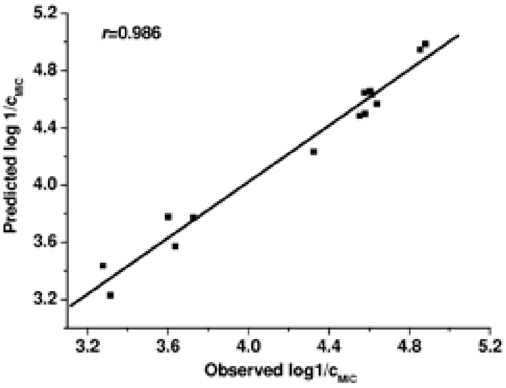


Fig. 1. **Plots of predicted versus experimentally observed inhibitory activity against *Saccharomyces cerevisiae***

In order to investigate the existence of a systemic error in developing the QSAR models, the residuals of predicted $\log 1/c_{MIC}$ were plotted against the observed $\log 1/c_{MIC}$ values (Fig. 2.). The propagation of the residuals on both sides of zero indicates that no systemic error exists in the development of regression models as suggested by Jalali-Heravi and Kyani[29].

From the derived model, it can be concluded that strong influence of the partition coefficient, $\log P$, is important for the antifungal activity and this parameter is usually related to pharmacological activity. This evidence was clearly described in lipid theory advanced by Meyer and Overton. According to this theory, log *P* is a measure of hydrophobicity which is important for the penetration and distribution of the drug, but also for the interaction of drug with receptors. Therefore, it can be suggested that lipophilic properties have to be checked for designing of potent antifungal agents as they are deciding factors for its activity.

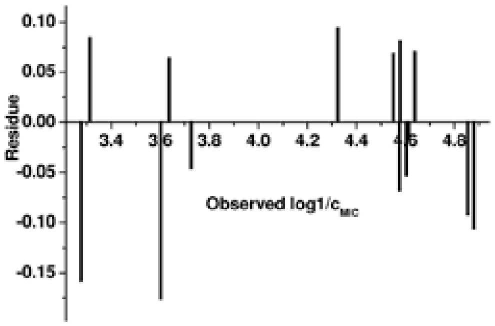


Fig. 2. **Plot of residual values against the experimentally observed log 1/c_{MIC} values**

Conclusions

QSAR analysis was performed to estimate the quantitative effects of the lipophilicity parameter, log*P*, of the different substituted 2-amino and 2-methylbenzimidazole derivatives on their antifungal activity against *Saccharomyces cerevisiae*. log *P* values were calculated for each molecule, and high-quality mathematical model relating the inhibitory activity, log 1/c_{MIC}, and log *P* was defined . For the estimation of the predictive ability of this model, the cross-validation statistical technique was applied. Comparison of the linear, quadratic and cubic relationships showed that the cubic equation was the most appropriate for prediction of antifungal activity of the investigated class of molecules. It is concluded that strong influence of the partition coefficient, log *P*, is important for the inhibitory activity and this parameter is usually related to pharmacological activity. The obtained mathematical model was used to predict antifungal activity of the benzimidazoles investigated and close agreement between experimental and predicted values was obtained. It indicates that this model can be successfully applied to predict the antifungal activity of these class of molecules.

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P82 QSAR MODELS FOR PREDICTING THE ANTIBACTERIAL ACTIVITY OF SOME 1-BENZYLBENZIMIDAZOLE DERIVATIVES

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Introduction

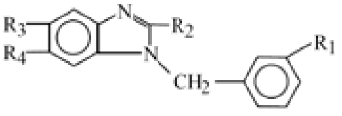
Quantitative structure-activity relationships (QSAR) represent an attempt to correlate structural or property descriptors of compounds with activities. These physicochemical descriptors, which include parameters to account for hydrophobicity, topology, electronic properties, and steric effects, are determined empirically or, more recently, by computational methods. Activities used in QSAR include chemical measurements and biological assays. For example, biological activity can be expressed quantitatively as in the concentration of a substance required to give a certain biological response. Additionally, when physiochemical properties or structures are expressed by numbers, one can form a mathematical relationship, or quantitative structure-activity relationship, between the two. The mathematical expression can then be used to predict the biological response of other chemical structures. QSAR currently are being applied in many disciplines, with many pertaining to drug design and environmental risk assessment. Using QSAR, it can be obtained an estimate of the activity of a chemical from its molecular structure only.[1–7] QSAR offers the possibility for screening a large number of chemicals in a short time and with low cost.

The benzimidazole nucleus, which is a useful structure for further research and for development of new pharmaceutical molecules, has received much attention in last decade. Because of their antimicrobial activities, new benzimidazoles have been synthesized and investigated for medical applications. Position and type of the substituents on the benzimidazole ring are responsible for the variety of biological activity. Many derivatives of benzimidazole are well known as antibacterial agents, as well as this class of compounds have been found to show antimicrobial activities against Gram-positive and Gram-negative bacteria, primarily because of the potential bio-activity of benzimidazole-based ligands.[8–15] So, the incorporation of the imidazole and benzimidazole nuclei is an important synthetic strategy in drug discovery.

Synthesis of benzimidazoles fused to another heterocyclic ring has attracted wide spread attention due to their diverse application as antioxidant[16,17], antifungal[18], antitubercular[19], anticancer[20,21] and antiallergic drugs[22]. Various benzimidazoles are also effective inhibitors of the growth of HIV-virus[23,24].

In view of above and in continuation of our studies on inhibitory activities of benzimidazole derivatives[4–7,11–14], as well as on correlation of molecular properties with activity, the objective of this investigations was to study the usefulness of QSAR in the prediction of the antibacterial activity of benzimidazole derivatives against Gram-negative bacteria *Pseudomonas aeruginosa*. The multiple linear regression (MLR) models have been developed as a mathematical equations which relate chemical structure to the inhibitory activity.

Table I
Structural formulae of the compounds



Cmpd	R_1	R_2	R_3	R_4
1	CH_3	H	CH_3	CH_3
2	Cl	H	CH_3	CH_3
3	F	H	CH_3	CH_3
4	OCH_3	H	CH_3	CH_3
5	CH_3	NH_2	H	H
6	Cl	NH_2	H	H
7	F	NH_2	H	H
8	OCH_3	NH_2	H	H
9	CH_3	NH_2	CH_3	CH_3
10	Cl	NH_2	CH_3	CH_3
11	F	NH_2	CH_3	CH_3
12	OCH_3	NH_2	CH_3	CH_3

Experimental

The investigated compounds (Table I) were synthesized by procedure described earlier[25].

Antibacterial Investigations

All the benzimidazole derivatives were evaluated for their *in vitro* growth inhibitory activity against bacteria *Pseudomonas aeruginosa* (ATCC 27853). Antibacterial activities of the compounds were tested by the disc-diffusion method under standard conditions using Mueller-Hinton agar medium as described by NCCLS[26].

The investigated isolate of bacteria was seeded in the tubes with nutrient broth (NB). It was taken 1 ml of seeded NB and homogenized in tubes with 9 ml of melted (45 °C) nutrient agar (NA). The homogenous suspension was poured out in Petri dishes. The discs of filter paper (diameter 5 mm) were ranged on cool medium. After cooling on formed solid medium, 2×10^{-5} dm^3 of the investigated compounds were placed by micropipette. After incubation of 24 hours in thermostat at 25–27 °C, inhibition (sterile) zone diameters (including disc) were measured (in mm). Inhibition zone diameter over 8 mm indicates the tested compound is active against microorganism. Every test was done in three replications.

Minimum inhibitory concentration (MIC) was performed by the agar dilution method according to guidelines established by the NCCLS standard M7-A5[27].

The MIC of tested benzimidazoles is defined as the lowest concentration of the compound at which no growth of the strain as observed in a period of time and under specified experimental conditions. Stock solutions of the compounds were prepared in dimethylformamide (DMF). Further dilutions were performed with distilled water. The concentration range of the compounds tested was between 6.25–125 μg ml^{-1}. The inoculated plates were than incubated at 35 °C for 16–20 h. A control using DMF without any test compound was included for each organisms. There was no inhibitory activity in the wells containing only DMF. The MIC values of the benzimidazoles tested were obtained as μg ml^{-1}. In order to classify the antibacterial activity, we established comparisons with antibacterial agents currently employed in therapeutic treatment. The MICs were compared with Ampicillin and Gentamicin which were screened under similar conditions as reference drugs.

Molecular Modeling

All molecular modeling studies were performed by using HyperChem 7.5 software (HyperCube Inc, Version 7.5) running on P-III processor[28]. HyperChem includes a model builder that turns a rough 2Dsketch of a molecule into 3D. The created 3-D models were cleaned up and subjected to energy minimization using molecular mechanics (MM$_2$). The minimization is executed until the root mean square (RMS) gradient value reaches a value smaller than 0.1 kcal mol^{-1}·A. The Austin Model-1 (AM-1) method was used for re-optimization until the RMS gradient attains a value smaller than 0.0001 kcal mol^{-1}·A using MOPAC. The lowest energy structure was used for each molecule to calculate molecular descriptors.

Descriptors Generation

The numerical descriptors are responsible for encoding important features of the structure of the molecules and can be categorized as electronic, geometric, hydrophobic, and topological characters. Descriptors were calculated for each compound in the data set, using the software HyperChem[28], Dragon[29], and CS Chem Office Software version 7.0(ref.[30]). Since there was a large number of desriptors for each compound, Pearson's correlation matrix was used as a qualitative model, in order to select the suitable descriptors for MLR analysis. The eight descriptors which were showing maximum correlation with inhibitory activity were chosen for further evaluation. The values of descriptors selected for MLR model are presented in Table II (molar refractivity (*MR*), polarizability (*P*), molar volume (*MV*), hydration energy (*HE*), total energy (*TE*), surface area grid (*SAG*), and partition coefficient (log*P*)).

Statistical Methods

The complete regression analysis were carried out by PASS 2005, GESS 2006, NCSS Statistical Softwares[31]. The Elimination Selection Stepwise regression (ES-SWR) algorithm was used to select the most appropriate descriptors. ES-SWR is a popular stepwise technique that combines Forward Selection (FS-SWR) and Backward Elimination (BE-SWR). It is basically a forward selection approach, but at each step it considers the possibility of deleting a variable as in the backward elimination approach, provided that the number of model variables is greater than 2.

Table II
Values of molecular descriptors used in the regression analysis

Cmpd	MR	P	MV	HE
1	87.25	30.78	811.60	–1.00
2	87.69	30.87	804.81	–1.86
3	83.10	28.85	777.39	–2.23
4	89.34	31.42	841.79	–3.68
5	81.56	28.46	744.54	–6.39
6	81.99	28.55	736.31	–7.16
7	77.40	26.53	704.51	–7.31
8	83.65	29.1	771.05	–8.95
9	90.12	32.13	844.29	–4.26
10	89.24	32.22	833.57	–5.38
11	85.97	30.2	802.16	–5.21
12	92.27	32.77	871.99	–6.83
	TE	DM	SAG	log*P*
1	27.17	3.98	490.32	4.24
2	26.87	3.974	429.54	4.31
3	27.02	3.976	477.14	3.91
4	28.92	3.978	507.17	3.63
5	26.35	4.464	458.41	3.44
6	26.03	4.427	450.08	3.52
7	26.07	4.428	433.17	3.12
8	27.94	4.429	470.97	2.83
9	27.67	4.428	503.71	4.42
10	27.18	4.409	498.16	4.49
11	27.46	4.410	480.77	4.09
12	29.38	4.406	517.74	3.80

Results

The substituted benzimidazoles were first evaluated for *in vitro* antibacterial activity against Gram-negative bacteria *Pseudomonas aeruginosa*. The values of MIC are summarized in Table III. The screening results reveal that all the compounds exhibited *in vitro* activity against the tested strain.

In an effort to determine the role of structural features, QSAR study was undertaken. A set of benzimidazoles consisting of 12 molecules was used for multilinear regression model generation.

The reference drugs were not included in model generation as they belong to a different structural series. Different physicochemical, steric, electronic, and structural molecular descriptors were used as independent variables and were correlated with antibacterial activity.

Developing a general model requires a diverse set of data, and, thereby a large number of descriptors have to be considered. Desriptors are numerical values that encode diff-

Table III
Antibacterial screening summary

Compound	$\log 1/c_{MIC}$ exp.	$\log 1/c_{MIC}$ predict.	Residuals
1	4.602	4.636	–0.034
2	4.637	4.636	0.001
3	4.609	4.518	0.091
4	4.328	4.373	–0.045
5	4.278	4.213	0.065
6	4.314	4.235	0.079
7	3.981	3.993	–0.012
8	3.704	3.732	–0.028
9	4.627	4.607	0.020
10	4.659	4.591	0.068
11	4.333	4.433	–0.100
12	4.352	4.372	–0.020
Ampicillin	4.446	–	–
Gentamicin	5.787	–	–

erent structural features of the molecules. Selection of a set of appropriate descriptors from a large number of them requires a method, which is able to discriminate between the parameters. Pearson's correlation matrix has been performed on all descriptors by using NCSS Statistical Software. The analysis of the matrix revealed 8 descriptors for the development of MLR model (Table II).

Mathematical models were formed by a stepwise addition of terms. A delition process was then employed where each variable in the model was held out in turn and using the remaining parameters models were generated. Each descriptor was chosen as input for the software package of NCSS and then the stepwise addition method implemented in the software was used for choosing the descriptors contributing to the antibacterial activity of benzimidazole derivatives.

The partition coefficient (log*P*) tends to correlate with antibacterial activity exclusively and the best monoparametric model was found to be the following:

$$\log 1/c_{MIC} = 0.518 \log P + 2.391 \qquad (1)$$
$$n = 12;\ r = 0.932;\ s = 0.085;\ F = 66.43$$

Addition of HE as an additional parameter to log*P*, increased the correlation coefficient from 0.932 to 0.951 (Eq.(2)).

$$\log 1/c_{MIC} = 0.415 \log P + 0.031\ \mathrm{HE} + 2.940 \qquad (2)$$
$$n = 12;\ r = 0.951;\ s = 0.010;\ F = 42.21$$

It should be noted that the addition of other parameters to log*P* and HE does not significantly improved the correlation coefficients. However, if quadratic values of descriptors were included in the stepwise multiple regression procedure, the best correlation was found as depicted in Eq.(3).

$$\log 1/c_{MIC} = -0.201 \log P^2 + 1.930 \log P - 0.001\ \mathrm{HE}^2 + 0.014\ \mathrm{HE} + 0.082 \qquad (2)$$
$$n = 12;\ r = 0.967;\ s = 0.008;\ F = 25.41$$

For the testing the validity of the predictive power of selected MLR model (3) the leave-one-out technique (LOO technique) was used. The developed model was validated by the calculation of following statistical parameters: predicted residual sum of squares (PRESS), total sum of squares deviation (SSY), cross-validated correlation coefficient (r^2_{CV}), and adjusted correlation coefficient (r^2_{adj}) (Table IV).

Table IV
Cross-validation parameters

Eq.(2)	
PRESS	0.151
SSY	0.942
PRESS/SSY	0.160
r^2_{CV}	0.840
r^2_{adj}	0.900

PRESS is an important cross-validation parameter as it is a good approximation of the real predictive error of the models. Its value being less than SSY points out that the model predicts better than chance and can be considered statistically significant. Thus, in view of this, model 3 is statistically significant. Further, to be a reasonable QSAR model, PRESS/SSY ratio should be lesser than 0.4. The data presented in Table IV indicate that for the developed model this ratio is 0.160. The high value of r^2_{CV} and r^2_{adj} are the essential criteria for qualifying the QSAR model 3 as the best one.

To confirm the predictive power of a model the inhibitory activitiy of 12 molecules included in the study was calculated by the model 3. The data presented in Table III show that the observed and the estimated activities are very close to each other. It indicates the good predictability of the established model 3. Fig. 1. shows the plots of linear regression predicted *versus* experimental values of the antibacterial activity of benzimidazoles investigated. To investigate the existence of a systemic error in developing the QSAR models, the residuals

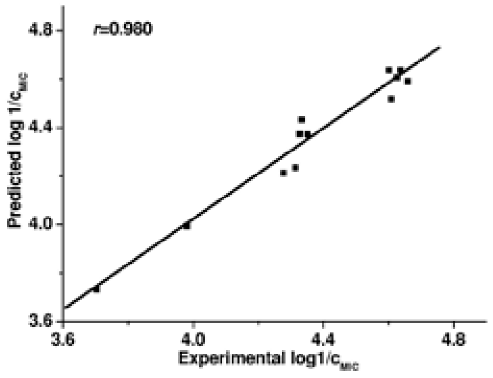


Fig. 1. **Plots of predicted versus experimentally observed inhibitory activity against *Pseudomonas aeruginosa***

of predicted values of inhibitory activity were plotted against the experimental values in Fig. 2. The propagation of the residuals on both sides of zero indicates that no systemic error exists in the development of regression models as suggested by Jalali-Heravi and Kyani[32]. It indicates that these models can be successfully applied to predict the antibacterial activity of these class of molecules.

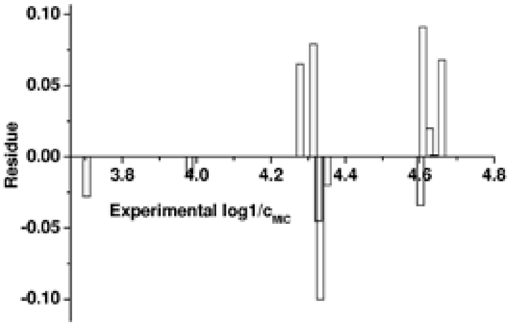


Fig. 2. **Plot of residual values against the experimentally observed log 1/c_{MIC} values**

Conclusions

From the results and discussion made above, we conclude that the 1-benzylbenzimidazole derivatives are effective in vitro against the Gram-negative bacteria Pseudomonas aeruginosa. Molecular modeling and QSAR analysis were performed to find the quantitative effects of the molecular structure of the compounds on their antibacterial activity. The problem of selecting the appropriate descriptor as input for theoretical model was overcome by Pearson correlation matrix, which can be used as a tool for identifying the appropriate descriptors when a large number of them with different features are available. An accurate mathematical model was developed for predicting the inhibitory activity of some benzimidazole derivatives. The validity of the model has been established by the determination of suitable statistical parameters. The established model was used to predict inhibitory activity of the benzimidazoles investigated and close agreement between experimental and predicted values was obtained. The low residual activity and high cross-validated r^2 values (r^2_{CV}) observed indicated the predictive ability of the developed QSAR model. It indicates that the antibacterial activity of series of 1-benyzlbenzimidazole derivatives can be successfully modeled using different molecular descriptors.

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P83 ANTIMICROBIAL INVESTIGATIONS OF NICKEL(II) COMPLEXES WITH SOME 1-BENZYLBENZIMIDAZOLE DERIVATIVES

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Introduction

Physiological activity and commercial applications of many benzimidazole derivatives have received much attention. Benzimidazole and its derivatives have different activities as they can act as bacteriostats or bactericides and fungicides.[1–10] Extensive biochemical and pharmacological activities have confirmed that these molecules are effective against RNA viruses and inhibit the formation of virus induced RNA polymerase, thereby preventing or retarding RNA synthesis various strains of microorganisms.[11–14] Antimicrobial activity of these class of compounds against *Helicobacter pylori*[15] and oral *Streptococci*[16] was also reported. Synthesis of benzimidazoles fused to another heterocyclic ring has attracted wide spread attention due to their diverse application as antioxidant[17,18], antifungal[19], antitubercular[20], anticancer[21,22], and antiallergic drugs[23]. Also, some of these compounds exhibited anti-HIV activity[24,25].

The complexes of transition metals with benzimidazole and related ligands have been extensively studied as models of some important biological molecules[26]. It was found that the complexes showed larger antimicrobial activitiy than the ligands applied alone.

Following our studies of the reactivity of benzimidazole derivatives with metallic halides[5–8], we evaluated the antibacterial activity of this type of complexes in this study. We report *in vitro* inhibitory activities of 1-benzylbenzimidazoles and their nickel (II) complexes against three gram-positive bacterial strains: *Bacillus cereus, Staphylococcus aureus* and *Sarcina lutea,* one gram-negative isolate: *Pseudomonas aeruginosa*.

Experimental

In the present paper we evaluated the antibacterial activity of nickel (II) complexes with the following starting ligands: 1-(3-chlorobenzyl)-2-aminobenzimidazole (L^1), 1-(3-fluoro-benzyl)-2-aminobenzimidazole (L^2), 1-(3-chlorobenzyl)-2-amino-5,6-dimethylbenzimidazole (L^3), 1-(3-fluorobenzyl)-2-amino-5,6-dimethylbenzimidazole (L^4) and 1-(3-methylbenzyl)-2-amino-5,6-dimethylbenzimidazole (L^5).

All the ligands were synthesized by Vlaović et al. according to a procedure described earlier.[27] Nickel (II) complexes were prepared following the same procedure described in our previous paper[7].

Antibacterial activity

All the nickel (II) complexes were tested for their in vitro growth inhibitory activity against Bacillus cereus ATCC 10876, Staphylococcus aureus ATCC 25923, Sarcina lutea ATCC 9341 and Pseudomonas aeruginosa ATCC 27853.

Antibacterial activities of the complexes were tested by the disc-diffusion method under standard conditions using Mueller-Hinton agar medium as described by NCCLS[28]. Each of the investigated isolates of bacteria was seeded in the tubes with nutrient broth (NB). 1 ml of seeded NB was homogenized in tubes with 9 ml of melted (45 °C) nutrient agar (NA). The homogenous suspension was poured out in Petri dishes. The discs of filter paper (diameter 5 mm) were ranged on cool medium. After cooling on formed solid medium, 0.02 ml of the investigated compounds ($\gamma = 1000$ µg ml^{-1}) were placed by micropipette. After incubation of 24 hours in thermostat at 25–27 °C, inhibition (sterile) zone diameters (including disc) were measured (in mm). Inhibition zone diameter over 8 mm indicates the tested compound is active against microorganisms. Every test was done in three replications. Antimicrobial activities of the free ligands against the same bacteria were tested in our previous studies[8].

Minimum inhibitory concentration (MIC) was performed by the agar dilution method according to guidelines established by the NCCLS standard M7-A5[29]. MIC was described as the lowest concentration of the compound that visibly inhibited colony's growth. Stock solutions of the compounds were prepared in dimethylformamide (DMF). Further dilutions were performed with distilled water. The concentration range of the compounds tested was between 60–750 µg ml^{-1} in two-fold dilution steps. The inoculated plates were than incubated at 35 °C for 16–20 h. A control using DMF without any test complex was included for each organisms. It was determined that the solvent had no activity against any of the test microorganisms.

Results

The results of antibacterial studies of the nickel (II) complexes with two series of 1-benzylbenzimidazole derivatives tested by the agar disc-diffusion method are summarized in Table I.

As can be seen from the data, all the investigated compounds displayed *in vitro* inhibitory activity against very persistent bacteria. The complexes investigated were found

Table I
In vitro antibacterial activity of complexes at a concentration of 1,000 µg ml^{-1}

Complex	Inhibition zone diameter [mm]			
	P.aeruginosa	*B. cereus*	*S. aureus*	*S. lutea*
$Ni(L^1)_2Cl_2$	22	26	26	29
$NI(L^2)_2Cl_2$	17	24	24	25
$Ni(L^3)_2Cl_2$	9	17	17	18
$Ni(L^4)_2Cl_2$	8	16	17	17
$Ni(L^5)_2Cl_2$	5	16	17	16

to be more active against Gram-positive than Gram-negative bacteria (*Pseudomonas aeruginosa*).

In the case of Gram-negative isolate only complexes of 2-aminobenzimidazole derivatives exhibited significant inhibitory activity. Nickel (II) complexes of L^3, L^4 and L^5 were slightly active against the *Pseudomonas aeruginosa*. In the case of *Bacillus cereus* and *Staphylococcus aureus* nickel (II) complexes of ligands L^1 and L^2 also express higher activity than another complexes. Gram-positive bacteria *Sarcina lutea* was persistent in all investigated cases, too. Nickel (II) complexes containing L^1 and L^2 were very highly or highly active, respectively. On the other hand, complexes of L^3, L^4 and L^5 were moderately active against the same bacteria.

In the next phase, MIC of the tested compounds was performed by the agar dilution method. From the results presented in Table II, it is seen that nickel(II) complex containing L^1 was active against *Pseudomonas aeruginosa* with a MIC value of 250 μg ml^{-1}, whilst $Ni(L^2)_2Cl_2$ was less toxic. However, $Ni(L^3)_2Cl_2$ and $Ni(L^4)_2Cl_2$ were same active, but complex of L^5 has the low activity against the same bacteria.

In the case of *Bacillus cereus* and *Staphylococcus aureus* complexes containing 2-aminobenzimidazole derivatives as ligands were more active (MIC = 125 μg ml^{-1}) than complexes of 2-amino-5,6-dimethylbenzimidazole derivatives. $Ni(L^3)_2Cl_2$ was equally active as $Ni(L^4)_2Cl_2$ with higher MIC value of 250 μg ml^{-1} against the same bacteria, whilst complex containing L^5 expressed MIC of 500 μg ml^{-1}.

On the other hand, all the complexes were more active against *Sarcina lutea.* The complex of L^3 with a MIC value of 125 μg ml^{-1} has the same activity as $Ni(L^4)_2Cl_2$, but complexes of 2-aminobenzimidazole derivatives were the most active. $Ni(L^5)_2Cl_2$ has the lowest activity against these two Gram-positive bacteria.

Comparing the activities of the tested complexes it was found that 1-substituted-2-aminobenzimidazole derivatives (L^1, L^2) formed the nickel (II) complexes which were more active than complexes of 1-substituted-2-amino-5,6-dimethylbenzimidazoles (L^3, L^4, L^5). Consequently, it is suggested that methyl groups at the 5 or 6 position decreases the general inhibitory activity of the tested complexes. Also, antibacterial results shows that if the benzimidazole nucleus was substituted with a 3-chlorobenzyl group at the N1 atom, the antibacterial activity was increased.

The differences found in the activities of the nickel (II) complexes and the non-complexed ligands obtained in our previous investigations[8], suggest that the coordinated Ni(II) may play a significant role in the antibacterial potency. A possible explanation may be offers by the chelation theory stating a relationship between decreasing polarizability of the metal and increasing lipophilicity of the complexes. This property is now seen as an important parameters related to membrane permeation in biological system. Many of the processes of drug disposition depend on the ability or inability to cross membranes and hence there is a high correlation with measures of lipophilicity. Moreover, many of the proteins involved in drug disposition have hydrophobic binding sites further adding to the importance of lipophilicity. The latter might promote inhibitory activity.

Moreover, the results of this study revealing that the compounds tested displayed higher activity against the Gram-positive than the Gram-negative one bacteria, likely point to the relevance of the structure of the bacterial cell wall in the antimicrobial potency of the substances. It is prospective because the cell wall is essential to the survival of many bacteria and some antibiotics are able to kill bacteria by inhibiting a step in the synthesis of peptydoglycan. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids, but in contrast, Gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins. These differences in cell wall structure can produce differences in antibacterial susceptibility and some antibiotics can kill only Gram-positive bacteria and is ineffective against Gram-negative pathogens.

Table II
MIC tested of complexes

Complex	MIC [μg ml^{-1}]			
	P.aeruginosa	*B. cereus*	*S. aureus*	*S. lutea*
$Ni(L^1)_2Cl_2$	250	125	125	62.5
$Ni(L^2)_2Cl_2$	500	125	125	62.5
$Ni(L^3)_2Cl_2$	750	250	250	125
$Ni(L^4)_2Cl_2$	750	250	250	125
$Ni(L^5)_2Cl_2$	1000	500	500	250

Conclusions

The antibacterial activity of the nickel (II) complexes with two series of 1-benzylbenzimidazole derivatives was tested against very persistent microorganisms: *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* and *Sarcina lutea*. All the complexes displayed *in vitro* inhibitory activity, but 1-substituted-2-aminobenzimidazole derivatives formed the nickel (II) complexes which were more active than complexes of 1-substituted-2-amino-5,6-dimethyl-benzimidazoles. The basic antibacterial activity of the benzimidazoles was produced by the presence of an amino group at the position 2 of the benzimidazole ring. Methyl groups at the 5 or 6 position decreases the general inhibitory activity of the relevant benzimidazoles. Also, the results indicated that tested complexes were more active against Gram-positive than Gram-negative bacteria. It may be concluded that the antibacterial activity of the compounds is related to cell wall structure of the bacteria. It is possible because the cell wall is essential to the survival of many bacteria and some antibiotics are able to kill bacteria by inhibiting a step in the synthesis of peptidoglycan.

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P84 CHEESE SPECIES IDENTIFICATION BY MULTIVARIATE ANALYSIS OF ELEMENTAL DATA

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Introduction

To guarantee cheeses' authenticity, responsible food control authorities need to dispose of reliable analytical traceability techniques that enable to check whether the products are correctly described and labelled, or not.

In the recent years, extensive literature data on the methods of analysis applicable to the detection of mixtures of milk from different species either in raw or even in processed milk products are available. A large number of methods are applicable to detect the presence of different milk species in cheeses, as well.[1–4]

Multivariate analysis represents valuable tool, making possible the categorization of different food samples via the consideration of many variables that can be measured, often in a single analytical level. In addition, multivariate analysis of chromatographic, electrophoretic or elemental data is considered to be the powerful method for the discrimination of cheeses based on the different geographical origin, varieties and quality or for the consideration and monitoring of cheese maturation.

As follows from previously published data, the concentration ranges of some elements in milk and cheese are strongly dependent on animal species and feeding, season of sample collection, environmental conditions and manufacturing processes, as well.[5–11] Coni et al. revealed, that the concentration of selected trace elements (e. g., of Al, Ba, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Pt, Sr and Zn) differ significantly for sheep and goat milks and for their related products[5]. The mineral composition of ewes', cows', and goats' milk and of samples of different pure-milk cheeses made from them was analysed by Martin-Hernandéz using the stepwise discriminant analysis, reaching the 76 % correct classification of cheeses according to milk species[6]. Another statistical study dealt with the comparison of metal composition of different types of milks based on the presence of Se, Fe, Cu, Zn, Na, K, Ca, Mg, brought 98% correct classification of the samples for each type of milk[7]. Discriminant analyses of some elemental data were successfully used by Koreňovská et al. for the identification of cheeses' region of origin.[8–10] Using the same approach, 94.1 % of Mahon cheese samples were correctly classified into traditional and industrial groups, and 89.7 % of samples into fresh, half-ripened, ripened and old-ripened groups. [11]

As follows from the above-presented data, the authentification of cheeses using the elemental markers requires reliable data, obtained by analytical methods of high specificity and sensitivity, e. g. by atomic absorption spectrometry (AAS).

In this paper, the identification and discrimination of cows', sheeps' and goats' cheeses from different Slovak regions using the selective elemental data and pattern recognition analysis is presented.

Experimental

Samples Characterisation

Commercially available samples of 54 cow hard cheeses of Emmental and Edam type, 116 sheep (40 hard and 76 bryndza type) and 20 goat cheeses, all of the Slovak origin were analyzed to the content of selected elemental markers (Ba, Cr, Cu, Hg, Mg, Mn, Ni and V) by AAS. These markers were chosen on the base of geochemical characterization of Slovak regions[12].

Reagents

All chemicals were of analytical grade. Nitric acid of suprapure quality and stock solutions of respective metal (Ba, Cr, Cu, Hg, Mg, Mn, Ni, and V at the concentration of 1.00 $g\,dm^{-3}$, each) were purchased from Merck (Darmstadt, Germany). Lanthanum chloride (5%) used as ionic suppressor was delivered from SMÚ (Bratislava, Slovakia).

AAS Conditions

Respective cheese sample (0.5 g) was digested in a mixture of 4 ml of 65 % HNO_3 and 0.5 ml H_2O_2, using the Milestone MLS 1200 MEGA (Sorisole, Italy) microwave digestion system.

Perkin Elmer 4100 atomic absorption spectrometer (Norwalk, CT, USA) equipped with a deuterium lamp background-correction system and HGA 700 graphite tube atomizer with pyrolytically coated graphite tubes and flame was used. The presence and concentration of Mg and Mn metals were determined from atomic spectrometry measurements, using an air/acetylene flame. Metal elements of Ba, Cr, Cu, Ni, and V were detected on graphite tube atomizer, as previously described by Koreňovská and Suhaj.[8–10]

The accuracy of results was verified by standard addition method. The accuracy of method used for metal elements determination was estimated by means of two reference materials (NCS ZC 73008, rice and NCS ZC 73013, spinage) to be 98–125 %. Recovery of the method was assessed by the analysis of fortified cheese samples; mean recoveries of elements reached 96–109 %. Finally, the combined standard uncertainty was 5.3–14 %.

Determination of Hg

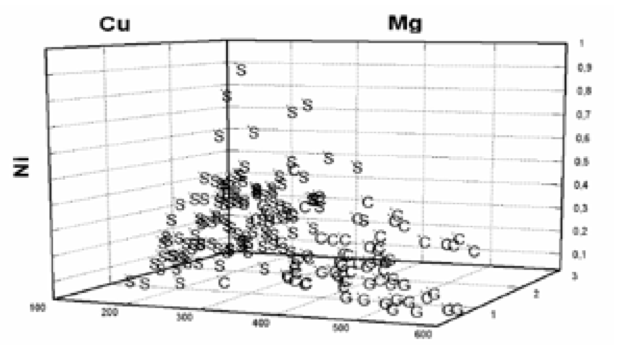
For the determination of mercury in cheeses, single – purpose mercury analyser AMA 254 (Altech, Prague, Czech Republic) was used. The BCR-150 skim milk powder (Brussels, Belgium) served as reference material.[8–10]

Statistics

Multivariate statistical methods, e.g., cluster analysis, factor analysis, and canonical discriminant analysis, were performed using the Unistat® software.

Results

Fig. 1. shows the preliminary predisposition of cows', sheeps' and goats' cheese samples under the study to natural grouping, obtained by hierarchical cluster analysis of determined elemental markers. Clusters were constructed using Ni, Cu and Mg variables, as they were found to have the most discriminating impact on cheeses distinction. As follows from data presented, samples are grouped into two major clusters: the first one correspond mostly to sheeps' cheeses and the second to cow and goat cheese samples, respectively.



Fig, 1. **Cluster graph of sheeps' (S), cows' (C) and goats' (G) cheeses, constructed using the Ni, Cu and Mg content as variables. Distance measure: Block; Method: Median**

Although there is a clear differentiation tendency, both groups of clusters contain some incorrectly classified samples. In order to achieve the better differentiation of examined cheese species, factor analysis using the principal component factoring with the varimax-rotation was performed to describe the main variations between the Ba, Cr, Cu, Hg, Mg, Mn, Ni and V content.

Using this approach, mathematical model explaining the mutual correlation between a large set of variables was constructed. As we found, first factor, related mainly to the content of Hg, Mg and Ni, explain 26 % of the total markers' variation; the second one (18.5 %) is strongly influenced by Cu and Mn variability, and the last factor (15.4 %) by the variability of Ba and V. These three factors sufficiently explain more than 60 % of overall elemental data variations. Visualisation of data obtained (Fig. 2.) suggest, that the most effective differentiation of cows', sheeps' and goats' cheeses can be achieved following the first factor axis.

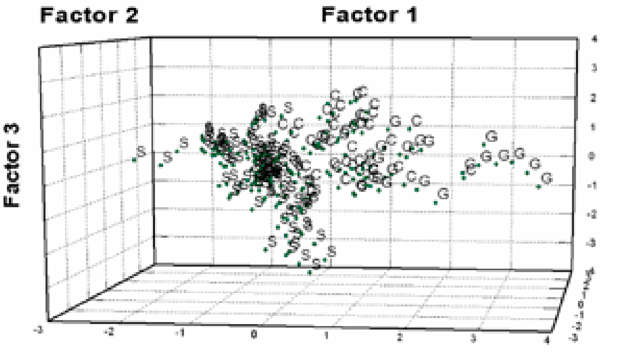


Fig. 2. **Principal component factoring of sheeps' (S), cows' (C) and goats' (G) cheeses; Rotation: Varimax; Variables selected: Ba, Cr, Cu, Hg, Mg, Mn, Ni and V**

Results of canonical discriminant analysis (Fig. 3.) demonstrated very high potential to distinguish the differences among the cows', sheeps' and goats' cheeses.

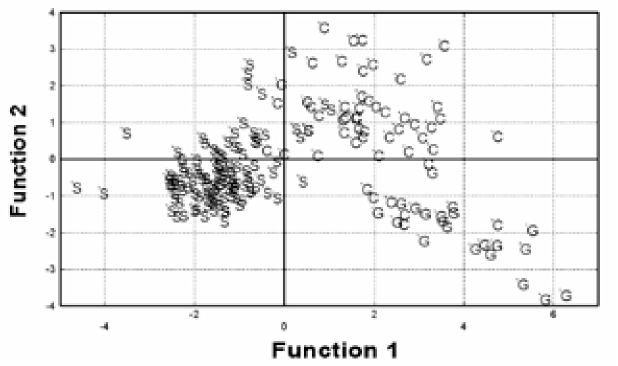


Fig. 3. **Canonical discriminant analysis of sheeps' (S), cows' (C) and goats' (G) cheeses; Plot of discriminant score; Variables selected: Ba, Cr, Cu, Hg, Mg, Mn, Ni and V**

Discriminant functions correctly classified 92.6 % of all samples according to the species' origin. First discriminant function reveals the highest canonical correlation (89.5 %) and explains up to 83.6 % of the total variance. As follows from the values of standardized coefficients, Mg, Mn and Ni markers most significantly influence the discrimination at first function, whereas for the second discriminant function, the presence of Cu and Hg is essential.

In addition, K^{th}-neighbour discriminant analysis provided 100 % correctness of samples classification at $k = 1$; and 91.1 % at $k = 2$. Stepwise discriminant analysis, which sorts the used markers according to their descending influence on the discrimination, gives the following order: Mg, Cu, Ni, Mn, Hg, V, Ba and Cr.

Some of the markers used in this work, e.g. Cu, Mg, and Mn, were previously successfully used for cheeses' origin authentification also by other authors.[5–7,13,14] Significant discrimination of cheeses' species presented in this work can be effectively explained by the geochemical differences in sheeps', cows' and goats' pasture soils. Sheeps' pastures are located in the mid-mountain regions whereas cows' and goats' ones are to be found predominantly in lowland agricultural areas. Thus, the content of minerals and other trace elements in feeding diet vary significantly, subsequently influencing their content in cattle milk and milk products.

Conclusions

Multivariate statistical methods of elemental markers analyses involving both the principal factor analysis and canonical discriminant analysis represent effective tools to discriminate Slovak commercial cows', sheeps' and goats' cheeses and to identify their origin. Results obtained enable the development of simple and rapid protocols for cheeses' classification and quality ensurance.

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P85 OPTIMALIZATION OF METHOD FOR QUANTIFICATION OF *STREPTOCOCCUS* MUTANS TO DENTAL MATERIALS

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Introduction

The attachment of certain microorganisms to specific surfaces in the human oral cavity and the resulting formation of dental plaque on teeth and dental materials are primary causes for oral diseases such as denture stomatitis, gingival inflammation, and secondary caries, which may consequently lead to unhealthy complications[1]. Secondary caries is the most frequent reason for the replacement of restorative materials including resin composites. That is reason, why antimicrobial agents are incorporated and bacterial adhesion has to be measured.

In the literature, several methods for quantification of deposited oral microorganisms were described, but a lot of them could be characterized as time, materials, instruments and financially – consuming.

The amount of sorbed bacteria could be expressed using radio-labelled microorganisms. Cells were radio-labelled using [3H] – thymidine[2–7], [3H] – uridine or [35S] – methionine. The amount of adsorbed bacteria was measured in a scintillation counter and was determined as radioactive counts per minute (CPM) of the labelled bacteria after washing them with buffer (KCl) to remove unbound bacteria.

Other tests for microorganisms quantification were carried on examine dental materials. Discs from this dental materials were inserted in petri dishes or tubes that contained cell suspension and than they were incubated for 24 hours. After incubation discs were removed and rinsed with distilled water or PBS. Adherent bacteria were fixed with methanol or glutaraldehyd and stained with acridine orange, crystal violet or modified Gram stain. Quantitative analysis was performed using a fluorescence microscope. The number of adherent cells in several random fields was counted on each sample and bacterial adhesion was expressed as percentage area coverage[8].

These methods were used the most often, but except them, other minor methods were tested. Capopreso at.al.[9] analysed bacterial adhesion to dental alloys spectrophotometrically in a microplate reader at 570 nm. The bacterial adhesion of each specimen was quantified as the ratio between the optical density at 570 nm and the surface area of the specimen. Blunden[9], Duskova[10,11] expressed the amount of deposited microorganisms as percentage weight gain. Wu-Yuan[12] and Wilbershausen[13] examined the attachment of oral bacteria by SEM. For SEM, the samples were fixed in formaldehyd or glutaraldehyd and dehydrated through a graded series of aceton or ethanol. Boeckh[14] placed bacterial suspensions in conical cavities within the material and after incubation, the suspensions were removed form the restoratives and the numbers of viable bacteria were counted.

F. Ozer at al. used for testing antibacterial activity Tooth cavity model. They prepared three cylindrical cavities in the flat surface of human extracted tooth. Cell suspension and brain heart infusion (BHI) broth were put in cavities and incubated for 24 h at 37 ºC. The number of *S. mutans* recovered was determined by the classical bacterial counting method using 5% sheep blood agar[8].

Experimental

The spectrophotometric Biuret method was used to quantified amount of bacteria *S. mutans* adhering to the polymer surface. In order to optimise Biuret method the composition of liquid culture medium, the amount of bacteria suspension used for the samples inoculation, the samples incubation time and methods for the bacteria releasing from the samples were tested.

Biuret Method

Under alkaline conditions substances containing two or more peptide bonds form a purple complex with copper salts in the reagent. Upon complexation, a violet color is observed. The absorbance of the Cu^{2+} protein complex is measured at 540 nm and compared to a standard curve. Bovine serum albumin is used as a standard. This method was employed for the quantification of the S. mutans in both culture medium and on the discs.

Biuret agent was prepared by dissolving 1.5 g $CuSO_4 \cdot 5H_2O$ + 6 g $C_4H_4O_6KNa \cdot 4H_2O$ in 500 ml H_2O. 300 ml 10% NaOH was added to the solution and distilled water to make 1,000 ml.

When studying the amount of bacteria in the suspension following procedure was used: 1 ml of the tested suspension was mixed with 4 ml of Biuret agent and 1 ml distilled water. Upon 10 seconds shaking period and 30 minute incubation, absorbance was measured against a blank at 540 nm.

To evaluate the amount of bacteria adhered to the disc, the bacteria had to be released from the disc. The samples were placed into 2 ml distilled water and ultrasonic bath were used. The time of ultrasonic bath was varied up to 8 minutes (2, 4, 6 and 8 minutes) in order to find out conditions when both the highest amount of bacteria is released and still no damage of the disc occurs. Further the procedure is same as in case of quantification of bacteria in suspension.

Dental Materials and Prepare of Samples

The discs were prepared from the commercially available microhybrid composites (Adoro, Ivoclar Vivadent), D3MA resin-based materials (Advanced Dental Materials) and ceramic materials (Ivoclar Vivadent) as reference material.

The material was placed in a metal mold between a layer of transparent foil and metal. It was cured in the Vectris curing machine for 20 minutes at 60 °C, 460 nm. After curing, the discs were removed from the mold and sterilized in autoclave.

Microbial Work

An oral cavity isolant *Streptococcus mutans* P2093 has been chosen as a tested microorganism. *S. mutans* is oral bacteria, that is assumed to be the main infander of the dental caries. The bacterial strain *S. mutans* used in this study was clinical isolates from child mouth (obtained from prof. MUDr. Martina Kukletová, CSc., Brno Masaryk University). The microorganism was stored in commercially available Petri dish with Blood agar IMA VLAC and IMA VLGP (Laboratory of Medical Microbiology, Třinec).

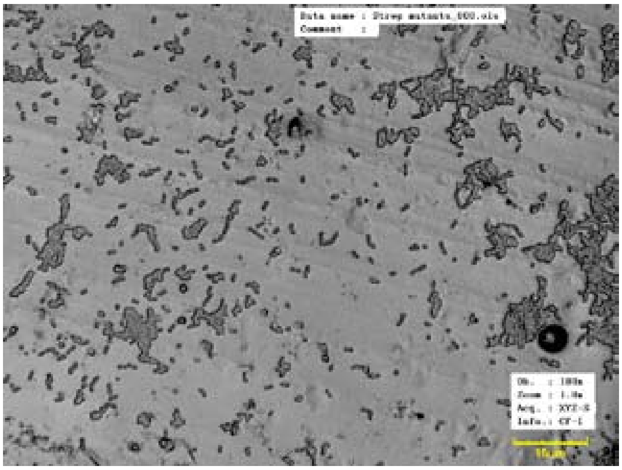

Fig. 1. ***S.mutans* growing on composite polymer disc**

The microorganisms were cultivated in liquid culture medium BHIB. In order to improve the bacteria growth a certain amount of further source of sugar was added into the medium. 0.1; 1; 5 and 10 wt% of sacharose and glucose were tested.

The samples were prepared by placing the sterilized disc in 5 ml BHIB medium in test tube and inoculating with bacteria suspension. The appropriate volume of bacteria suspension and incubation time had to be tested. First one was tested by inoculation of 0.2; 0.4; 0.6; 0.8 a 1.0 ml of bacteria suspension into 5 ml BHIB and evaluation of bacterial growth. The incubation time was varied up to 28 hours and appropriated incubation time was evaluated from the growing curve.

Results

First of all the appropriate composition of the culture medium was investigated. 0,1; 1; 5 and 10 wt% of sacharose and glucose were added to BHIB and the influence of additives on bacteria growth were evaluated see Fig. 2. Best growing results were obtained for the medium with 1 wt% of glucose.

When testing the influence of inoculated volume on bacteria growth, the volume was varied up to 1.0 ml. The results are presented in Table I. The highest absorbance was measured when using 0.2 ml of bacteira suspension. This volume was used for further testing.

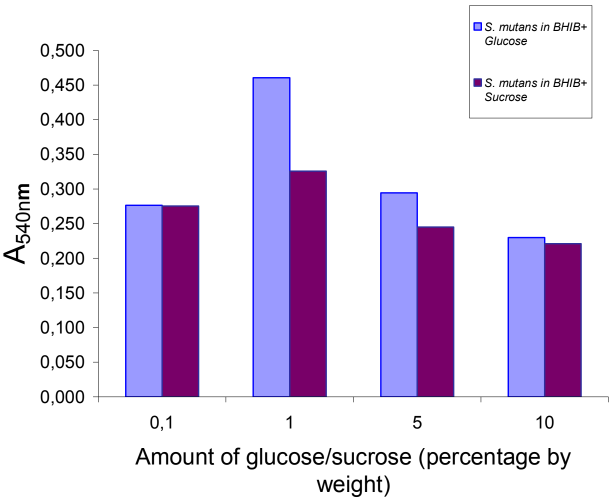

Fig. 2. ***S. mutans* growing in BHIB with glucose or sacharose**

Table I
Influence of suspension volume on S. mutans growth

Inoculated volume [ml]	Absorbance [540 nm]
0.2	0.290
0.4	0.288
0.6	0.285
0.8	0.286
1.0	0.270

Growing curve (Fig. 3.) presents influence of incubation time on the bacterial growth respectively on absorbance. It could be seen that the amount of bacteria increases with increasing of the incubation time until it reached maximal value. In our experiment the maximal absorbance resp. amount of bacteria was obtained for the samples incubated for 18 hours. When incubating the bacteria in medium for longer time the nutriens in medium are exhausted. This results in dying out of bacteria and thus decreasing of absorbance.

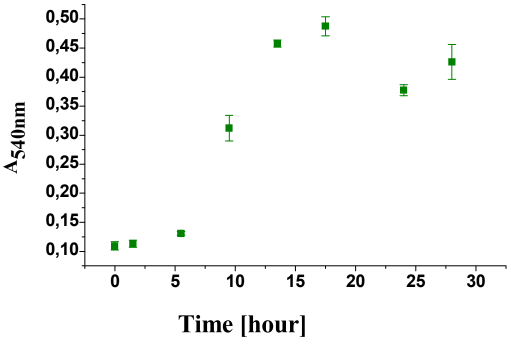

Fig. 3. ***S.mutans* growing curve**

Finally appropriated method for releasing the bacterial cells from disc had to be worked out. The ultrasonic bath was used and the working time was varied up to 8 minutes. The amount of released bacteria and effect of ultrasonic waves on the tested material was evaluated see Fig. 4. The best results were obtained for the working time of 6 minutes. Longer working time causes irreversible material damage while shorter treatment time did not released all bacteria.

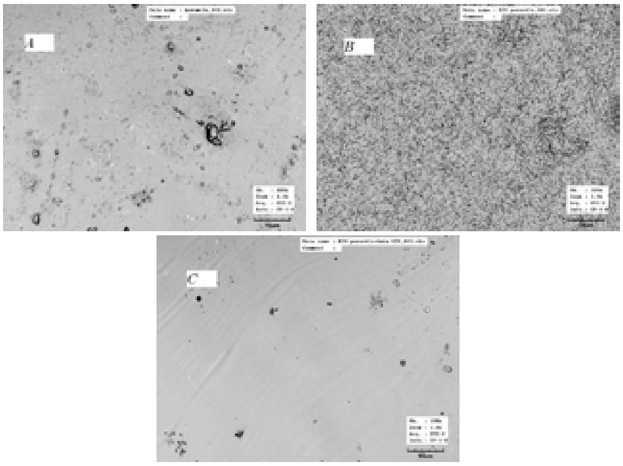

Fig. 4. **Ceramic materials: A = without *S. mutans*, B = with *S. mutans*, C = after ultrasonig bath for 6 minutes**

The main goal of this work was to find out appropriate method for the studying of the microbial adhesion to new dental materials. The spectrofotometric techniques based on Biuret reagent were employed. The method was optimised in order to fulfill requirements for further material testing.

According to our measurement the BHIB medium with 1 wt% of glucose gives the best growing results. Samples placed in BHIB-glucose medium was further inoculated with 0,2 ml of *S. mutans* suspension. This volume fulfil both requirement – low volume and good growing results. Further the appropriate incubation time was investigated. The highest absorbance was obtained for the samples incubated for 18 hours.

Finally time neccessary for releasing bacteria from the disc samples in ultrasonic bath was evaluated. The best results were obtained after 6 minutes. The highest amount of bactery was released while the disc still remained undamaged.

This optimised method will be employed for testing new dental materials. Our further studies will be focused on testing various antimicrobial additives that will improve microbial resistance of dental restorative materials.

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P86 BROMINATED FLAME RETARDANTS PROFILES IN BREAST MILK AND ADIPOSE TISSUE SAMPLES: CASE STUDY CONCERNED WITH CZECH POPULATION

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Introduction

Brominated flame retardants (BFRs) are chemicals that are extensively used in various polymers and especially in electronic equipment, plastics, electronic, textiles and other furniture[1]. Due to their persistence and bioavailability in combination with their large consumption and disposal, they are becoming emerging environmental contaminants. Now they have become ubiquitous in the environment and magnify in the biological tissue as seen in classical organochlorine compounds (OCs).

The Czech population body burden of halogenated persistent organic pollutants (POPs) is of a great concern due to incautious handling of hazardous chemicals in the past time. A monitoring of human matrices, including polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) has been employed for this purpose in the recent decade.

The main aim of the presented study was to investigate levels and profiles of (*i*) BFRs represented by PBDEs and HBCD and (*ii*) "classical" organochlorine contaminants occurring in the adipose tissue of people living in the Czech Republic and in human breast milk from Czech mothers. To our knowledge, this is the very first study reporting PBDEs levels in adipose tissue samples of Czech citizens together with similar studies conducted elsewhere in the world.

Experimental

Breast Milk

Samples of breast milk were obtained from 56 Czech women living in Olomouc region (located in the north-east part of the Czech Republic) during the autumn 2007 in the cooperation with the Gynaecological-maternity Clinic, Faculty Hospital in Olomouc. The age of mothers participating in this study ranged from 21 to 45 years (mean and median 29 year). The breast milk was expressed manually into the glass bottles and stored at –18 °C until analysis according to WHO methodology.[2] The analytical procedure used for analysis of human breast milk samples was described in detail in our earlier study.[3,4]

Human Adipose Tissue

Adipose fat tissue samples for determination of selected POPs were collected from patients (n = 98) who underwent a tumescent liposuction for aesthetic reasons. The mean age of the group was 35.5 years with a range of 17–60 years. The analytical procedure used for analysis of adipose tissue samples has been described in detail in our earlier study[5]; therefore, there is only a brief summary of procedure steps. Approximately 5 g of adipose fat tissue sample was homogenized with anhydrous sodium sulphate (20 g) and and extracted in a Soxhlet apparatus for 8 h using a hexane : dichlormethane mixture (1 : 1, v/v, 150 ml). The extract was rotary evaporated at 40 °C and residues were weighted for a lipid determination. An aliquot of isolated fat (cca 750 mg) was dissolved in 10 ml of an internal standard (PCB 112) solution (cyclohexane: ethylacetate, 1 : 1, v/v). Sample extracts were then purified on a Bio Beads S-X3 column using cyclohexane : ethylacetate (1 : 1, v/v) as a mobile phase. A fraction corresponding to a elution volume of 14–30 ml was collected.

Results

The first data on the occurrence of BFRs in Czech humans employing breast milk as a bioindicator matrix were reported by Kazda et al. in 2004[4], however, the information on the other major POPs, such as PCBs and DDTs was not provided.

An overview of the most abundant PBDE and PCB congeners together with OCP levels in both types of examined matrices collected in the Czech Republic within presented study is shown in Table I. The results clearly document ubiquitous occurrence of PBDEs emission sources in the environment of general Czech population.

BDE congeners 47 (tetrabromo-), 153 (hexabromo-) and 183 (heprabromo-) were predominant and accounted up to 80 % of the total PBDEs. Other relatively abundant representatives of this group were congeners BDE 99 and 100 (both pentabromo-). BDEs 28, 49, 66, 85 and 154 were detected only in several samples, mostly close to the limit of quantification.

Interestingly, PBDE profile in human adipose tissue found in our present study was not identical to that one observed in breast milk samples. While in the latter matrix BDE 47 was the most abundant congener followed by BDE 153, opposite ratio of these major PBDEs was found in adipose tissue samples (see Fig. 1.). Biotransformation and accumulation kinetic properties of individual PBDE congeners after human exposure may contribute to these differences. As shown in Fig. 1., a similar trend was observed in a recent Japanese study[6], while this was not reported in a study conducted in Sweden, which documented, in both human milk and adipose tissue, the levels of BDE 47 to be approximately 4-times higher than BDE 153. The use of Penta-BDE technical mixture with a majority of BDE 47 was probably dominating in this country[7]. In this Scandinavian country this difference could be attributed to different uses of these products or a different in the diet of individuals. On the other hand, no significant differences were found by a comparison PCB profiles in both examined matrices.

Table I
Levels of PBDEs, PCBs and OCPs determined in breast milk and adipose tissue collected in the Czech Republic during the year 2007 (ng g^{-1} lipid)

Analyte	Breast milk (n = 56)		Adipose tissue (n = 98)	
	Mean	Median	Mean	Median
BDE 28	0.31	0.12	0.1	0.05
BDE 47	1.52	1.11	1.1	0.7
BDE 49	0.22	0.14	0.05	0.05
BDE 66	0.13	0.13	0.1	0.05
BDE 85	0.19	0.12	0.05	0.05
BDE 99	0.73	0.53	0.5	0.2
BDE 100	0.3	0.17	0.5	0.3
BDE 153	0.54	0.21	1.3	1
BDE 154	0.14	0.11	0.2	0.1
BDE 183	0.32	0.15	0.7	0.4
BDE 209	2.93	1.52	5.4	<2
HBCD	0.22	0.13	1.2	<0.5
PCB 28	2.3	1.56	2	1.8
PCB 52	2.36	1.64	1.6	1.3
PCB 101	2.73	2.34	4.2	1.9
PCB 118	17.84	13.43	17.7	14.3
PCB 138	143.61	119.84	121.6	110.1
PCB 153	229.81	202.76	233.6	219.8
PCB 180	192.98	166.67	245	230.7
HCB	40.78	34.72	120.4	72.1
b-HCH	11.28	8.31	23.9	17.5
p,p´-DDE	178.95	148.81	582.5	478.8
p,p´-DDD	4.26	3.94	1.7	1.3
p,p´-DDT	22.04	16.64	24.9	20.4

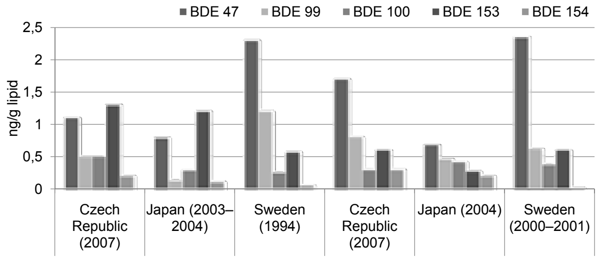

Fig. 1. **PBDE congener profile in both examined matrices from various countries**

Generally, based on available studies, PBDE levels in breast milk and adipose tissue samples collected within this study were comparable to those reported in other European countries but considerably lower (by almost one order of magnitude) than results from the United States, probably due to a less extensive use of this group of BFRs in common goods and products[6-11].

As compared to PBDEs, the levels of OCs (PCBs and OCPs) were up to 2–3 orders of magnitude higher, obviously, due to a relatively longer history of their intensive use in a wide range of areas. Although PCBs, DDT and several other persistent OCs were banned many decades ago, and, in spite of their decline witnessed in the Czech Republic alike worldwide, these POPs are still persisting in very high quantities in the environmental compartments. Not surprisingly, their transfer into the food chain still continues, what was documented also in this study. The OCs pattern found in Czech breast milk was: PCBs > DDTs > HCB > HCHs. The following PCBs profile was found: CB 153 > CB 138 > CB 180 > CB 118 > CB 101 ~ CB 52 ~ CB 28. The dominating PCB congeners No. 153, 138 and 180 contributed together to the total PCBs content by almost 80 %.

Regarding to other chlorinated POPs, p,p´-DDE was their major representative in all analyzed samples. The contamination input in the food chain obviously occurred many years ago since the parent compound, p,p´-DDT, was significantly lower. Other OCPs like HCB (hexachlorobenezene) and β-HCH (hexachlorocyclohexane), were apparently in all samples at levels above LOQs (see Table I).

The levels of PCBs were similar to those found in other European countries. While no age dependency was found for PBDEs, an increase of PCB and OCP levels with age was observed. Different exposure routes of donors were documented by the absence of the relationship between PCBs and OCPs.

Conclusions

This study reports the residue levels of PBDEs and HBCD together with "classical" OCs in human adipose tissue and breast milk samples obtained in the Czech Republic.

The results clearly show the ubiquitous occurrence of BFRs in the general Czech population, BDE 153 and BDE 47 being the most abundant congeners in examined biotic samples. Despite its broad use, BDE 209 was detected in only a few adipose tissue samples.

The mean values of PBDEs in human fat from Czech donors did not largely differ from those recorded from other European countries

Also PCB and OCP levels were comparable to those reported in similar studies conducted outside of the Czech Republic. It should be noted that the production and use of PCBs has been banned for three decades, whereas the use and production of PBDEs still continues. In spite of the fact, that penta- and octa-BDE technical mixtures were banned in the EU four years ago, humans are still exposed in their daily lives from food and emissions from various products into their environment.

This research was supported by grants from the Ministry of Education, Youth and Sports of the Czech Republic – BIO-BROM (2B06151) and MSM 6046137305.

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P87 IODINE IN MILK ON THE CZECH MARKET

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Introduction

Iodine is an essential trace element. Biological role of this trace element is related to the hormonal system of thyroid gland where iodine is a constituent of thyroid hormones. Therefore iodine is very important element also for human population. Natural sources of iodine are e.g. seaweed, seafood and also plants grown on iodine-rich soil[1].

Iodine deficit is typical problem for areas where there is low content of iodine in the diet especially in areas where seafood consumption is low[2]. It is well known that Europe and therefore also the Czech Republic belong between countries with known iodine deficiency. Usual public health protection measure is fortification of salt with iodine.

Many studies have been published about problem of dietary intake of iodine for the Czech population. Summary study about situation in the Czech Republic published Scientific Committee on Food in 2007[3].

The National Institute of Public Health in Prague, Centre of Hygiene of Food Chains in Brno is involved in “The Project on Dietary Exposure to Selected Chemical Substances”[4]. The objective of the project is to describe the dietary exposure of the population of the Czech Republic. In the framework of this project iodine has been monitored since 1998.

Since year 2000 to 2003 has been observed increasing trend of iodine intake. This effect causes mainly usage of fortified salt in kitchen and during industrial food processing. Doses observed in the 2003 reached 3.59 $\mu g\,kg^{-1}$ b.w./day (230 μg/person/day)[5]. This value matches 153 % of recommended daily intake by Czech law (Regulation 446/2004).

As a biomarker of short term (24 hours) intake of iodine the urinary level is mostly used. The National Institute of Public Health in Prague and some other institutes monitor urinary level of iodine for many years. It was recognized that average iodine concentration in adult’s urine increased from 85 $\mu g\,dm^{-3}$ to 251 $\mu g\,dm^{-3}$(ref.[6]) during years 1995–2005.

This increase can be attributed to some specific foods in our diet. High concentrations of iodine were measured in fish, meat products, instant soups, milk and milks products. It was recognized that significant exposure sources are mainly milk and milks products, bread, meat products and eggs (descending order to total exposure dose).

Probabilistic modelling was used to estimate usual daily intake of iodine from various kinds of foodstuffs for age groups in the Czech population[3,7]. It was observed that milk is the most important source of iodine for various age groups in the Czech Republic. Results of that modelling (it did not take added salt after cooking into account) also support an idea that high intake of iodine can be a certain risk for young children (< 10 years) where milk and dairy produces represent very important parts of their diet[7].

That was the reason why our laboratory focused its research on iodine concentration in marketed consumer milk.

Experimental

Twice a year, in May and November, 24 samples of milk (12 semi-skimed and 12 skimed milks) were purchased from markets in twelve towns in the Czech Republic. These samples were analyzed as one composite sample. Milk samples have been also analysed as individual samples since 2007 to get better imagination about distribution of iodine concentrations.

For determination of iodine was used Sandell-Kolthoff method. The principle of this method is in catalytic function of iodide in oxidation-reduction reaction Ce(IV) and As(III).

Samples are transferred to refractory glass tube. Solutions of potassium hydroxide and zinc sulphate are added. These mixtures are stirred and tubes are placed in an oven at 105 °C to dry the samples and then in a cold muffle furnace where are left until temperature gradually rises to 600 °C. The tubes are taken out. At this stage the food samples are not completely digested. The samples are soaked with 0.5 ml of water and then placed in a cold muffle furnace and digestion process is repeated. Now the samples are completely digested. Into each tube containing the digest, 6 ml of water is added and stirred to dissolution of the water-soluble part of the digest. The suspension is transferred into a centrifuge tube and centrifuged for 10 min at 2500 $rev\,min^{-1}$.

Aliquot of the supernatant liquid is transferred to the glass tube and refilled with water to 2 ml. Then 2 ml of arsenic(III) solution is added and the tube is placed in the cooling bath (10 min). Then 2 ml of cerium(IV) solution is added and the tube is placed in the water bath at 40 °C (20 min) and then in the cooling bath (10 min). Then 0.5 ml of brucine is added and the tube is placed in an oven at 105 °C (15 min). After adding of every solution thorough stirring is important.

Finally, a light absorption is measured by spectrometer at 430 nm. Limit of quantification is 15 $\mu g\,kg^{-1}$. This method is accredited according to CSN EN ISO/EC 17025:2005 by the Czech Accreditation Institute.

Results

Average concentration of iodine in milk analysed since 1998 to 2007 is 268 $\mu g\,kg^{-1}$. The results are shown in Fig. 1. Average concentration of iodine in milk composite samples has been higher since 2003.

In 2007, after publishing of results clarifying a key role of milk in iodine intake, we analyzed all of 24 samples from the Czech market individually. Results for semi-skimed milks are shown in Fig. 2. and for skimed milk in Fig. 3. Semi-skimed milk from town No. 7 (May, 07) has not been determined.

Average content of iodine in semi-skimed milk was 344 $\mu g\,kg^{-1}$ in May and 277 $\mu g\,kg^{-1}$ in November. Average

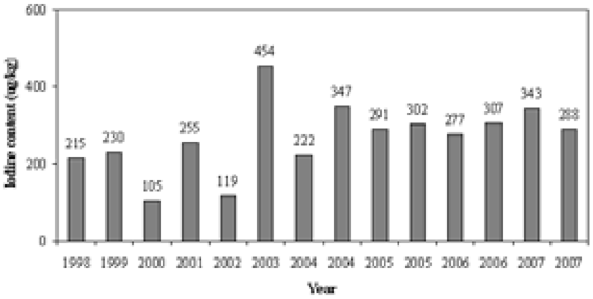

Fig. 1. **Average iodine content in milk (1998–2007)**

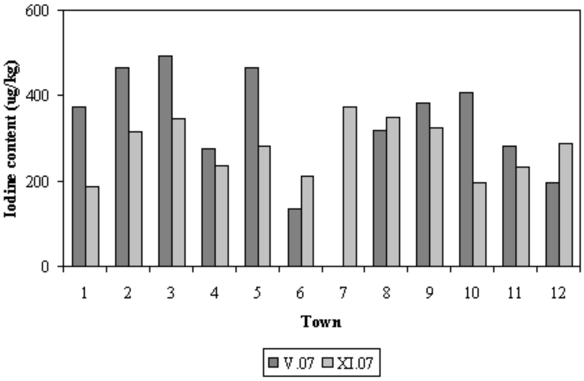

Fig. 2. **Iodine content in semi-skimed milk (2007)**

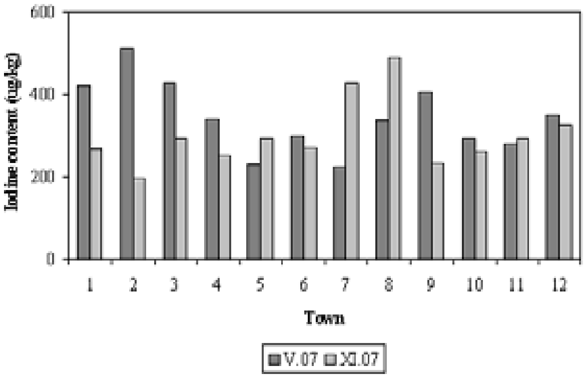

Fig. 3. **Iodine content in skimed milk (2007)**

content of iodine in skimed milk was 343 μg kg^{-1} in May and 299 μg kg^{-1} in November. These data can signalize decreasing trend of iodine content in milk available at the Czech market in 2007 in comparison to previous years. Semi-skimed milk contain slightly higher average amount of iodine than skimed milk. Fig. 2. and Fig. 3. show relatively high concentration variability between individual milk samples. Maximal concentration of iodine in milk was 509 μg kg^{-1} (skimed milk, XI/07, town No. 2) and minimal concentration was 135 μg kg^{-1} (semi-skimed milk, V/07, town No. 6) in year 2007.

Conclusions

The dietary intake of iodine has monitored since 1998. The monitoring of the dietary exposure requires information about iodine content in individual foodstuffs and extends of their consumption[7]. At the present information is available and therefore we can calculate both a point and a probabilistic estimate of intake for different age categories of the Czech population.

It was found that milk is the most important source of iodine which can present certain health risk for high consumers mainly for children. According to the EC Regulation No. 1459/2005 allowed amount of iodine in complete feed for dairy cows and layers decreased from 10 mg kg^{-1} to 5 mg kg^{-1}. Results of feed control are not known at the moment in the Czech Republic. Then samples of milks have been analysed individually since 2007 to clarify possible exposure doses dominy from milk.

Average content of iodine in milk (period 1998–2007) was 268 μg kg^{-1}. Over the last years was this value about 300 μg kg^{-1}. Average content of iodine in milk in May and November 2007 was 343 μg kg^{-1} and 288 μg kg^{-1} respectively. We found large variability of concentrations between individual milk samples. High concentration variability can be problem for food operators and also for consumers due to not standard iodine content (e.g. production of infant and baby milk formulas). Optimal content of iodine in milk in context of overall dietary exposure is about 200 μg kg^{-1}. It would be advantage for all to keep variability of iodine content in individual milk samples as much as close to this value.

Optimal iodine intake is important for good health of Czech population. The most endangered group is children. These are reasons why monitoring of iodine content in individual samples of milk from the Czech market will continue.

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P88 MONITORING OF MERCURY CONTENT IN THE FOOD BASKET FOR THE CZECH POPULATION DURING 1994–2007

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Introduction

Mercury belongs to group of heavy metals. In nature mercury can be found in various forms (organic and inorganic compounds, elementary Hg^0) with different toxicity. For human organism all mercury forms can be toxic. The most important and the most danger forms are alkyl-mercury compounds like methylmercury (MeHg) and inorganic compound like Hg^{2+}. The hazard is connected also with cumulative properties of mercury. The highest concentrations are measured in kidneys, liver and spleen.

Less frequent acute intoxication causes vomit, diarrhea and abdominal ache. More important is a risk of chronic intoxication. Exposure can lead to cerebral paralysis, cephalonia, deafness, blindness and high blood pressure (Minamata disease)[1]. MeHg as the most danger mercury form comes through blood-brain barrier and via placenta can be transported into fetal brain.

Main exposure routes for mercury are inhalation and digestion... Disputative question is long-term influence of amalgam fillings. Mercury is very well absorbed after oral exposure (95 % MeHg is absorbed). Due to potential health risks it is necessary to monitor the mercury content in foodstuffs of vegetable and as well as animal origin. Plants and animal contamination can be caused by growing in contaminated environment (industry wastes and exhalation). Important source of mercury exposure especially in seaside countries are fish.

Many studies have been published about the determination of mercury in foods. Centre for the Hygiene of Food Chains in Brno has been involved in the monitoring of the content of total mercury in foods (see "The Project on Dietary Exposure of the Czech Population to Selected Chemical Substances" [2,3]). This project has been started in 1994 and the third period runs in this time. The aim of this monitoring programme is an assessment of dietary exposure, comparison with toxicological reference points, and characterization of health risks. To achieve these goals it is necessary to perform a systematic monitoring of the mercury content in the food basket of the Czech population as well as to know consumption of individual foods.

This work summarizes the mercury content found in individual foods in our laboratory during past 14 years. Also their contribution to the total dietary exposure and validation by using of biomarkers are included.

Methodology

The analyzed foods were selected according to the knowledge of their consumption by the average person in the Czech population[4,5]. In total 143 kinds of foodstuffs were selected and studied in specific time periods. Food samples were collected in 4 regions (12 sampling towns) and delivered to Centre of Hygiene of Food Chains in Brno. This organization represents 572 food samples that have been processed during two-year period. Some kind of foodstuffs (more frequently consumed) are sampled repeatedly and measured every year (e.g. milk or potatoes even 2 × per year and town), others (less frequently consumed) are sampled and measured only once in the whole two-years period. In total 880 individual samples were measured in this period. After delivery of samples into the Centre of Hygiene of Food Chains in Brno, the samples undergo the culinary treatment in pre-analytical laboratory so that they are analyzed in the same state as they are consumed. The samples are then homogenized and delivered into the central analytical laboratory for further treatment and analyses.

In analytical laboratory mercury content (as a total mercury) is determined by AAS method using principle of selective system AMA 254 with solid sample dosing without previous analytical preparation. Optimized method provides adequate sensitivity for the determination of the mercury in foods. The limit of quantification is 0.1 $\mu g\,kg^{-1}$ for a minimal sample weight 200 mg. The degree of recovery ranged from 95 to 105 %. The RSD of repeatability does not exceed 10 %. This method is accredited according to ČSN EN ISO/IEC 17025:2005. To ensure accuracy of the results matrix reference materials as well as various internal test materials are used. In addition our laboratory participates in proficiency testing (FAPAS, IMEP etc.).

Results

The content of total mercury was monitored in 143 kinds of food representing so called food basket of the Czech population. Based on previous experience only fifteen kinds of food with the highest measured mercury content were selected and visualized in Fig. 1. This figure shows foods with their corresponding concentration values. The dietary exposure is influenced not only by the content of mercury in the given food, but also by the quantity of consumed foods To calculate the dietary exposure doses it is also necessary to take into account the changes caused by the culinary treatment of the given food which are described by the so called culinary factor[3]. The influence of both the consumption and culinary factor on the final exposure dose is demonstrated by the following figure. The fifteen most significant exposure sources of total mercury are given in Fig. 2. In both figures (1.,2.) concentration data are used from the last monitoring period 2006–2007.

Foodstuffs listed in Fig. 1. and Fig. 2. are traditionally highlighted in first places during whole period of monitoring program (1994–2007). Fig. 3. shows the changes in measured content of total mercury [$\mu g\,kg^{-1}$] in five selected most

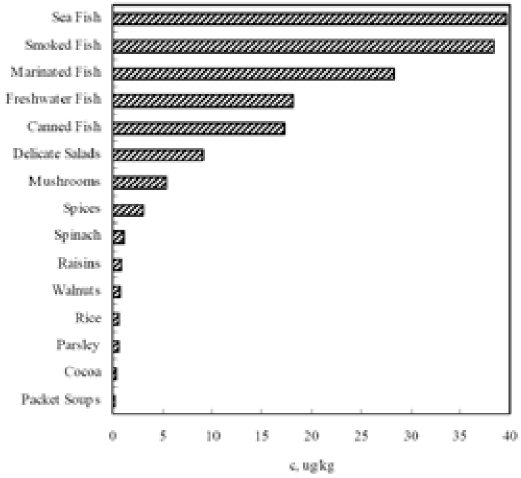

Fig. 1. **Total mercury concentration in selected foods in the Czech Republic in 2006/2007**

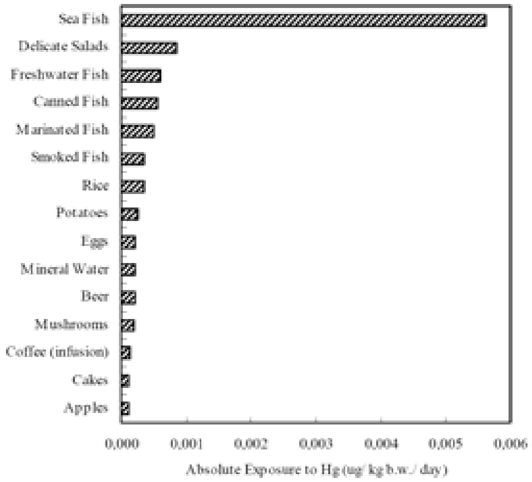

Fig. 2. **Dietary exposure sources for total mercury in the Czech Republic in 2006/2007**

contaminated foods in time (years) and their contribution to the exposure doses is plotted in Fig. 4.

The trend of exposure doses to total mercury is shown in Fig. 5. There are included exposure doses for different population groups in the Czech Republic during monitoring period 1994–2007. The point estimate of health risk indicates fluctuating trend with a slight tendency to increasing. The most exposed group are children due to higher usual intake of foods per kg of their body weight.

For assessment of human exposure and better health risk characterization are often used also biomarkers (human blood, urine or hair). This special approach is also used by the National Institute of Public Health in the monitoring project called "Biological monitoring"[6,7]. In Fig. 6. the behaviour of the total dietary exposure with time is shown for monitored period 1994–2007. Also in this figure the levels of mercury in blood samples in selected years for adult Czech population in the same period are shown.

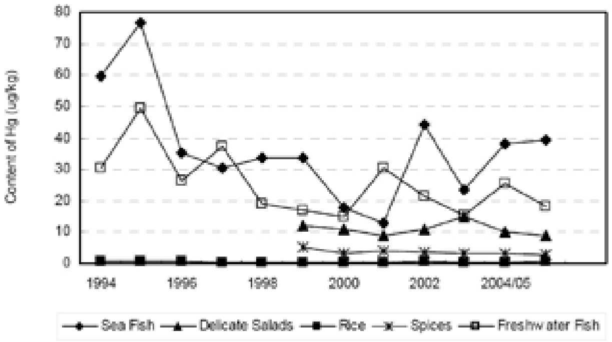

Fig. 3. **Content of total mercury in selected foods**

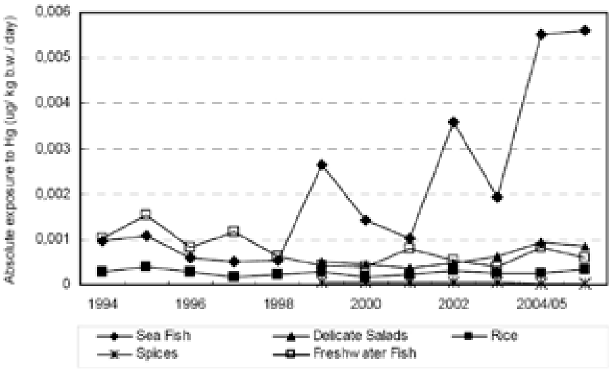

Fig. 4. **Contribution of selected foods to the overall exposure doses for total mercury**

Discussion

The results from whole monitored period (1994–2007) support an idea that foods containing the highest amounts of total mercury are fish and fish products, delicate salads, mushrooms, spices and rice (Fig. 1.). With regard to the significance of foods as dietary exposure sources the most important foods are as well fish and fish products, delicates salads and rice. (Fig. 2.). It support an idea that the most hazardous foods from exposure point of view are fish meat and fish products. 80–90 % of total mercury in fish is expected as MeHg in muscle[8,9]. Then total mercury content is suitable indica-

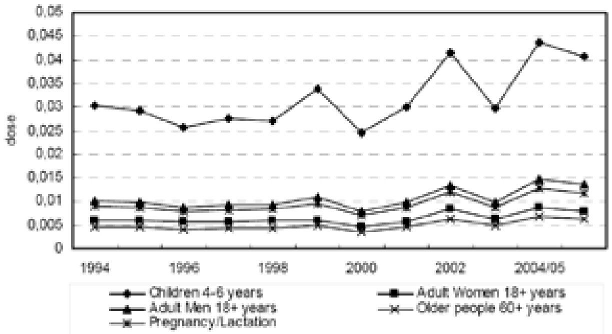

Fig. 5. **Exposure doses: total mercury [μg kg^{-1} b.w. day^{-1}] (consumption models according to the food guide pyramide)**

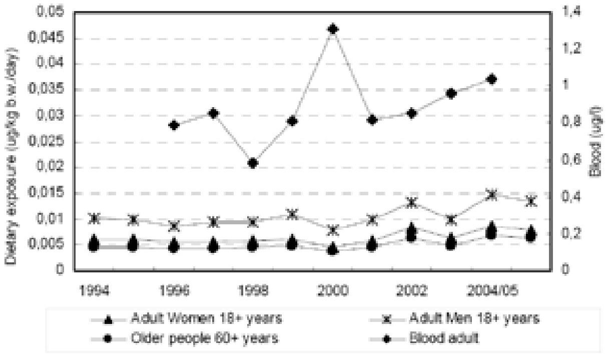

Fig. 6. **Comparison of measured overall dietary exposure doses of total mercury and levels measured in Blood (point estimate)**

tor of possible exposure to MeHg in this case. Other groups of foods are not of so important for exposure of the Czech population.

The estimate of the dietary exposure from the most significant samples (sea fish) show tends to increase. This tendency to increase may be due to the trend of growing consumption this commodity given by development of business and import from seaside countries. The monitoring also showed an increasing tendency for the dietary exposure to mercury which was in correspondence with the levels of mercury in human blood in the same period. In the Fig. 5. are included data found for adult. The same trend can be seen for children too.

Conclusion

The content of total mercury in the samples of the food basket of the Czech Republic has been monitored since 1994. Produced data enabled the calculation of both - the exposure dose coming from individual samples and the total dietary exposure dose. The average value of the total dietary exposure in the period 1994–2007 is 0.0126 μg kg^{-1} b.w. day^{-1} It represents 0.0882 μg kg^{-1} b.w. week^{-1}.

In 1978 the FAO/WHO JECFA evaluated total mercury and set PTWI at 5 μg kg^{-1} b.w. week^{-1} from which MeHg should not be higher than 3.3 μg kg^{-1} b.w. week^{-1}. US EPA defined RfD 0.1 μg kg^{-1} b.w. day^{-1}. PTWI set by FAO/WHO JECFA for MeHg was revised into 1,6 μg kg^{-1} b.w. week^{-1}(ref.[9,10,11]) in June 2003. According to longitudinal monitoring results it was proved that average value of overall dietary exposure to total mercury has not been breaking toxicological reference points set by FAO/WHO JECFA or US EPA in none of different population groups in the Czech Republic. Potentially risky groups are children and pregnant women or nursing mothers due to highest sensitivity of embryo/neonates in combination with dietary habit involving highly contaminated kinds of fish (mainly predator fish meat). Therefore it is important regularly evaluate dietary habit of the Czech population, analyse possible food sources of (at least) total mercury and do relevant advices to consumers. We should not forget that hazard of mercury in fish is in unknown extent balanced by benefits of some important nutrients coming from that food sources.

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P89 THE INFLUENCE OF SURFACE CHARACTERISTICS ON BACTERIAL CELL ADHESION

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Introduction

Bacterial cell adhesion is the first step in the formation of multicellular structure called biofilm. Biofilm is a dynamic community of cells which display distinct properties from the planktonic cells. These can be utilized in bioremediation technologies[1], however the stability of adhesion must be ensured. For this purpose, the principles of initial adhesion must be investigated and understood.

Experimental

- Microorganism.

Gram-positive pollutant degrading bacteria *Rhodococcus erythropolis* CCM 2595 was obtained from the Czech Collection of Microorganisms (Masaryk University Brno, Czech Republic).

- Cultivation and biomass determination.

Cells were cultivated in 200 ml of medium in shaked Erlenmeyer flasks. The growth of suspended cells was monitored as optical density at 400 nm (O.D.). Either a complex medium nutrient broth (HiMedia, India) or minimal medium was used (KH_2PO_4 0.17 g dm^{-3}, K_2HPO_4 0.13 g dm^{-3}, $(NH_4)_2SO_4$ 0.71 g dm^{-3}, $MgCl_2$ 0.34 g dm^{-3}, $MnCl_2$ 1 m g dm^{-3}, $CaCl_2$ 0.26 m g dm^{-3}, $FeSO_4$ 0.6 m g dm^{-3}, Na_2MoO_4 2 m g dm^{-3}, pH 7). In minimal medium phenol or glucose was used as the only carbon and energy source.

- Cell hydrophobicity.

Hydrophobicity of cells was determined by the MATH test[2].

- Cell fatty acids.

For fatty acids determination the cells were harvested by centrifugation, washed and lyophilized. Fatty acids were esterified to methyl esters, extracted to hexan and analysed by GC-FID.

- Adhesion monitoring.

The Flow cell 81 (BioSurface Technologies, USA) was used for assessing the adhesion of cells. Materials with different hydrophobicity and other properties were evaluated. Three types of glass were employed: microscope slide (labeled glass in following text), coated glass and hydrophobized glass. Also polymeric materials silicone and teflon were evaluated. The glass and silicone materials were prepared at the Department of Polymers, Faculty of Chemical Technology, Institute of Chemical Technology, Prague.

- Material hydrophobicity.

The material hydrophobicity (except teflon) was determined by the Surface Energy Evaluation system (Department of Physical Electronics, Faculty of Science, Masaryk University, Czech Republic).

Results

The External Conditions Influence on Cell Hydrophobicity

Cell wall hydrophobicity reflects the cell physiological state and is one of the most important cell characterictics that determine the ability to adhere[3]. Cell hydrophobicity can be influenced by the type of the source of carbon and energy.

In our study we investigated the effect of medium composition (complex and minimal media, optimal and stressful cultivation conditions). We found that the initial phenol concentration 0.3 g dm^{-3} can be considered as optimal and that phenol concentration 0.7 g dm^{-3} partially inhibits the growth and can be called stressful (data not shown). Concentration 1.0 g dm^{-3} caused considerable inhibition of the growth. Nutrient broth was chosen as a complex medium. Also glucose (as a C source) in minimal medium was tested. During the experiments, hydrophobicity of cells in different growth phases was determined to ascertain the influence of this factor, which according to literature, can be considerable[4].

The *Rhodococcus erythropolis* cells were proven to be highly hydrophobic in all monitored media (see Fig. 1.). The medium composition influence on variation of cell hydrophobicity was significant. The changes in cell hydrophobicity during the growth (exponential, stationary phase) were not considerable and therefore the subsequent experiments were carried out with cells in stationary phase.

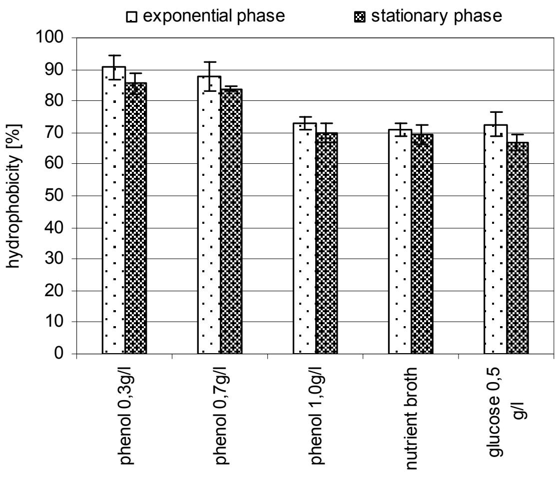

Fig. 1. **The dependence of *R. erythropolis* cells hydrophobicity on media composition**

The fatty acid composition of cells cultivated in media with different phenol concentration is presented in the Table I. Results indicate that there is dependency of fatty acid composition on initial phenol concentration.

Table I
Cell envelope fatty acid composition. Cells were harvested by centrifugation and lyophilized fatty acids were esterified to methyl esters, extracted to hexan and analysed by GC-FID

	Fatty acid (methyl ester)	t_R [min]	Carbon atoms	Phenol [g dm^{-3}]	
				0.3	0.7
	not identified	7.6	x		
14 : 0	tetradecanoate	13.3	14		4.8
i-15 : 0	13-methyltetradecanoate	14.9	15		
	not identified	17.8	x		
16 : 1	cis-9-hexadecanoate	17.9	16	8.3	12.7
16 : 0	hexadecanoate	18.3	16	41.8	51.7
	not identified	19.4	x	3.2	
17 : 0$^{\Delta}$	cis-9,10-methylenhexadecanoate	20.6	17	8.8	
17 : 0	heptadecanoate	20.9	17		
18 : 1^{9}	cis-9-oktadecanoate	22.7	18		
18 : 0	oktadecanoate	23.4	18	2.1	8.0
	not identified	24.2	x	33.6	24.5
	not identified	26.8	x	2.1	

Cell Adhesion

The determination of material hydrophobicity and other surface characteristics influence on cell adhesion was the aim of our work. Five materials were tested, both hydrophilic and hydrophobic. The hydrophilic materials were: glass without any modifications (strictly hydrophilic) and coated glass with slightly less hydrophilic surface. The hydrophobic materials were: hydrophobized glass and silicone with equal hydrophobicity, to ascertain the influence of surface moieties. The last material was teflon, known for its extreme hydrophobicity[5] and antiadhesion properties.

Experiments were carried out using cells precultivated in Erlenmeyer flasks. The hydrophobicity of these cells was approximately 85 % (Fig. 1.). Initial adhesion experiments (see Table II) were carried out in the presence of phenol (0.3 g dm^{-3}). The adhesion was monitored after one hour and after 24 hours. The results confirmed that hydrophobic cells do not adhere to hydrophilic surface. The results also verified our assumption that beside hydrophobicity there are other important factors in the process of cell adhesion. The teflon was proven to be an unfavourable material for cell adhesion.

Table II
The adhesion of *Rhodococcus erythropolis* cells on different materials after one and twenty-four hours

Experiment duration [h]		1	24
material	material hydrophobicity	colonized area [%]	
glass	26.4 ± 6.6	0	0.2
coated glass	55.9 ± 8.2	4.9	3.2
hydrophobized glass	97.0 ± 2.0	12.1	12.9
silicone	97.0 ± 3.6	39.5	40.1
teflon	108 [5]	7.6	4.9

The colonized area was three times higher on silicone than on the hydrophobized glass with the same hydrophobicity. The influence of the experiment duration was not significant.

Also the adhesion of cells with different inoculation origin was evaluated (see Table III). The cells were precultivated in inoculum medium (nutrient broth or mineral medium with phenol) and then transferred to experiment medium. This was proven to be substantial in cell attachment. The cells adhered the most to silicone, but only in medium with phenol concentration 0.3 g dm^{-3}. When transferred from either nutrient broth or minimal medium to minimal medium with phenol concentration 0.7 g dm^{-3}, cells adhered considerably less.

Table III
The influence of inoculum cultivation conditions on Rhodococcus erythropolis cells adhesion on different materials

		Experiment set-up		
inoculum medium		n.broth	n.broth	phenol 0.7 g dm^{-3}
experiment medium		phenol 0.3 g dm^{-3}	phenol 0.7 g dm^{-3}	phenol 0.7 g dm^{-3}
material	material hydrophobicity	colonized area [%]		
glass	26.4 ± 6.6	0	0.2	0.04
silicone	97.0 ± 3.6	39.5	4.5	9.2
teflon	108	7.6	5.5	6.0

Conclusions

In our study the influence of cultivation conditions on cell hydrophobicity and cell adhesion was confirmed. It was found that not only hydrophobicity of materials plays important role in colonization, but also other surface characteristics are significant.

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P91 SELECTION OF SUITABLE OF MICROORGANISMS FOR PREPARATION OF LACTIC ACID FERMENTED CABBAGE JUICE

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Introduction

The vegetable juices processed by the lactic acid fermentation introduce change in the beverage assortment. During fermentation, a great amount of another substances useful for health is produced by lactic acid bacteria and by other microorganisms[1]. Most probiotic microorganisms are lactic acid bacteria such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Streptococcus lactis*[2].

The term *probiotic* refers to live microorganisms that survive passage through the gastrointestinal tract and have beneficial effects on the host[3].

Prebiotics are an alternative for probiotics or their cofactors. They are defined as non-digestible or low-digestible food ingredients that benefit the host organism by selectively stimulating the growth or activity of one or a limited number of probiotic bacteria in the colon[4]. Inulin and oligofructose are the most studied and well-established prebiotics[5]. Inulin is a polydisperse fructan and it is a mixture of oligomers and polymers of chain of fructosyl units with one glucosyl unit at the end of a chain. The non-digestibility of inulin is the reason for the reduced caloric value of this natural polysaccharide.

Inulin is frequently used as an additive in functional ood articles, especially as substitute for lipid compounds and upplement for sugar. It can also be used in products with increased dietary fibre content, e.g. bread or food product, with a bifidogenic effect[6].

The conditions for lactic acid fermentation based on a cabbage juice and four probiotic lactic acid bacteria has been studied. All cultures showed good biochemical activity in the cabbage juices. Addition of prebiotic preparation into the cabbage juices has been studied also.

Experimental

Bacterial Strains and Growth Conditions

Lactobacillus plantarum CCM 7039, *Lactobacillus amylophillus* CCM 7001, *Lactobacillus amylovorus* CCM 4380 and *Bifodobacterium longum* CCM 4990 (obtained from Czech Collection of Microorganisms, Faculty of Sciences, Masaryk University, Brno, Czech Republic) were cultivated at 30 °C 2 day in the three medium (medium A-MRS broth, medium B-MRS broth with addition 2 % prebiotic preparation and medium C-MRS broth, the glucose was substituted prebiotic preparation). For fermentation we used night culture with initial concentration 10^6 CFU per milliliter. The pH of MRS medium was adjusted to 5.8 ± 0.2, and the medium was autoclaved 120 °C for 20 min.

Analysis of Growth

Cellular growth was followed by plate counting throughout the fermentation. The number of CFU per milliliter were obtained through enumeration on MRS agar after anaerobic incubation at 37 °C for 48 h.

Analysis of Metabolite Production

The concentration of lactic acid, citric acid and acetic acid were determined through high-performance liquid chromatography with RI detector (K-2301. Knauer). An Polymer IEX H form (250 × 8 mm, Watrex) column was used with 9 mM of H_2SO_4 as the mobile phase at a flow rate of 1 ml min^{-1}. The column temperature was kept constant at 50 °C. Before injection the samples were filtred (pore size 0,20 μm). All samples were analyzed triplicate. The measurement of pH was performed using a LABOR-pH-meter CG-834 SCHOTT, Germany[7].

Fermentation of Cabbage Juices

The fresh raw material (cabbage – *Brassica oleracea* L. convar. *capitata* (L.) *Alef. var. alba*, variety HOLT was purchased in a local market in Slovakia. Fresh sample juices were obtained by extracting the juice from cabbage using a kitchen juicer. Subsequently, the juices were gauze-filtered, fortified by adding 2% D-glucose and 0.5% NaCl, and inoculated with a culture of the lactic bacteria. Thus treated juices were poured into 250 ml graduated flasks, which were stoppered with sterile stoppers and left to ferment in a thermostat for 168 hours at 21 °C. During the fermentation, samples at specified intervals were taken for analytical determinations.

Results

In this study we applied *Lactobacillus plantarum* CCM 7039, *Lactobacillus. amylophillus* CCM 7001, *Lactobacillus amylovorus* CCM 4380 for preparation of lactic acid fermented cabbage juices. The suitability of *Bifidobacterium longum* CCM 4990 was also tested because study of Lukačová et. al., describes this bacterium as a potential probiotic culture[8].

In this work, we also tested influence lactic acid bacteria on the prebiotic preparation Frutafit® IQ (90–95 % instant inulín).

At the first, we tested the grow lactic acid bacteria in the tree MRS medium. We find out, that the lactic acid bacterie during the 48 h of cultivation has suitable condition for grow in the MRS medium with glucose and 2% inulin than in the MRS medium (without glucose) with 2% inulin. The Fig. 1. describe the grow *Lactobacillus amylophilus* CCM 7001 in the free various medium. The Fig. 2. describe decrease of pH during cultivation of *Lactobacillus amylophilus* CCM 7001.

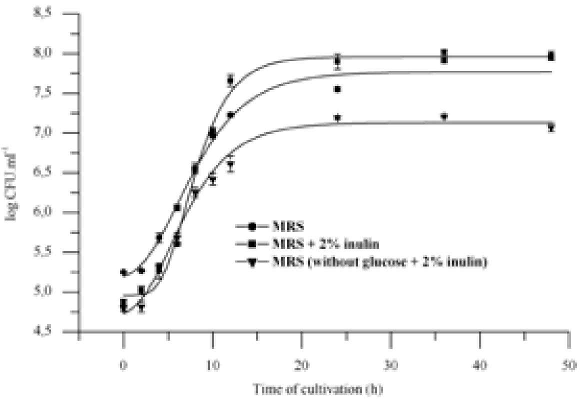

Fig. 1. **The growth of *Lactobacillus amylophilus* CCM 7001 in the tree various medium**

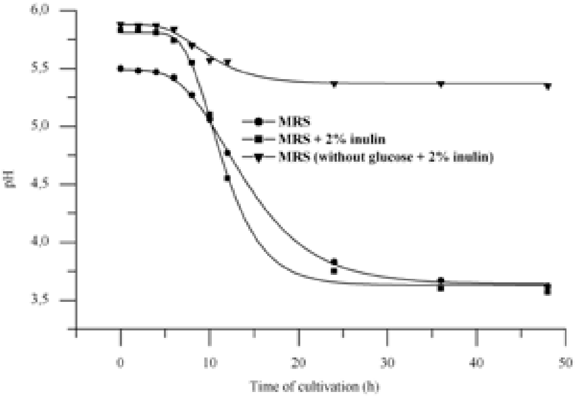

Fig. 2. **The pH of *Lactobacillus amylophilus* CCM 7001 in the tree various medium**

From this result we conclude that the *Lactobacillus plantarum* CCM 7039, *Lactobacillus. amylophillus* CCM 7001, *Lactobacillus amylovorus* CCM 4380 and *Bifodobacterium longum* CCM 4990 were not capable utilised inulin from Frutafit® IQ during 48 h of cultivation.

During the fermentation, pH of the juices decreases usually from 6–6.5 to 3.8–4.5. A rapid decrease of pH at the beginning of fermentation has decisive importance for the quality of the final product[9]. At the beginning of fermentation process, the cabbage juices had pH 6.3–6.1. At the end of fermentation process (after 168 h) the samples had pH 4.2–3.86. Formation of lactic acid is considered to be the key factor of the preserving effect in lactic acid fermentation[10]. After 168 h of fermentation, the cabbage juices containing 5.5–11.7 g dm^{-3} of lactic acid.

Conclusions

From the results it could be concluded that cabbage could be an effective raw material for prepared lactic acid fermented juices inoculated with microorganisms *Lactobacillus plantarum* CCM 7039, *Lactobacillus. amylophillus* CCM 7001, *Lactobacillus amylovorus* CCM 4380 and *Bifidobacterium longum* CCM 4990.

Also the results show, that tested probiotic bacteria and addition of 2 % prebiotic preparation were suitable for preparation lactic acid fermented cabbage juice with functional properties.

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P92 USING OF HYDROCOLLOIDS FOR DELAYNG STALING OF BAKERY PRODUCTS

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Introduction

One group of the most extensively used additives in the food industry are the hydrocolloids[1]. The most well know and applied in the industry polymers included in this kind of substances are alginates, carrageenans, agar, guar gum, arabic gum, methyl cellulose and carboxymethyl cellulose[2]. These compounds used in food products and processing can serve as processing aids, provide dietary fiber, impart specific functional properties or perform a combination of these roles. Guar and xanthan gums, for instance, have been used at 7 % and 2 % levels, respectively, in bread to provide dietary fiber for therapeutic purposes. At a lower level of incorporation, gums have served as additives to improve the quality of bread[3].

In the baking industry, hydrocolloids are of increasing importance as bread improvers as they can induce structural changes in the main components of wheat flour systems along the breadmaking steps and bread storage[1]. The hydrocolloids are added to bakery products for control water absorption and consequently dough rheology[4], improving their shelf life by keeping the moisture content and retarding the staling[5]. It is well known the effect of hydrocolloids on starch pasting properties[5,6]. These starch properties, that include gelatinization temperature, paste viscosity and retrogradation of the starch, affect baking and final quality and staling behaviour of baking products[6].

The present study was done to examine the effect of different hydrocolloids on retarding the staling process of baked goods.

Experimental

A wheat flour T650 (containing 11.20 ± 0.2 % of proteins, 0.68 ± 0.001 % of ash in dry matter and 31.55 ± 0.4 % of wet gluten in dry matter) and wholemeal spelt flour (containing 17.30 ± 0.3 % of proteins, 2.21 ± 0.002 % ash in dry matter and 40.99 ± 0.5 % of wet gluten in dry matter) and of local origin was used in the study. Blend flour was obtained mixing of wheat and wholemeal spelt flours in ratio 85:15.

The hydrocolloids used were: guar gum, gum arabic, xanthan gum and methyl 2-hydroxyethyl celullose. The dough was prepared according to formulation, which was 100 % blend flour, salt 2 %, sugar 1 %, yeast 4 %, sunflower oil 2.5 %, hydrocolloid 1 % on flour weight basis and water to farinographic consistency 400 BU (Brabender Units). All raw materials were procured from the local market in Slovakia.

The ingredients were mixed during 6 minutes in farinographic mixing bowl. After 20 min fermentation, the dough was divided into 100 g loaves, formed on dough former, proofed 45 min and baked in an electric oven during 12 min at 230 °C. Baking trials were performed in triplicate.

Determination of Crumb Hardness

Crumb hardness was measured on freshly baked loves (2 h after baking) and on loaves that were stored for 24, 48 and 72 hours at ambient temperature using a manually operating penetrometer AP 4/1 when 1 penetrating unit represented 0.1 mm.

Results

Bakery products have a very short shelf life and their quality is highly dependent on the period of time between baking and consumption[7].

Firming of bread crumb during storage is a common phenomenon and leads to a crumbly texture, and lower consumer acceptance. Staling of bakery products is generally defined as an increase in crumb firmness and a parallel loss in product freshness[8]. Firming is the preferred parameter used to evaluate staling development[9].

In this study, the anti-staling potential of various hydrocolloids was investigated. We concluded that all from applied hydrocolloids expect methyl 2-hydroxyethyl cellulose reduced firmness during 72 h storage period, given softer crumb than control samples (without hydrocolloids). The reason for the softer texture with gum treatments might be that the hydrophylic gums were holding more water, which led to higher moisture content in the final baked product and as a consequence, retrogradation of the starch and bread firming is retarded[10]. Guar gum had the greatest effect in decreasing crumb hardness (see Fig. 1.). This hydrocolloid has a softening effect due to a possible inhibition of the amylopectin retrogradation, since guar gum preferentially binds to starch[11]. Effect addition of different concentration of guar gum (from 0 to 2 %) on shelf life of baked products was also

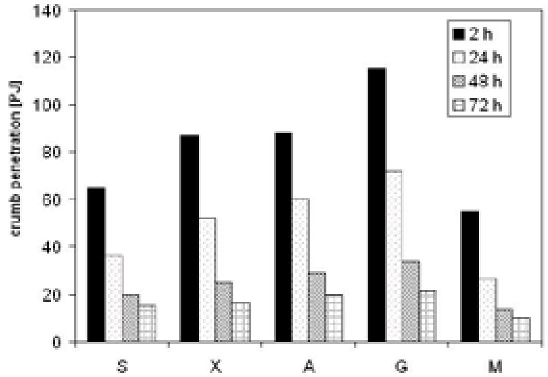

Fig. 1. **Effect hydrocolloids on crumb penetration (S-control sample, × – xanthan gum, A – gum arabic, G – guar gum, M-methyl 2-hydroxyethyl celullose**

studied. From results concluded that addition 0.75–2 % of this compound significantly delaed baked goods staling process. Expect these positive effects, the incorporation of guar gum also improved palatability of final products.

Conclusions

Nowadays, the use of additives has become a common practice in the baking industry. Baked goods staling evaluated during 72 h storage period through bread firmness values showed, that loaves prepared with hydrocolloids extent products contained celullose derivate were soften when control sample. Guar gum was showed as good bakery improver owing to its good crumb softening effects.

The incorporation of hydrocolloids into the loaves also influenced rheological parameters of dough and sensory acceptance of final products in different ways.

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P93 INFLUENCE OF PH ON THE RHEOLOGY PROPERTIES OF TOMATO KETCHUPS

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Introduction

A large part of the world tomato crop is processed into tomato paste, which is subsequently used as an ingredient in many food products, mainly soups, sauces and ketchup[1]. Tomato ketchup is concentrated dispersion of insoluble matter in aqueous medium[2].

By definition, rheology is the study of the deformation and flow of matter. So, rheological properties are based on flow and deformation responses of foods when subjected to stress[3]. The flow properties of food products significantly influence not only technology processes during the production, but the texture of final products, too[4]. Tomato ketchup viscosity is very important from both engineering and consumer viewpoints[5].

The aim of this work was investigated the influence of pH on the rheological properties of 3 samples of the formulated tomato ketchups at pH levels 3.5, 4.0 and 4.5. This pH range is most common for tomato ketchups. In recent years rheological properties of food products have become increasingly important in the formulation of food and the optimization of food processing.

Experimental

Three samples of tomato ketchup were prepared in laboratory conditions. The tomato paste with tomato soluble solids 30 % and guar gum cames from the company Kolagrex Int., s.r.o., Kolárovo, Slovak Republic. Some ingredients such as Isosweet and Resistamyl 348 were from Amylum Slovakia, s.r.o., Boleráz, Slovak Republic and other were purchased from local market.

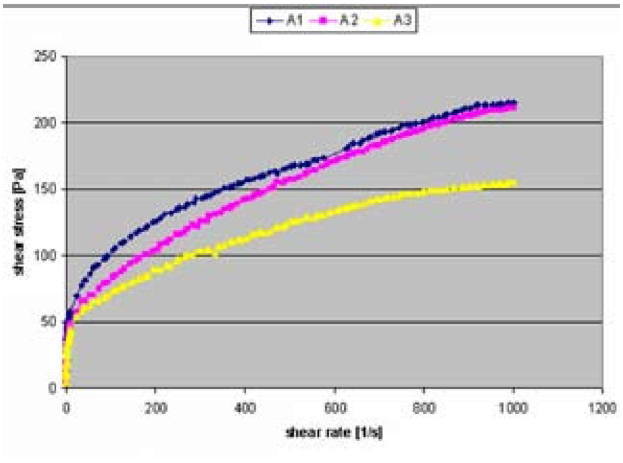

Fig. 1. **Flow curves of the studied ketchup samples at shear rate 0–1,000 s^{-1} and temperature 25 ± 1 °C**

Tomato Ketchup Preparation

The 100 g of tomato ketchup samples were prepared following the recipe: 35 g of tomato paste (TSS 30 %) mixed with ingredients typically used in ketchup preparation (15 g of isosweet, 11.5 g of vinegar, 1.5 g of salt, 2 g of resistamyl 348, 0.1 g of guar gum and 34.9 g of water). The pH level of the mixture was modified at level 3.5 (A1), 4.0 (A2) or 4.5 (A3) and followed by stirring for 5 min. The mixture was heated and stirred constantly until temperature between 80–85 °C reached. Ketchup samples were then stored at ambient temperature (25 °C) for 24 h before analyses.

Rheological Measurement

The rheological measurements of this study were carried out using the rotational viscometer (controlled rate mode) HAAKE Viscotester VT 550 (Haake, Karlsruhe, Germany) with coaxial cylinder measuring sensor systems MV DIN 53019. The Viscotester VT 550 was computer-operated with the application software RheoWin Job Manager and data evaluation software RheoWin Data Manager (ThermoHaake, Karlsruhe, Germany).

Flow curves (the graphical correlation between shear stress τ and shear rate D) were measured at a continuously increasing shear rate ramp over 3 min from 0 to 1000 s^{-1}. The apparent viscosity:

$$\eta_a = \tau/D \tag{1}$$

The thermal stability of the studied samples was measured at the low shear rate of 10 s^{-1} after 3 min of shearing.

Results

From the shape of the flow curves, which are demonstrated on the Fig. 1., it is possible to get the important information about rheological character of each ketchup sample. All 3 samples behave as non-Newtonian fluids, of which shear stress and apparent viscosity was changed with rate of dynamic strain. Rheograms confirmed their pseudoplastic characters too.

Viscosity curves (Fig. 2.) were constructed by conversion of values of flow curves. From their shape it is possible to set viscosity of the product at specific shear rates to which will be product exposed during dispersion, mixing, pumping, and filling to container as well as during application with consumer. The highest apparent viscosity during shear rate range 10–1,000 s^{-1} was determined in ketchup sample A1 with pH 3.5. Decreasing in the apparent viscosity was followed by sample A2 with pH 4.0 and then by sample A3 with pH 4.5.The requirement putting on some food products exposed to the specified temperature range is that its viscosity changed minimally. The effect of temperature on viscosity of ketchup samples is showed in Fig. 3. Ketchups should have

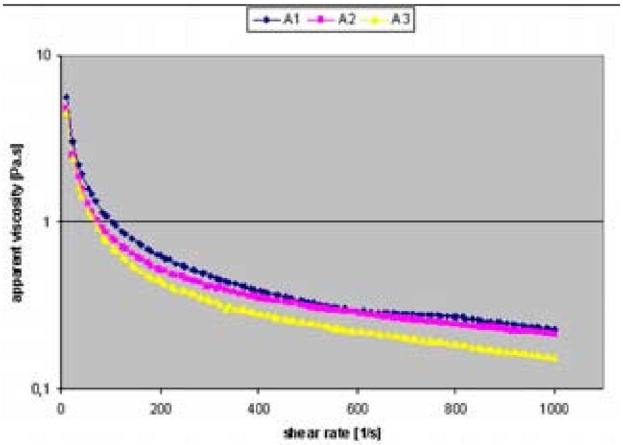

Fig. 2. **Viscosity curves of the studied ketchup samples at shear rate 10–1,000 s^{-1} and temperature 25 ± 1 °C**

small viscosity sensitivity between refrigerator and room temperature[6]. The smallest change in the apparent viscosity about 11.4 % was found in sample A1 with pH 3.5.

Conclusions

Rheological properties are next to colour and flavour the important criterion of sensory evaluation of the food product and its acceptation by consumer. The necessity to set the flow behaviour of product so that their characteristics, standard and quality would be maintained during transportation, storage and application is important from aesthetical and practical point too.

Three samples of the formulated ketchup with different pH level were investigated. The pH level of analysed tomato ketchups varied from 3.5 to 4.5, the most common pH range in ketchups.

The results indicated that studied samples of tomato ketchup behave as non-Newtonian fluids with pseudoplastic characteristics. The apparent viscosity was decreased with increasing pH level of tomato ketchups samples at all temperature range from 5 °C to 90 °C and at all shear rate range from 10 s^{-1} to 1,000 s^{-1}.

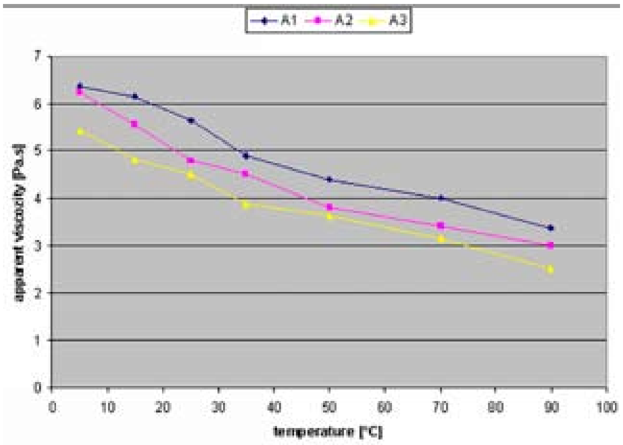

Fig. 3. **Effect of temperature on viscosity of the studied ketchup samples at shear rate D = 10 s^{-1} and temperature range from 5 ± 1 °C to 90 ± 1 °C**

This work has been supported by APVT No. 20-002904.

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P94 DISTRIBUTION OF DIAZINON IN VARIOUS TISSUES AND ITS EFFECT ON SERUM CHOLINESTERASE AFTER AN INTRAPERITONEAL ADMINISTRATION

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Introduction

Diazinon (O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) belongs to the group of organophosphate insecticides used to control cockroaches, fleas and ants. It is also used to control a wide variety of sucking and leaf eating insects and it has also veterinary use[1].

Diazinon has toxic effect on nervous system. This effect is achieved through the inhibition of acetylcholinesterase[2]. This enzyme has a key role in process for controlling of nervous signal transfer. Lack of acetylcholinesterase causes accumulation of acetylcholine. Accumulation of this neurotransmitter on the connections between nerves and muscles causes uncontrolled muscular contraction and algospasmus, between nerves and glands causes continual secretion of these glands, while acetylcholine cumulation between certain nerve cells in a brain causes sensory behavior disorders[3].

Diazinon is also able to cause oxidative stress of the organism through the forming of free radicals that are formed during the increased overextension of the organism by pesticides[4,5].

LD 50 (letal dose for 50 % of tested animals) for diazinon is very different depending up to the species of used laboratory animal. For rats, this value was established to be 1250 mg kg^{-1} after peroral administration and for laboratory mice 80–135 mg kg^{-1} of diazinon after peroral administration. ADI value (acceptable daily intake) was established on 0.002 mg kg^{-1} of diazinon per day[3,6]. Typical symptoms of poisoning are weakness, headaches, tightness in the chest, blurred vision, nonreactive pinpoint pupils, salivation, sweating, nausea, vomiting, diarrhea, abdominal cramps, and slurred speech. Some researches pointed out that diazinon has also mutagenic effect, however current evidence is inconclusive[3,7]. When it comes for a carcinogenic effect, diazinon is not considered to be a carcinogenic compound[7].

Metabolism and excretion rates for diazinon are rapid. The half-life of diazinon in animals is about 12 hours. The product is passed out of the body through urine and in the feces. The metabolites account for about 70 % of the total amount excreted. Diazinon does not belong to the group of chemicals that is characteristic with the long-lasting cumulation in human or animal tissues. However, in some cases could be diazinon detected in the samples of fat tisse, because this tissue has certain ability to cumulate organophosphate insecticides for relatively short period of time.Cattle exposed to diazinon may store the compound in their fat over the short term[8].

In this assay, we studied the effect of diazinon intraperitoneal administration on rat serum cholinesterase catalytic activity and the distribution of diazinon in various tissues of organs of experimental animals.

Experimental

Laboratory rats in the age of 135 days were randomly divided into 2 groups. Each group consisted of 10 males. Animals in the first group were administrated with diazinon (Sigma, USA) 20 mg kg^{-1} b.w. intraperitoneally in physiological solution. The second group served as a control group and was administrated only with the physiological solution. 24 hours after the administration of tested substance, animals were sacrificed, blood samples were taken from hearts and samples of livers, kidneys, muscles and fat tissue were taken during the autopsy.

Catalytic activity of cholinesterase was determined with using of Bio-La-Test®. The amount of diazinon in tissues was determined with using of gas chromatography with mass spectrometry.

Basic statistical characteristics – arithmetic mean, standard deviation and variation coefficient were calculated for cholinesterase catalytic activity and for amount of diazinon in each group. Obtained data were then processed in order to determinate statistical significance of the results. The Student's t-test was finally used for establishment of statistical significance.

Results

No deaths were observed in any of groups of experimental animals. However, animals from diazinon treted group approximately 12 hours after the administration of diazinon showed symptoms connected with depression of cholinesterase activity and did not react on external stimuli. Results of the determination of cholinesterase activity are presented in Table I.

Table I
Cholinesterase activity ($\mu\text{kat dm}^{-3}$) in different groups

Group of animals	Cholinesterase activity activity (X ± SD)	Variation coefficient [%]
Dizinon group	$1.81 \pm 0.79^{*}$	44
Control group	3.69 ± 0.51	14

X – arithmetic mean, SD – standard deviation,
*p-value < 0.05

Changes of cholinesterase catalytic activity were observed in diazinon treated animals. We observed significant decrease of cholinesterase catalytic activity from 3.69 $\mu\text{kat dm}^{-3}$ in control group to 1.81 $\mu\text{kat dm}^{-3}$ in experimental group. Muscular weakness, confuse and lethargy were typical symptoms that were observed on experimental animals in our assay.

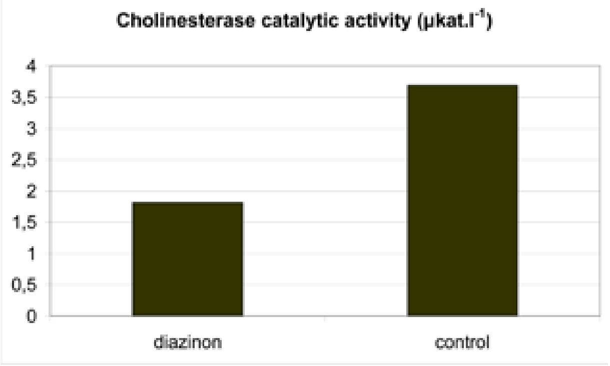

Fig. 1. **Cholinesterase activity in different groups**

Results of the determination of diazino amount in various tissues of control animals in comparison with experimental group of animals are presented in Table II.

Table II
Amount of diazinon in various tissues [mg kg^{-1}]

Tissue	Amount of diazinon [mg kg^{-1}])		
	Control	Experimental (X ± SD)	Variation coefficient [%]
Liver	0	0	0
Kidney	0	0.072 ± 0.036	49
Muscle	0	0.067 ± 0.041	62
Fat	0	3.717 ± 3.749*	100

X – arithmetic mean, SD – standard deviation,
*p-value<0.05

After analyzing samples of livers, kidneys, muscles and fat tissue for amount of diazinon we observed significant increasing of dizinon amount in samples of fat tissue. Inconsiderable incease of diazinon amount was detected in samples of kidneys and musled. On the other hand we did not detect any amount of diazinon in liver samples of experimental animals.

Conclusions

Itraperitoneal administration of diazinon in amount of 20 mg.kg^{-1} caused significant depression of cholinesterase catalytic activity. Significantly increased amount of diazinon was detected only in samples of fat tissue. On the other hadn, we did not detect any amount of diazinon in samples of liver.

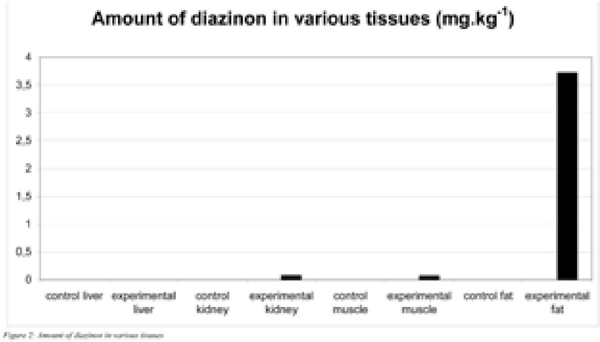

Fig. 2. **Amount of diazinon in various tisuues**

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P95 IDENTIFICATION OF BACTERIAL STRAINS OF *LACTOCOCCUS LACTIS* SPECIES IN HARD CHEESES USING PCR

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Introduction

Lactococci are the most prominent group of lactic acid bacteria applied in dairy fermentations. Strains of *L. lactis* ssp. *lactis* have been used in the manufacture of different types of cheese. Moreover, some lactococcal strains produce bacteriocins which show activity against food pathogens. Differentiation of these strains on the basis of phenotypic tests is time-consuming and can lead to misclassification. Due to the wide use in dairy industry and different technological properties, fast and reliable PCR-based methods were developed which enable identification of *L. lactis*[1] and distinction between the two subspecies *lactis* and *cremoris*[2]. False-negative results can occur due to the presence of extracellular PCR inhibitors in real tested samples of dairy products.[3–5] The problem of pure DNA preparation can be resolved by means of various isolation and purification methods. Solid phase systems based on non-selectively adsorbing DNA have been developed. It was shown that PCR-ready DNA can be isolated using magnetic microspheres P(HEMA-*co*-EDMA) containing carboxyl groups[4,5] in the presence of high concentrations of PEG 6000 and NaCl.

The aim of this work was to develop a method for PCR-ready DNA isolation from different hard cheese samples. Carboxyl-functionalised magnetic poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) microspheres (P(HEMA-*co*-EDMA)) were used for DNA isolation. The quality of extracted DNA was checked by PCR amplification.

Material and Methods

Chemicals and Equipment

Primers for PCR were synthesised by Generi-Biotech (Hradec Králové, Czech Republic); *Taq*I DNA polymerase was from Bio-Tech (Prague, Czech Republic), DNA ladder 100 bp from Malamité (Moravské Prusy). The PCR reaction mixture was amplified on an MJ Research Programme Cycler PTC-100 (Watertown, USA).

Magnetic nonporous poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) P(HEMA-*co*-GMA) microspheres containing carboxyl groups were prepared according to the previously described procedure[6] in the Institute of Macromolecular Chemistry (Academy of Sciences of the Czech Republic) in Prague. Magnetic particles were separated on a Dynal MPC-M magnetic particle concentrator (Oslo, Norway).

Methods

The type strain *Lactococcus lactis* subsp. *lactis* CCM 1877^T from the Czech Collection of Microorganisms was used as a control strain. It was cultivated on MRS agar with 0.5 % of glucose at 30 ºC for 48 h. The cells from 1.5 ml culture were centrifuged (14,000 g 5 min^{-1}), washed in water, and resuspended in 500 µl of lysis buffer (10mM Tris, pH 7.8, 5mM EDTA pH 8.0, lysozyme 3 mg ml^{-1}). After 1 hour, 12.5 µl of 20% SDS and 5 µl of proteinase K (10 µg ml^{-1}) was added and incubated at 55 °C overnight. DNA was extracted using phenol methods[7], precipitated with ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.8).

The DNA from hard cheese samples was isolated from crude cell lysates from cheese filtrates by the phenol extraction procedure[7] (control) and by magnetic microspheres (see later). Magnetic microspheres P(HEMA-*co*-EDMA) containing carboxyl groups (100 µl, 2 mg ml^{-1}) were added to the crude cell lysates (100 µl) together with 5M NaCl (400 µl), 40% PEG 6000 (200 µl) and water to a volume of 1,000 µl (200 µl). After 15 minutes of incubation at laboratory temperature, the microspheres with bound DNA were separated using magnet, washed in 70% ethanol, and DNA was eluted into 100 µl of TE buffer.

Species-specific PCR primers PALA4 and PALA14 (targeted on *acm A* gene encoding *N*-acetylmuramidase specific to *Lactococcus lactis*, 1131 bp long PCR products)[1] were used for the identification of *Lactococcus lactis* species. The PCR mixture contained 1 µl of each 10mM dNTP, 1 µl (10 pmol µl^{-1}) of each primer, 1 µl of Taq 1.1 polymerase (1 U µl^{-1}), 2.5 µl of buffer (1.5mM), 1–3 µl of DNA matrix, and PCR water was added up to a 25 µl volume. The amplification reactions were carried out using the following cycle parameters: 5 min of the initial denaturation period at 94 °C (hot start), 60 s of denaturation at 94 °C, 60 s of primer annealing at 45 °C, and 60 s of extension at 72 °C. The final polymerisation step was prolonged to 10 min. PCR was performed in 30 cycles. The PCR products were separated and identified using electrophoresis in 1.5% agarose gel. The DNA on the gel was stained with ethidium bromide (0.5 µg ml^{-1}), observed on a UV transilluminator (305 nm), and documented.

Results and Discussion

Pre-PCR processing procedures have been developed to remove or reduce the effects of PCR inhibitors from hard cheese samples. Ten different cheese samples were used. The method of DNA isolation using magnetic microspheres was evaluated. Different amounts of cheese and different procedures of their homogenisation were tested at first. The best results were achieved with cheese samples (1 g of cheese 1.5 ml^{-1} of sterile water) homogenised in a grinding mortar. The hard pieces of cheese samples were removed using filtration through sterile gauze. The fat layer was removed from the filtrates by pipetting. The cells in the filtrates (1.5 or 3 ml) were centrifuged (10,000 g 5 min^{-1}), washed with 1 ml of sterile water, and resuspended in 1 ml of lysis buffer with lysozyme and treated with laboratory temperature (see above). Then, the procedure of DNA isolation using magnetic microspheres was used (see above). In PCR 1 and 3 μl of DNA matrix were used. The results with 3 μl of DNA matrix are shown in Fig. 1. In all samples prepared from 3 ml of filtrate, PCR products specific to *Lactococcus lactis* were amplified.

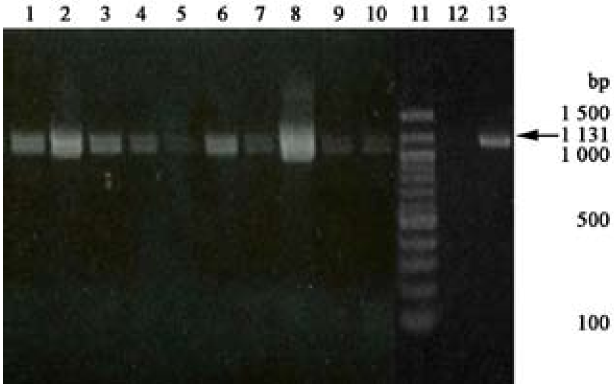

Fig. 1. **Agarose gel electrophoresis of PCR products obtained after amplification of *Lactococcus lactis* DNA (3 μl) isolated from hard cheese samples by magnetic microspheres. Lanes 1-10: hard cheese samples, lane 11: DNA standards (100 bp ladder), lane 12: negative control without DNA, lane 13: positive control with purified *Lactococcus lactis* subsp. lactis CCM 1877T DNA (c_{DNA} = 10 ng μl^{-1})**

Conclusion

The evaluated procedure of hard cheese sample preparation for DNA isolation using magnetic microspheres was verified on 10 different cheese samples with success. The quick and more simple method of DNA isolation with magnetic microspheres gave results comparable to those with DNA isolated using phenol extraction.

The financial support of the National Grant Agency for Agricultural Research (NAZV) grant No. 1G 57053 and a long-term research programme of the Ministry of Education, Youth, and Sports of the Czech Republic (MSM 0021622415) are gratefully acknowledged. We thank Mr. Ladislav Červený for his kind language revision.

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P96 IDENTIFICATION OF BACTERIAL STRAINS OF *STREPTOCOCCUS THERMOPHILUS* SPECIES USING PCR

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Introduction

Most molecular diagnostic studies of lactic acid bacteria have been focused on the strains of *Lactobacillus* and *Bifidobacterium*[1–4] genera due to their probiotic potential and commercial exploitation in food industry. Undeservedly less attention has been devoted to the identification of *Streptococcus thermophilus* in the literature. *Streptococcus thermophilus* is namely one of the most widely used lactic acid bacteria in the dairy industry as a starter culture component in the manufacture of fermented dairy products, such as yoghurt and cheese, and in probiotic preparations. Identification of *Streptococcus thermophilus* is therefore important. Classical methods based on biochemical characterisation are in many cases insufficient. For this reason, specific PCR primers for identification *of Streptococcus thermophilus* cells were described in the literature[5,6].

The aim of this work was to isolate PCR-ready DNA from yoghurt and milk drink samples containing PCR inhibitors. Carboxyl-functionalised magnetic poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) microspheres (P(HEMA-*co*-EDMA)) were used for DNA isolation. The quality of extracted DNA was checked by PCR specific to *Streptococcus thermophilus*.

Material and Methods

Chemicals and Equipment

Primers for PCR were synthesised by Generi-Biotech (Hradec Králové, Czech Republic); *Taq*I DNA polymerase was from Bio-Tech (Prague, Czech Republic), DNA ladder 100 bp from Malamité (Moravské Prusy). The PCR reaction mixture was amplified on an MJ Research Programme Cycler PTC-100 (Watertown, USA).

Magnetic nonporous poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) P(HEMA-*co*-GMA) microspheres containing carboxyl groups were prepared according to the previously described procedure[7] in the Institute of Macromolecular Chemistry (Academy of Sciences of the Czech Republic) in Prague. Magnetic microspheres were separated on a Dynal MPC-M magnetic particle concentrator (Oslo, Norway).

Methods

The strain *Streptococcus thermophilus* CCM 4757 from the Czech Collection of Microorganisms was used as a control strain. It was cultivated on MRS agar with 0.5 % of glucose at 30 ºC for 48 h. The cells from 1.5 ml culture were centrifuged (14,000 g 5 min^{-1}), washed in water, and resuspended in 500 µl of lysis buffer (10mM Tris, pH 7.8, 5mM EDTA pH 8.0, lysozyme 3 mg ml^{-1}). After 1 hour, 12.5 µl of 20% SDS and 5 µl of proteinase K (10 µg ml^{-1}) were added and incubated at 55 °C overnight. DNA from these crude cell lysates was extracted using phenol method[8], precipitated with ethanol and dissolved in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.8).

Altogether 2 g of yoghurt (milk drink) samples resuspended in 2 ml water was centrifuged (12,000 g 5 min^{-1}) to sediment the bacteria cells. The cells in the sediments were washed with 1 ml of sterile water and lysed. DNA from yoghurt samples was isolated from crude cell lysates by the phenol extraction procedure (control) (see above) and by magnetic microspheres (see later). Magnetic microspheres P(HEMA-*co*-EDMA) (100 µl, 2 mg ml^{-1}) containing carboxyl groups were added to the crude cell lysates (100 µl) together with 5M NaCl (400 µl), 40% PEG 6000 (200 µl) and water to a volume of 1,000 µl (200 µl). After 15 minutes of incubation at laboratory temperature, the microspheres with bound DNA were separated using magnet, washed in 70% ethanol, and DNA was eluted into 100 µl of TE buffer.

Species-specific PCR primers were used for the identification of *Streptococcus thermophilus* species[9]. The PCR mixture contained 1 µl of each 10 mM dNTP, 1 µl (10 pmol µl^{-1}) of each primer, 1 µl of Taq 1.1 polymerase (1 U µl^{-1}), 2.5 µl of buffer (containing 1.5 mM Mg^{2+} ions), 1–5 µl of DNA matrix, and PCR water was added up to a 25 µl volume. The amplification reactions were carried out using the following cycle parameters: 5 min of the initial denaturation period at 95 °C (hot start), 60 s of denaturation at 95 °C, 60 s of primer annealing at 58 °C, and 60 s of extension at 72 °C. The final polymerisation step was prolonged to 10 min. PCR was performed in 30 cycles. The PCR products were separated and identified using electrophoresis in 1.5% agarose gel. The DNA on the gel was stained with ethidium bromide (0.5 µg ml^{-1}), observed on a UV transilluminator (305 nm), and documented on a TT667 film using a CD34 camera.

Results and Discussion

Fermented diary products (8 white and fruit yoghurts and 2 milk drinks) from the market were used for the analysis. Pre-PCR processing procedures have been developed to remove or reduce the effects of PCR inhibitors from tested samples. The quality of DNA in crude cell lysates of bacteria from yoghurt (milk drink) samples was proved (see Material and methods) using agarose gel electrophoresis. DNA in amounts from approximately 70 to 350 ng was detected. Then, the method of DNA isolation using magnetic microspheres was evaluated. The amounts of eluted DNA in all samples tested were smaller than the detection limit for gel electrophoresis, but these amounts were sufficient for PCR. Detection of specific 968 bp long PCR products is given in Fig. 1. in which PCR product intensities for different amounts of DNA matrix are compared. PCR products of higher inten-

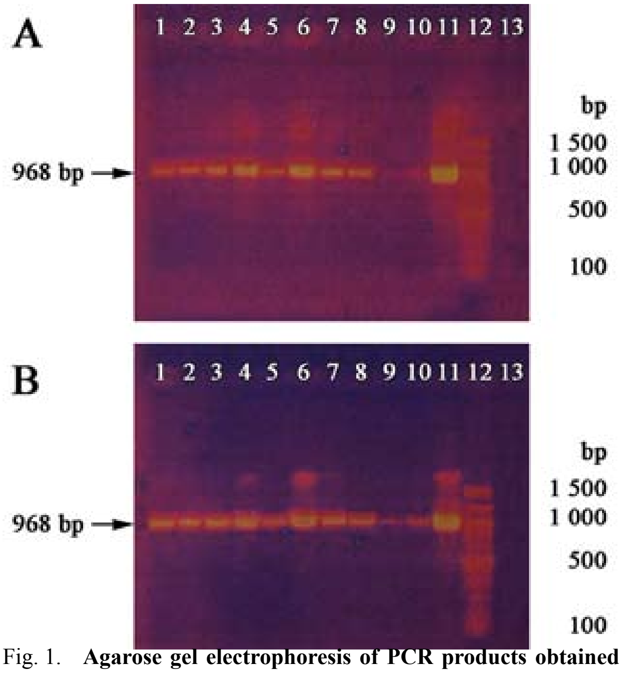

Fig. 1. **Agarose gel electrophoresis of PCR products obtained after amplification of *Streptococcus thermophilus* DNA isolated from yoghurt and milk drink samples by magnetic microspheres. Lanes 1-8: yoghurt samples, lanes 9,10: milk drinks, lane 11: positive control with purified Streptococcus thermophilus CCM 4757 DNA ($c_{DNA} = 10$ ng µl^{-1}), lane 12: DNA standards (100 bp ladder), lane 13: negative control without DNA. A: 1 µl of DNA matrix, B: 2 µl of DNA matrix**

sity were detected using higher amounts of DNA matrix. From these results it follows that the negative influence of PCR inhibitors in tested products on the PCR course was thus eliminated. DNA was isolated in a quality suitable for PCR.

Conclusion

The method of DNA isolation from yoghurt samples by magnetic microspheres was evaluated. Using the proposed DNA isolation process, PCR products specific to *Streptococcus thermophilus* were amplified in all samples. The results presented here show that the simple method proposed is suitable for the isolation of whole DNA from yoghurt and milk drink samples, which can be used for PCR.

The financial support of the National Grant Agency for Agricultural Research (NAZV) grant No. 1G 57053 and a long-term research programme of the Ministry of Education, Youth, and Sports of the Czech Republic (MSM 0021622415) are gratefully acknowledged. We thank Mr. Ladislav Červený for his kind language revision.

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P97 STUDY OF OXIDATION STABILITY OF SELECTED VEGETAGLE OILS

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Introduction

Vegetable oils are obtained from different parts of oilseed plants by cold expression and subsequent extraction and purification. Since long ago, oils belong to among basic cosmetic preparations and use in pharmaceuticals, food industry and other industrial purposes. They embody the whole series of authenticated positive efects on human organism and they are used as a part of food supplements[1].

Food lipids undergo a chain of changes due to ripening, harvesting, processing and storage. These changes are caused by several factors including browning reactions, microbial spoilage and lipid autoxidation. Lipid oxidation is a free-radical chain reaction that causes a total change in the sensory properties and nutritive value of food products[2].

Oxidation processes influence quality of oils and fats. Characteristic changes associated with oxidative deterioration include development of unpleasant tastes and odours as well as changes in color, viskosity, density or solubility[3]. The others of the effects of lipid oxidation can be loss of vitamins, and damage to proteins[2].

Differential scanning calorimetry (DSC) is the technique used for establishing the oxidative stability of oils and fats and characterizion of their physical properties[4,5]. Oxidative stability and deterioration of oils depend on initial composition, concentration of minor compounds with antioxidant or prooxidant characteristics, degree of processing, and storage conditions[6]. Quality and stability of vegetable oils are important factors that influence its acceptability and market value.

The induction period (IP) is measured as the time required to reach an endpoint of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation[7].

Experimental

Samples

Different vegetable oils from various plant origins were used in this study. These were Grape Seed oil refined, Crude Linseed oil, Castor oil, Almond oil refined, Soya Bean oil refined, Avocado oil refined, Apricot Kernel oil refined, Corn oil refined and Olive oil refined.

Samples were obtained from M + H, Míča and Harašta, s.r.o. After opening the bottles with oils there were stored at 4 °C.

Method

Differential Scanning Calorimetry (DSC) of each sample in oxygen atmosphere were performed several times until reproducible results were obtained. For this purpose Shimadzu DSC-60 (Kyoto, Japan) was used connected through TA-60WS with computer, where the data were collected.

The furnace was calibrated by using transition temperatures of fusion of indium and zinc (melting point: 156.6 °C for indium, 419.6 °C for zinc).

Samples were measured out 2.5 µl (0.2, 0.5 $°\mathrm{C\,min^{-1}}$), 5.0 µl (1.0, 3.0, 5.0, 7.0 $°\mathrm{C\,min^{-1}}$) and 10 µl (10.0, 15.0 $°\mathrm{C\,min^{-1}}$) and measured in open aluminum pan. Flow rate of oxygen was set at 15 $\mathrm{ml\,min^{-1}}$ and rate of heating 0.2, 0.5, 1.0, 3.0, 5.0, 7.0, 10.0 and 15.0 $°\mathrm{C\,min^{-1}}$ from room temperature (25 °C) to 300 °C was applied. Obtained results were treated by means of enclosed software TA-60.

Results

For the using vegetable oils, the kinetic parameters important to the determination of induction period were obtained for non-isothermal DSC measurements. The onset temperatures of oxidation for various using oils were measured with scan rates 0.2, 0.5, 1.0, 3.0, 5.0, 7.0, 10.0 and 15.0 $°\mathrm{C\,min^{-1}}$.

The parameters A and B were obtained by a comparison of experimental and theoretical values of onset temperatures of the oxidation peaks using the program TIND. The values of kinetic parameters A and B are listed in the Table I.

Table I
Values of the kinetic parameters A and B

Oils	A[min]	B[K]
Castor	2.72×10^{-13}	1.38×10^{4}
Olive	6.62×10^{-13}	1.32×10^{4}
Soya Bean	1.54×10^{-11}	1.16×10^{4}
Avocado	4.69×10^{-11}	1.12×10^{4}
Grape Seed	4.43×10^{-12}	1.19×10^{4}
Almond	5.26×10^{-11}	1.10×10^{4}
Corn	1.00×10^{-10}	1.08×10^{4}
Apricot Kernel	1.29×10^{-10}	1.06×10^{4}
Crude Linseed	8.99×10^{-12}	1.12×10^{4}

The temperature dependence of the induction period can be expressed according to the Equation (1):

$$t_i = A \exp (B/T) \qquad (1)$$

Providing that parameters A [min] and B [K] were obtained by minimizing the sum of squares between experimental and theoretical values of heating rate and T [K] is specific temperature, induction period t_i [min] can be determined.

Induction period values (t_i) of various using vegetable oils calculated for temperature 25 °C and 100 °C are shown in Table II.

Table II
Values of the induction periods – extrapolation to 25 °C (t_i^{25}) and extrapolation to 100 °C (t_i^{100})

Oils	t_i^{25} [min]	t_i^{100} [min]
Castor	3.00×10^7	2.81×10^3
Olive	1.28×10^7	1.70×10^3
Soya Bean	1.34×10^6	5.29×10^2
Avocado	1.03×10^6	5.37×10^2
Grape Seed	9.87×10^5	3.22×10^2
Almond	5.93×10^5	3.52×10^2
Corn	5.44×10^5	3.80×10^2
Apricot Kernel	3.31×10^5	2.65×10^2
Crude Linseed	1.79×10^5	9.49×10^1

In the case low temperature (25 °C), sequence of stability vegetable oils was falled: castor > olive > soya bean > avocado > grape seed > almond > corn > apricot kernel > crude linseed.

Enormously high induction period values, especially castor and olive oil, appeared unreal. By this reason, the extrapolation on high temperature (100 °C) was applied.

From the determined values it is obvious that castor and olive oil had the longest induction period and apricot kernel and crude linseed oil had the shortest induction period. In the case soya bean and avocado oil, it was obtained the similar results and the values of the both oils differed about 8 minutes.

Induction periods for individual oils were determined from the known values onset temperatures and parameters A and B. Equation 1 was used for calculation. Dependences of the experimental calculated induction periods on the temperatures are demonstrated in Fig. 1.

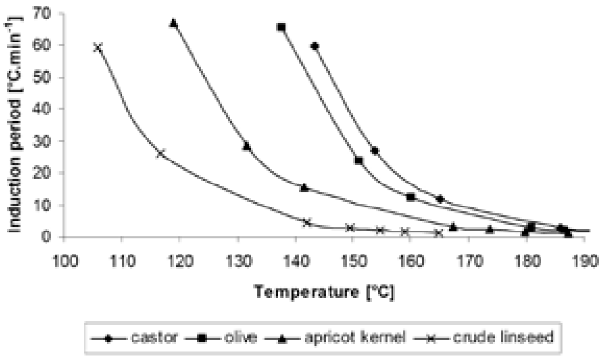

Fig. 1. **Temperature dependences of the induction periods for the most stable (castor and olive oil) and the least stable (apricot kernel and crude linseed oil) vegetable oils**

Conclusions

The order of stability of vegetable oils was identified by statistical comparison of record gained from various temperature extrapolation and reciprocal comparison of stability of given oils. The order of the most stable oils (castor and olive oil) and the least stable oil (linseed oil) depending on extrapolation and reciprocal comparison did not differ. The non-isothermal method DSC was employed with the aim to determine the induction period of vegetable oils.

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P98 ANTIOXIDANT PROPERTIES OF GRAPE SKINS EXTRACTS INVESTIGATED BY EPR AND UV-VIS SPECTROSCOPY

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Introduction

The growing attention is recently focused on the improvement of human health by the consumption of food or food supplements rich in antioxidants. Grape skins contain a plenty of different flavonoids, e.g., quercetin, catechins, flavonols, anthocyanidins, phenolic acid derivatives and other compounds. Anthocyanins, as the most abundant of them, are associated with the colour of several aerial and subterranean organs in many plants. In grapevines, anthocyanins are accumulated in leaves during senescence and are responsible for the colouration of grape skins in red and rosé cultivars, and in the grape pulp, respectively. These pigments are water soluble and reveal the beneficial effects on human health, including the enhancement of visual acuity, but evinced also anticarcinogenic, antimutagenic or anti-inflammatory action. The reduction of coronary heart disease and thus the phenomenon known as the "French Paradox" is attributed to them, as well.[1-5]

Main interest is paid to the isolation of phenolic compounds from grapes and wine. A great effort is given to choose a suitable extraction system for anthocyanins isolation, as they are highly reactive compounds and exceptionally sensitive to pH changes[6,7]. Supercritical fluid extraction with carbon dioxide and pressurized liquid extraction with acidified water, sulphured water or acidified organic solvents were successfully applied for the extraction of different phenolic compounds from grapes and wines in the past.[8-11]

In this contribution, the complex study of grape skin methanolic extracts, prepared by Pressurized Fluid Extraction (PFE)[12,13] from two wine grape varieties, St. Laurent and Alibernet from Velké Pavlovice and Mikulov sub-regions (South Moravia region, Czech Republic) is presented.

Experimental

Respective methanolic extracts were prepared from three different amounts (0.5 g, 1.0 g and 1.5 g) of either crude dried grape skins (St. Laurent) or lyophilized grape skin powders (Alibernet) using the PFE extraction at different temperature (40 °C–120 °C) and pressure of 15 MPa[12, 13].

Antioxidant activity of extracts was tested by EPR spectroscopy using the Bruker portable EPR spectrometer e-scan with accessories in Fenton system (H_2O_2/Fe^{2+}), generating reactive hydroxyl radicals ($^{\bullet}OH$) followed by spin trapping technique, using the 5,5-dimethylpyrroline-N-oxide (DMPO) as spin trap[14]. In addition, radical scavenging activity of extracts was assessed applying 2,2-diphenyl-1-picrylhydrazyl ($^{\bullet}$DPPH) free radical and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) assays. All the experiments were performed at 298 K, using the same quartz flat cell. EPR spectra were processed similarly as previously described elsewhere[15].

Total phenolic compounds' content (TPC) of individual extracts was determined using the Folin-Ciocalteu assay and their tristimulus colour values (CIE Lab) were estimated, using the UV-VIS spectrophotometer Specord (Carl Zeiss, Jena, Germany)[16,17].

In addition, pH values of all extracts were also measured using the combined glass electrode.

All the data obtained were subsequently correlated and discriminated, using the multivariate statistics, involving the canonical discriminant analysis, principal component analysis, and canonical correlation analysis, respectively.

Results

EPR Experiments

Antioxidant properties of grape skin extracts were tested in experimental systems, in which free radicals were generated via Fenton reaction. Fig. 1. shows typical time evolution of EPR spectra recorded in system containing respective

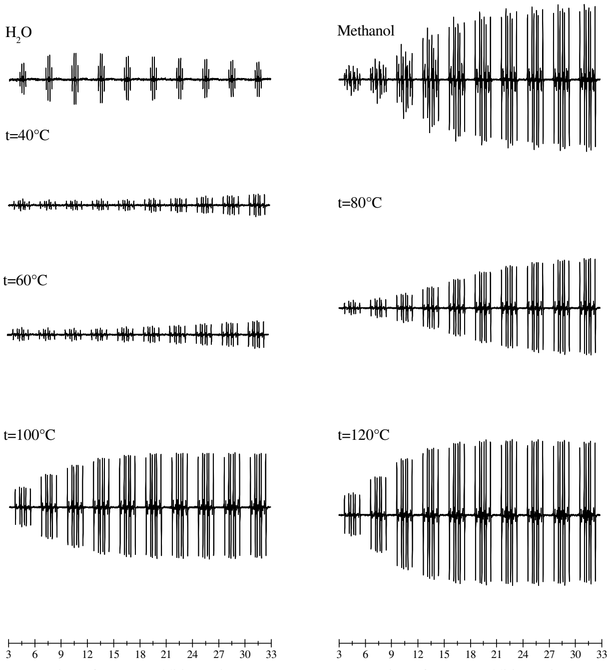

Fig. 1. **The time evolution of EPR spectra recorded in system containing deionised water, methanol (reference) and methanolic extracts of St. Laurent variety prepared from 1.5 g of crude grape skins at 40–120 °C, respectively; Fenton's reagents and DMPO spin trap. Spectra were recorded at 298 K using magnetic field sweep, SW = 8 mT**

grape skin extract of St. Laurent wine variety and Fenton's reagents, in the presence of DMPO spin trap. As follows from simulation analysis, in the presence of pure water, dominantly the formation of $^{\bullet}$OH radicals is observed, whereas in pure methanol (reference sample), the formation of different types of carbon-centred radicals is to be found, in accord with our previous experiments and already published data.[14,15,18] The addition of grape skin extracts into system resulted in the decrease of spin adduct concentration as a result of competitive reactions between the antioxidants, generated free radicals and spin trap.[15,18,19]

Staško et al., suggested previously the expression of antioxidant activity of Tokay wines as the relative amounts of radicals scavenged[19]. Using this approach, we can conclude that antioxidant properties of St. Laurent variety extract is significantly lower than that of Alibernet (66 % and 89 %, respectively, for extracts prepared from 1.0 g at 40 °C). As we moreover proved, antioxidant activity of extracts is in both cases strongly dependent on extraction temperature, falling down to 9 % for St. Laurent extract prepared from 1.0 g at 120 °C, whereas that of Alibernet descended practically to zero.

$^{\bullet}$DPPH and ABTS$^{\bullet+}$ radicals assays are traditionally used for food samples radical-scavenging ability (RSA) evaluation. They were previously successfully used, e. g., for tea or wine samples characterisation.[15,18-20] As we confirmed, extracts of both varieties prepared at 40 °C demonstrated significant ability to terminate $^{\bullet}$DPPH and ABTS$^{\bullet+}$.

Pellegrini et al. suggested the effective comparison of RSA for different food products, based on Trolox-equivalent antioxidant capacity (TEAC) calculation[21]. Following this approach, TEAC value of each extract was calculated for reactions with both $^{\bullet}$DPPH and ABTS$^{\bullet+}$. As follows from the results obtained, the increasing extraction temperature TEAC$_{\mathrm{ABTS}^{\bullet+}}$ values of St. Laurent variety (Fig. 2.) are slightly lower in comparison to that of Alibernet, only slightly influenced by extraction temperature. On the other hand, TEAC$_{\mathrm{DPPH}}$• values differ significantly, in the case of St. Laurent extracts prepared from 1.0 g, they range from 1.50 to 1.75, whereas in the case of Alibernet from 3.7 to 4.0.

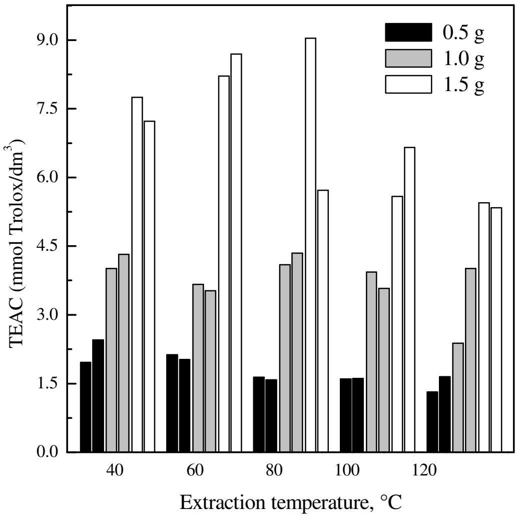

Fig. 2. **Trolox equivalent antioxidant capacity (TEAC) of St. Laurent extracts, prepared at different temperature from different mass of crude grape skins in the reaction with ABTS$^{\bullet+}$**

UV-VIS Experiments

The TPC content of all extracts under study was monitored and expressed as Gallic acid equivalent (GAE)[16]. The results obtained indicate that the content of polyphenols in Alibernet variety extracts is more than two times higher than that of St. Laurent. This would explain generally higher antioxidant and radical-scavenging activities of Alibernet extracts. Significant correlation (>85%, P>0.01) between the TPC and TEAC for both types of extracts was achieved, in dependence on the mass of grape skins used for the extract preparation. As follows from data presented in Table I, correlation is better for St. Laurent varieties, probably due to different composition of polyphenols.

Table I
Correlation matrices between the TPC content and radical-scavenging abilities of grape skin extracts

	St. Laurent		
	TPC	TEAC$_{\mathrm{ABTS}^{\bullet+}}$	TEAC$_{\bullet\mathrm{DPPH}}$
TPC	1	0.9778	0.9518
TEAC$_{\mathrm{ABTS}^{\bullet+}}$	0.9778	1	0.9060
TEAC$_{\bullet\mathrm{DPPH}}$	0.9518	0.9060	1
	Alibernet		
	TPC	TEAC$_{\mathrm{ABTS}^{\bullet+}}$	TEAC$_{\bullet\mathrm{DPPH}}$
TPC	1	0.8650	0.9220
TEAC$_{\mathrm{ABTS}^{\bullet+}}$	0.8650	1	0.6909
TEAC$_{\bullet\mathrm{DPPH}}$	0.9220	0.6909	1

As a result of growing extraction temperature from 40 °C up to 120 °C, approx. 20% and 40% decrease of the TPC content in extracts of St. Laurent and Alibernet varieties was noticed, respectively.

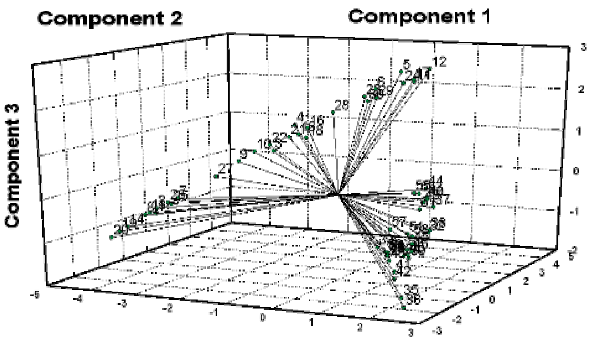

Fig. 3. **Classification – recognition of both wine grape varieties (1–30 – St. Laurent, 31–60 – Alibernet) based on principal component analysis**

The influence of extraction temperature on CIE L^*, a^*, b^* colour characteristics or on pH values was only negligible.

Statistical Evaluation

All the above mentioned characteristics were used for the classification and mutual recognition of both wine grape varieties using the principal component analysis (Fig. 3.) and canonical discrimination analysis[22].

Both statistical approaches provided practically 100% correct classification of both varieties. As also follows from discriminant analysis, all the monitored characteristics are dependent on the mass of grape skins used for respective extract preparation.

Additional experiments are in progress to evaluate the influence of solvent on the above-mentioned characteristics as well as to analyze the profile of polyphenols in both grape skin varieties.

Conclusions

Multi-experimental analysis performed with methanolic extracts prepared from grape skins of two varieties (St. Laurent and Alibernet) by PFE, revealed their significant antioxidant and radical-scavenging abilities, in significantly positive correlation with the TPC content. The influence of extraction temperature on radical – scavenging abilities, tristimulus values or TPC content is non-significant.

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P99 THE IMPACT OF FERMENTATION PROCESS ON AMINO ACID PROFILE OF GRÜNER VELTLINER WHITE WINE

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Introduction

Amino acids represent 30–40 % of total wine nitrogen. It is generally accepted, that they may act as nutrients for both yeast and malolactic bacteria during the wine fermentation. In the yeasts' growing phase, they can be either partially or totaly metabolised, excreted by living yeasts at the end of the fermentation or released by proteolysis during the autolysis of the dead yeasts. In addition, amino acids can serve as substrate for the production of aroma compounds [3–5] or biogenic amines[6,7] in wine. On the contrary, their production by enzymatic degradation of the grape proteins was described[1,2].

There is no doubt, that their content in wines is dependent on the fertilization and climatic conditions, duration of skin maceration in the must, field treatments and on other factors[2,6–8]. Recently, amino acids' content in wines is studied extensively for several reasons, e. g, due to the differentiation of Champagnes and sparkling wines[2], the determination of geographic origin, variety and vintage[2,7,8], the assessment of substances with enological interest (e.g., of aroma compounds)[3–5], or toxicological interest (biogenic amines etc.)[6,7,9,10], the monitoring of nitrogen metabolism during the fermentation[5,11] or their changes during the ageing of wines[12,13].

The determination of amino acid in wines is usually employed by HPLC with precolumn derivatization using *o*-phthaldialdehyde (OPA), fluorenylmethyl-chloroformate (FMOC) or 6-aminoquinolyl-N-hydroxysuc-cinimidyl carbamate (AQC).[2,4,6–8,10–14] Ion-exchange chromatography with post-column derivatization with ninhydrin reagent[1,9,15] or comprehensive two-dimensional gas chromatography[16] was successfully applied, as well.

In this contribution, the impact of spontaneous and inoculated fermentations on amino acids profile of Grüner Veltliner (*Vitis vinifera*) white wine is presented, applying an ion exchange chromatography with post-column derivatization with ninhydrin and spectrophotometric detection system.

Experimental

Wine Sample

Grüner Veltliner (*Veltlínské zelené*) white wine variety from Velké Pavlovice sub-region (South Moravia region, Czech Republic) was investigated. Wine samples were prepared by two different technological processes. In the first one, the spontaneous fermentation was used, whereas in the second, wine was prepared by inoculated fermentation. The sampling procedure was performed every other day continuously, starting from cider up to the final young wine.

Chromatographic Conditions

Respective wine sample was analysed to free amino acids (FAA) content after its filtration and dilution in Li-citric buffer (pH 2.2), employing the AAA T339 amino acid analyser (Mikrotechna, Prague, Czech Republic) equipped with Ostion LG ANB ion-exchange resin column. Amino acids separation was achieved by stepwise gradient elution using the Li-citric buffer system. Post-column derivatization with ninhydrin reagent and spectrophotometric measurement was used for their determination.

Results

Concentration profile of the following 36 compounds potentially present in wine samples under study was investigated: cysteic acid (CystAC), tautine (Tau), phosphoethanolamine (PE), urea, aspartic acid (Asp), hydroxyproline (Hpro), threonine (Thr), serine (Ser), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), α-aminoadipic acid (α-AAD), proline (Pro), glycine (Gly), alanine (Ala), citrulline (Cit), α-aminobutyric acid (α-ABA), valine (Val), cysteine (Cys), methionine (Met), cystathionine (Cysne), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), α-alanine (α-ALA), β-aminobutyric acid (β-ABA), γ-aminobutyric acid (GABA), ethanolamine (EA), ammonium (NH_3), ornithine (Orn), lysine (Lys), histidine (His), 1-methyl histidine (1-met His), 3-methyl histidine (3-met His), arginine (Arg).

Results obtained showed, that total FAA concentration in wine samples prepared by spontaneous fermentation ranged from 302.30 mg dm^{-3} up to 1,578.25 mg dm^{-3}, whereas in those prepared by inoculated fermentation, from 289.64 mg dm^{-3} up to 1,371.41 mg dm^{-3}.

In the sample prepared by a spontaneous fermentation, the total concentration of free amino acids slowly increased during the first three days from 1,299.75 g dm^{-3} to the maximum of 1,371.47 mg dm^{-3}, then in next six days of fermen-

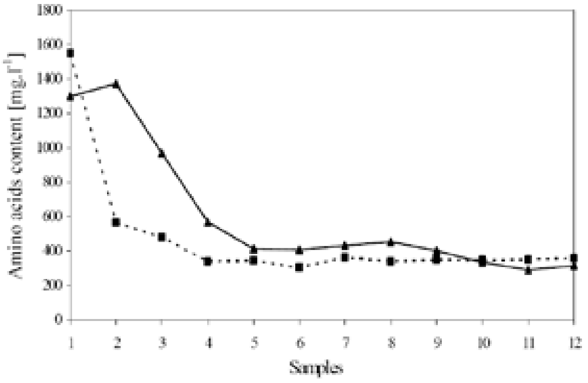

Fig. 1. **Total free amino acids content monitored during 24 days in the wine samples prepared by spontaneous (▲) and inoculated (■) fermentation processes**

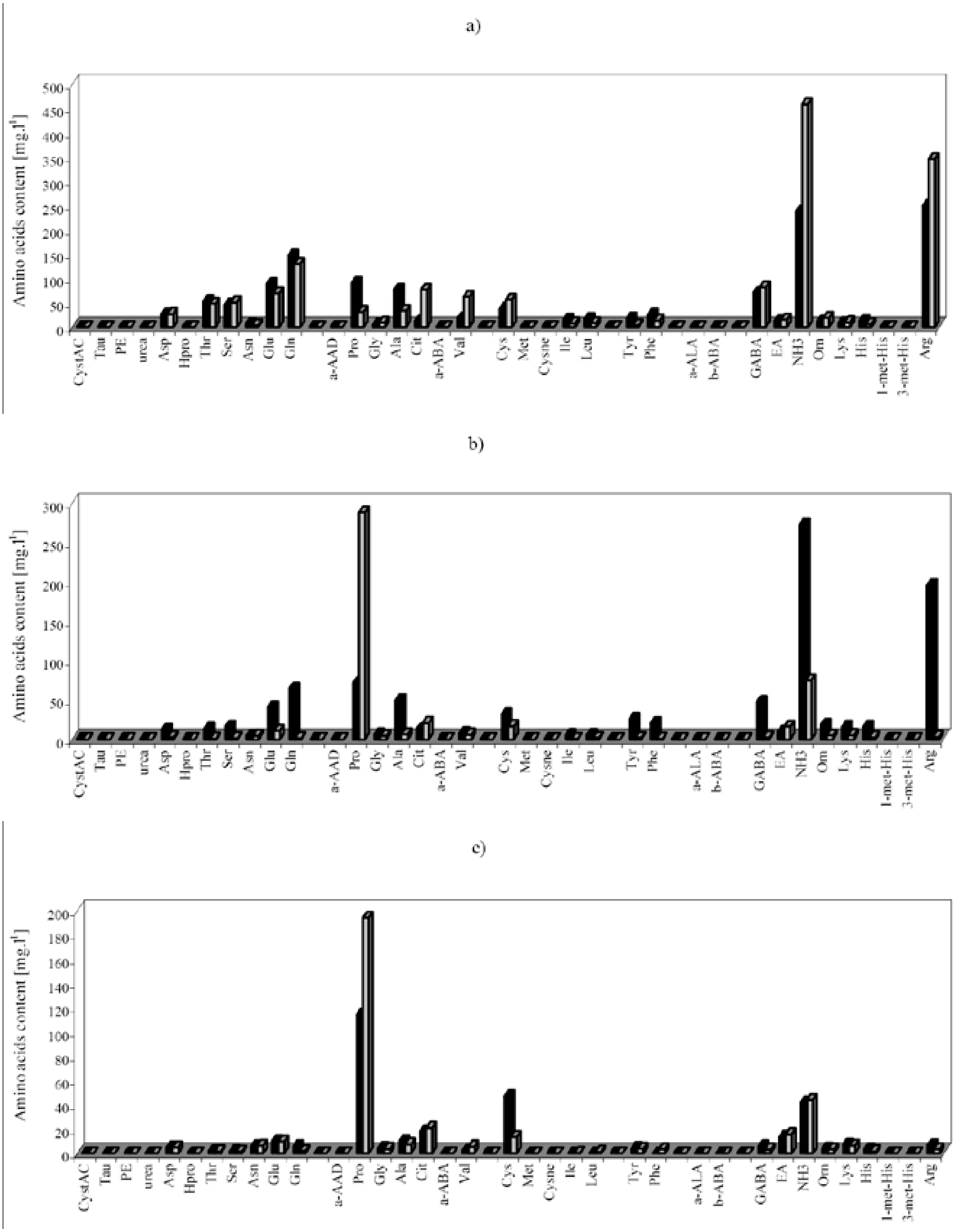

Fig. 2. **The content of individual amino acids in a) cider, b) semi – fermented product, c) young wine prepared by spontaneous (black fill) and inoculated (gray fill) fermentation**

tation, the gradual decrease was noticed, falling down to 412.92 mg dm^{-3}. The continous fermentation proces caused their decrease to 289.64 mg dm^{-3} (Fig. 1.). The content of some amino acids, e.g. of Asp, Thr, Ser, Glu, Gln, Val, Ile, Leu, Ala, GABA, Orn, His and Arg revealed exactly the same decreasing trend as described above, whereas the content of Asp, Thr, Ser, Glu, Gln, Ala, Leu and Ile decreased after seven days of fermentation practically to zero.

In the so-obtained young wine, the total amino acids content was found to be 313.38 mg dm^{-3}. Proline was identified as the most abundant of them (113.86 mg dm^{-3}), followed by cysteine (47.18 mg dm^{-3}) and citrulline (18.32 mg dm^{-3}). Majority of amino acids identified in young wine reached the concentration level of 3–15 mg dm^{-3}, with the exception only for Tyr, Ser and Phe with the concentration lower than 3 mg dm^{-3}.

In the sample prepared by inoculated fermentation, a rapid decrease of total amino acids concentration from 1,578.25 mg dm^{-3} to 567.37 mg dm^{-3} was observed right at the begining of the fermentation process. During the next four days, only slow decrease of the amino acids content was detected, as also depicted on Fig. 1.

The same amino acids as in the case of spontaneous fermentation were found to influence the decrease of the total FAA content. On the other hand, the concentration of Asp, Tyr, Gly, Lys, Orn and EA amino acids remained practically unaffected by the fermentation.

Total amino acids content in young wine after twenty four days of fermentation was 358.13 mg dm^{-3} (Fig. 1., Fig. 2.).

The results obtained are in good agreement with the amino acid values determinated in other wine varieties and viticultural regions[15,22].

Conclusions

Twenty-three different amino acids were positively identified in Grüner Veltliner white wine. For the first time, the dependence of the amino acids' profile on wine-making technologies is presented. The type of fermentation influences also the concentration of amino acids. This approach could be effectively used for the classification and identification of wines.

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P100 CHARACTERIZATION OF NEUTRAL LIPID COMPOSITION OF *NIGELLA SATIVA* L. SEEDS OIL

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Introduction

Nigella sativa L. is an annual herbaceous plant which belongs to the family of *Ranunculaceae,* widely cultivated in Middle East, Asia and Northern Africa. The seeds, known as black cumin, are used for culinary purposes, as a spice for pastry and bakery. *Nigella* seeds and oil are used in arabian folk medicine for the treatment of asthma, as carminative, diuretic or lactogogue[1,2]. More recently *Nigella sativa* fixed oil showed gastroprotective, hepatoprotective and antitumour effects *in vivo*[3,4]. The biological effects of the oil are mainly atributable to thymoquinones, known as powerfull antioxidants. Other components such are polyunsaturated fatty acids, phytosterols and tocopherols can contribute to the beneficial effects of the black cumin seeds.

The lipid composition, including lipid classes, fatty acids and sterols of *Nigella* seeds of various geographical origins were reported[5,6,7]. The fixed oil contains high levels of unsaturated fatty acids and an unusual fatty acid – 20 : 2 (11c, 14c) – considered as a chemotoaxanomic marker for *Nigella* species. The main sterols reported in black cumin seeds were β-sitosterol, stigmasterol and campesterol[6,7]. The aim of this work was the study of lipid fraction in the oil of *Nigella* seeds cultivated in Romania, as a potential nutraceutical or ingredient of functional food.

Experimental

Extraction and Lipid Fractionation

Total lipids were extracted with chloroform : methanol (2 : 1; v/v), evaporated to dry and kept –20 °C in use. Neutral lipids were separated by TLC with a solvent mixture of hexane: ethyl ether: acetic acid (95 : 15 : 1).

Fatty Acids Analysis

The fatty acids methyl esters of total lipids and lipid fractions (FAME) were obtained by transesterification with BF_3/methanol. A Shimadzu GC 17A with FID detector and a Crompack Silica 25 MXO capillary column (25 m × 0.25 mm i.d., film thickness 0.25 µm) was used. The temperature program was: 5 min at 150 °C, 4 °C min^{-1} to 235 ° C (hold 5 min). The injector temperature was 260 °C and the detector temperature –260 °C. The carrier gas was helium.

Sterols Analysis

A part of total lipid extract was saponified by refluxing with 1M KOH ethanol/water (8 : 2, v/v) solution for 1 h. The unsaponifiables were then extracted first with petroleum ether and diethyl ether.

The sterols were derivatized BSTFA containing 1% TMCS in pyridine and separated on fused silica capillary column coated with 5% phenyl/95% dimethylpolysiloxane (30 m × 0.25 mm i.d., film thickness 0.25 µm; Rtx-5; Restek Corporation, Bellefonte, PA, USA). The same GC system mentioned above was used, with the following temperature program: 5 min at 200 °C, 10 °C min^{-1} to 300 °C (hold 20 min).

FAME and sterols peaks were identified by comparison of their retention times with those of commercially available standards (Sigma). All extractions and GC-FID runs were performed in triplicate and mean values were calculated.

Results

Nigella seeds are characterized by high oil content, ranging between 30–40 % of fresh weight. The Romanian cultivar investigated has a total lipid (TL) content of 30.1 g 100 seeds^{-1}. For German cultivar a value of 39.2 % oil in fresh seeds was reported[8]. The major fraction is the neutral lipids, representing 96.6 % of the oil while the polar lipids (PL) count for 3.4 %. The total sterols were found at 87 mg 100g^{-1} seeds, value which is lower than those reported for German cultivar but higher than for the Tunisian one (Table I)[6,7].

Table I
Nigella seeds oil composition

Fraction	Amount
Neutral lipids	29.1 ± 0.05 g 100 g^{-1} seeds
Polar lipids	0.9 ± 0.01 g 100 g^{-1} seeds
Total sterols	87.2 ± 0.02 mg 100 g^{-1} seeds
Total oil	30.1 ± 0.05 g 100 g^{-1} seeds

In the total lipids extract the major fatty acid was linoleic acid – 56.6 %, followed by oleic acid – 23.5 %. The most important saturated fatty acid was palmitic acid – 12.66 %. Saturated fatty acids represent 16 %, monounsaturated fatty acids – 23.8 % and polyunsaturated fatty acids account for more than 59 % of total lipids. The unusual eicosadienoic acid 20 : 2 (11c, 14c) was found at 2.5 %, value which is close to that reported for other cultivars.

The fatty acids composition of triacylglycerols (TAG) was closed to that of the TL. Diacylglycerols (DAG) and monoacylglycerols (MAG) contains lower palmitic and linoleic acid and higher oleic and eicosadienoic acid. The esterified sterols (SE) and the polar lipds (PL) contain the highest concentration of saturated fatty acids: palmitic (more than 17 %) and stearic (more than 4 %). The PL class also has the highest level of eicosadienoic acid. Miristic, linolenic and gadoleic acids were present in negligible amount in all lipid classes (Table II). Only traces of arachidic acid 20 : 0 were found. The sterol fraction contains: 37 % β-sitosterol, 11 % stigmasterol and 10 % campesterol as major compunds.

Tabulka II
Fatty acids composition of lipid classes [% of total fatty acids]

Fatty acid	TL	TAG	DAG	MAG	SE	PL
14:0	0.20	0.21	0.20	0.20	0.22	0.24
16:0	12.66	12.21	11.43	11.36	17.59	17.88
18:0	3.15	1.12	3.35	3.67	4.57	4.33
18:1 n-9	23.52	21.93	24.49	24.98	22.84	20.26
18:2 n-6	56.61	57.05	51.34	50.21	46.52	47.28
18:3 n-6	0.22	0.21	0.26	0.3	0.73	0.44
20:1 n-9	0.26	0.26	0.19	0.27	0.29	0.26
20:2 n-6	2.52	2.39	3.36	4.227	2.14	4.75

Conclusions

Black cumin seeds oil is exceptionally rich in biologically active polyunsaturated fatty acids, mainly linoleic acid. The presence of lipid-lowering phytosterols increases the nutritional value of the oil. There are small but non significant variations in the oil content and fatty acids composition in seeds of various origins which can be explained by genetic and environmental factors.

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P101 IDENTIFICATION OF VIABLE *LACTOBACILLUS* CELLS IN FERMENTED DIARY PRODUCTS

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Introduction

Lactobacillus and *Bifidobacterium* species are commonly found in foods and are members of the gastrointestinal tract of humans and animals. They are the most commonly used group of lactic acid bacteria (LAB) in the production of human probiotics. Methods for qualitative and quantitative control of probiotic products are required due to the growing interest in their commercial exploitation. Differentiation of viable and non-viable cells of LAB from a product is still problematic. Culture-dependent enumeration is relatively time-consuming and the results may be influenced by poor viability or low densities of the target organisms[1]. Rapid and reliable methods are needed for routine determination of initial cell counts in the inoculum or for viable cell estimation during the time period of storage. Therefore, culture-independent analysis as an alternative and/or complementary method for quality control measurements of probiotic products was developed. The combination of PCR and the ethidium monoazide (EMA-PCR) dye is a new method for selective distinction between viable and dead bacterial cells[2-4]. The general application of EMA is based on EMA penetration into dead cells with compromised cell-membrane (cell-wall) integrity. EMA is covalently linked to DNA by photoactivation and this DNA cannot be amplified in PCR. Viable cells remain intact and only DNA from these cells can be amplified and gives a PCR product.

The aim of this work was to optimise and use EMA-PCR for distinction between viable and dead *Lactobacillus* cells in real samples of dairy products (yoghurts).

Materials and Methods

Chemicals and Equipment

The primers for PCR were synthesised by Generi-Biotech (Hradec Králové, Czech Republic); *Taq*I DNA polymerase was from Bio-Tech (Prague, Czech Republic); DNA ladder 100 bp (Malamité, Moravské Prusy), and EMA from Sigma (St. Louis, USA). The *Lactobacillus paracasei* CCDM strain (obtained from the Czech Collection of Dairy Microorganisms, CCDM, Tábor, Czech Republic) was used for DNA isolation. The dairy products (white yoghurts before and after expiration date) were obtained from the market. The PCR reaction mixture was amplified on an MJ Research Programme Cycler PTC-100 (Watertown, USA).

Methods

Bacterial cells of *Lactobacillus paracasei* were cultivated at 37 °C aerobically in liquid MRS medium for 24 h or on MRS agar up to 48 hours, respectively. Altogether 1 ml of the cells was washed by water and resuspended in 500 µl lysis buffer (10 mM Tris-HCl, pH 7.8, 5 mM EDTA, pH 8.0, lysozyme 3 mg ml^{-1}), and incubated at laboratory temperature for 1 h; 10 µl proteinase K (1 µg ml^{-1}) and 2.5 µl SDS (20 %) (end concentration 0.5 %) was added and the mixture was incubated at 55 ºC for 18 h. DNA was extracted from crude cell lysates with phenol[5] and dissolved in TE buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA, pH 8.0). The concentration of DNA was estimated spectrophotometrically and DNA was dissolved to a concentration of 10 µg ml^{-1}.

Yoghurt samples (Klasik white yoghurt OLMA Olomouc from the trade network before and after expiration date, 1 g) was resuspended in 1 ml sterile water. The cells were washed twice with sterile water and treated with EMA (0.1 mg ml^{-1}) for 10 min at laboratory temperature. Photoactivation was performed using light exposure (halogen lamp, 500 W) for 5 minutes on ice. Then the cells were washed with 1 ml of water to remove EMA from the sample. The cells without EMA treatment were used as control. Afterwards the cells were lysed by boiling (10 min) and crude cell lysates (5 µl) were used as DNA matrix in EMA-PCR.

PCR was performed with LBLMA 1 and R16 primers specific to the *Lactobacillus* genus[6], which enabled the amplification of a 250 bp long amplicon. Briefly: the PCR mixture contained 0.5 µl of each 10 mM dNTP, 0.5 µl (10 pmol µl^{-1}) of each primer, 0.5 µl of Taq 1.1 polymerase (1 U µl^{-1}), 2.5 µl of buffer (1.5 mM), 1–5 µl of DNA matrix, and PCR water was added up to a volume of 25 µl. The amplification reactions were carried out using the following cycle parameters: 5 min of the initial denaturation period at 94 °C (hot start), 60 s of denaturation at 94 °C, 60 s of primer annealing at 55 °C, and 60 s of extension at 72 °C. The final polymerisation step was prolonged to 10 min, the number of cycles was 30. The EMA-PCR products (250 bp) were detected using agarose gel electrophoresis (1.8 %) in 0.5 × TBE buffer (45 mM boric acid, 45 mM Tris-base, 1 mM EDTA, pH 8.0). The DNA was stained with ethidium bromide (0.5 µg ml^{-1}), observed on a UV transilluminator (305 nm), and photographed on a TT667 film using a Polaroid CD34 camera.

Results and Discussion

The ability of EMA to covalently bind to DNA and to inhibit PCR was confirmed using purified DNA isolated from *Lactobacillus paracasei* cells. As a result of EMA activity, DNA treated with EMA was not amplified in PCR in contrast to DNA without EMA treatment. The results are shown in Fig. 1. The method developed was applied for the discrimination of viable and dead *Lactobacillus* cells from dairy products (yoghurt). The results are shown in Fig. 2. and Table I. Non-expired or shortly expired (12 days) yoghurts contained both dead and viable *Lactobacillus* cells because intensities of EMA-PCR products amplified from EMA treated and

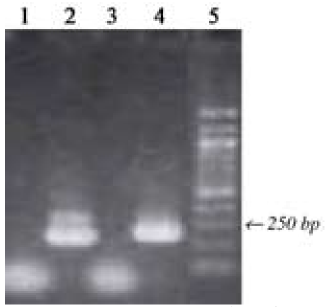

Fig. 1. **Agarose gel electrophoresis of EMA-PCR products obtained after amplification of purified *Lactobacillus* DNA: lane 1: EMA treatment, lane 2: no EMA treatment, lane 3: negative control without DNA, lane 4: positive control with purified *Lactobacillus* DNA (c_{DNA} = 10 ng μl^{-1}), lane 5: DNA standard (100 bp ladder)**

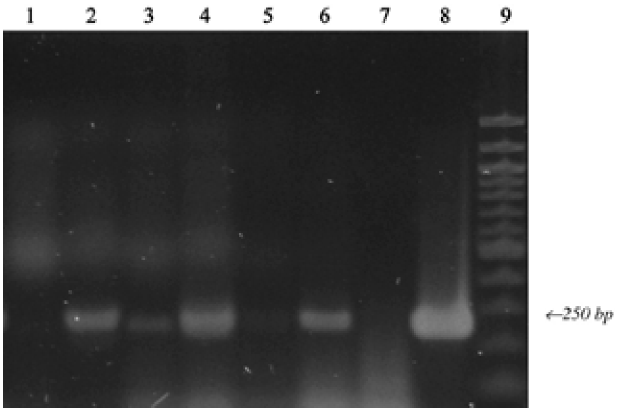

Fig. 2. **Agarose gel electrophoresis of EMA-PCR products obtained after amplification of *Lactobacillus* DNA from yoghurt: lanes 1, 3, and 5: cells with EMA treatment, lanes 2, 4, and 6: cells without EMA treatment, lane 9: DNA standard (100 bp ladder); lanes 1 and 2: 32 days after yoghurt expiration, lanes 3 and 4: not expired, lanes 5 and 6: 12 days after yoghurt expiration, lane 7: negative control without DNA, lane 8: positive control with purified *Lactobacillus* DNA (c_{DNA} = 10 ng μl^{-1})**

not treated cells deffered. On the contrary, in longer expired yoghurts only dead cells were detected. This is in agreement with the presumption that non-expired yoghurt samples contain more viable cells.

Table I
Identification of viable and dead *Lactobacillus* cells in yoghurt samples

Yoghurt expiration [days]	EMA treatment	EMA-PCR product	Viability of cells
Non-expired	+	++	viable and dead
	–	+++	
12	+	+	viable and dead
	–	++	
32	+	–	dead
	–	+++	

Conclusions

The results presented here show that the method proposed (ethidium monoazide treated cells in combination with polymerase chain reaction) is suitable for the distinction of viable and dead *Lactobacillus* cells. The method developed was applied for the analysis of real dairy products (yoghurts). More viable cells were detected in non-expired yoghurt samples.

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P102 DYNAMICS OF SELECTED PESTICIDES APPLIED TO APPLE PROTECTION DURING PRE-HARVEST AND POST-HARVEST PERIOD

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Introduction

Pesticides represent an important group of agrochemicals used for food crop protection. However, their residues can often be detected in treated crops at the harvest time. Because of the health hazard, this issue is of high concern, mainly when crops containing residues are intended for baby food production. Infants and young children are the most vulnerable group of human population; therefore, uniform maximum residue limit (MRL) 0.01 mg kg^{-1} has been established in EU for any pesticide residue occurring in processed baby food. Currently, most of producers require raw materials complying with this (low) residue limit, although it applies to processed product. Under real-life conditions, this may pose a problem, since many pesticide preparations even when used in accordance with Good Agriculture Practice (GAP) may leave residues exceeding the "baby food" MRL[1].

Apples are the key raw material for production of fruit-based baby food. Unfortunately apples can be attacked by various disorders not only during pre-harvest period but also during storage. Sometimes incipient infection is too small to be seen prior to fruit storage but may develop under favourable conditions (high humidity) as a result of sporulation from older lesions. Particular treatment strategy should be applied to apples intended for storage because the decline of pesticide residues within a post-harvest time might be slower (depending on the storage conditions) than during the pre-harvest period when the drop is relatively rapid due to various environmental factors[2].

Experimental

Field Experiments

Four different apple varieties, Golden Delicious, Gloster, Idared and Melrose, were examined in this study. The same treatment schedule was applied to all studied varieties. The overview of pesticide preparations and some relevant characteristics of active ingredients are listed in Table I.

In the first phase, the experimental apple trees obtained specific pesticide treatment from April to June, see Table I. In the second phase, the orchard was divided into four sections (S1–S4). In the S1 no pesticides were used, while in S2–S4, fungicides against storage pests were applied according to the scheme shown in Table II.

The first set of samples was taken for analysis at the time of harvest (October 10, 2007) and the next two after three (January 8, 2008) and five (March 19, 2008) months of cold storage (1–3 ºC).

Table I
Schedule of the pre-harvest treatment

Application date	Pesticide preparation/ dosage [per hectare]	Active ingredient	Mode of action	Safety period [days]
April 12, 2007	Chorus 75 WG/0.2 kg	cyprodinil	fungicide	28
	Cascade 5 EC/1.5 kg	flufenoxuron	insecticide	-
April 19, 2007	Clarinet 20 SC/1.5 dm^3	fluquinconazole	fungicide	28
		pyrimethanil	fungicide	
April 26, 2007	Thiram Granulo/3.0 kg	thiram	fungicide	14
May 3, 2007	Dithane Neotec/3.0 kg	mancozeb	fungicide	21
	Calypso 480 SC/0.2 dm^3	thiacloprid	insecticide	14
May 11, 2007	Delan 700 WDG/0.6 dm^3	dithianon	fungicide	21
	Score 250 EC/0.2 dm^3	difenoconazole	fungicide	49
May 18, 2007	Polyram WG/3.0 kg	metiram	fungicide	21
	Insegar 25 WP/0.3 kg	fenoxycarb	insecticide	60
May 25, 2007	Integro/0.5 dm^3	methoxyfenozide	insecticide	14
June 1, 2007	Baycor 25 WP/1.0 kg	bitertanol	fungicide	35
	Score 250 EC/0.2 dm^3	difenoconatole	fungicide	49
June 7, 2007	Domark 10 EC/0.4 dm^3	tetraconazole	fungicide	14
June 14, 2007	Syllit 400 SC/1.7 dm^3	dodine	fungicide	21
	Trebon 10 EW/0.5 dm^3	etofenprox	insecticide	28
June 26, 2007	Mythos 30 SC/1.0 dm^3	pyrimethanil	fungicide	28
	Mospilan 20 SP/0.25 kg	acetamiprid	insecticide	28

Table II
Schedule of the treatment against storage diseases

Code	Pesticide preparation [kg ha^{-1}] active ingredient – safety period	Application date
S1	–	–
S2	Delan 700 WG (0.7) dithianon – 21 days	September 19, 2007
S3	Thiram Granuflo (3.0) thiram – 14 days	September 26, 2007
S4	Zato 50 WG (0.15) trifloxystrobin – 14 days	September 26, 2007

Analytical Methods

Most of pesticides were liquid chromatography (LC) amenable, so for isolation of 14 from 17 examined analytes the QuEChERS method[3] was applied. Crude extract (without cleanup step realized with primary-secondary amine (PSA)) was examined by LC coupled with tandem mass spectrometry (MS/MS). Limits of quantitation (LOQ) for these pesticides were 0.002 mg kg^{-1}, only for dodine LOQ was 0.005 mg kg^{-1}. Ethylenbisdithiocarbamates (EBDC), in this study represented by mancozeb, metiram and thiram, were determined as a sum by solid phase micro-extraction (SPME) of their degradation product (carbon disulfide), identification/quantitation was performed by gas chromatography coupled with mass spectrometry (GC-MS), LOQ was 0.001 mg kg^{-1}.

Results

At the time of harvest, residues of some pesticides (acetamiprid, cyprodinil, flufenoxuron, fluquinconazole, fenoxycarb, tetraconazole and thiacloprid) used for treatment (Table I) were not detected. Other pesticides exceeding the method LOQ could be grouped into three categories based on their concentration levels: *(i)* compounds with residues below 0.01 mg kg^{-1} at the time of harvest (i.e. difenoconazole, dithianon, methoxyfenozide, EBDC), *(ii)* compounds with residues exceeding the "baby food" limit at the harvest time, but declining below the limit at the end of the storage period (i.e. bitertanol, etofenprox) and *(iii)* compounds with residues not significantly declining and exceeding the 0.01 mg kg^{-1} all the time (i.e. dodine, pyrimethanil). No generalization on the relationship between residues dynamics and apple variety could be drawn. The highest amount of bitertanol residues occurred in variety Golden Delicious (Fig. 1. A) and etofenprox residues was the highest in variety Idared (Fig. 1. B) in all experiments. Worth to notice that the changes of pesticides classified as concentration category *(iii)* were not dependent on apple variety.

Treatment for post-harvest disease protection was carried out before the harvest in compliance with required safety period. For this purpose three fungicide preparations with different active ingredients (dithianon, thiram and trifloxystrobin) were tested (Table II). Thiram, although applied only 14 days before the harvest, did not exceed "baby food" limit,

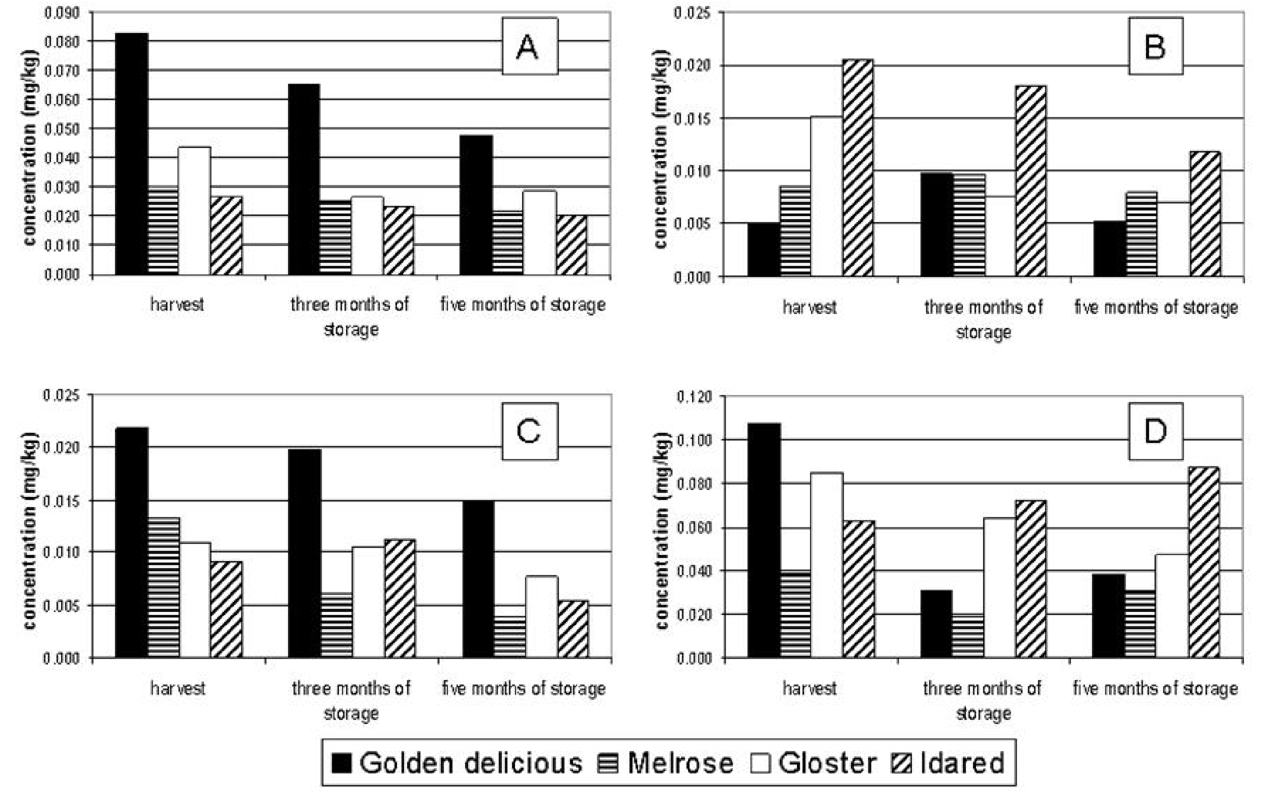

Fig. 1. **The dynamics of selected pesticides during cold storage (1–3 °C); A: bitertanol, B: etofenprox, C: trifloxystrobin, D: dithianon**

whereas residues of dithianon and trifloxystrobin exceeded 0.01 mg kg^{-1} during the entire storage period. As shown in Fig. 1. C, residues of trifloxystrobin were the highest in variety Golden Delicious. Different concentration trend was observed with dithianon residues, the lowest levels were detected in variety Melrose (Fig. 1. D) and similar dynamics of its residues could be observed although in apples treated five months before storage.

Conclusions

The presented study has documented relatively large variation of pesticide residues in various apple varieties obtained the same treatment. Interestingly the residues tended to be the highest in Golden Delicious while in Melrose were in most experiments the lowest. This may be associated with different wax content in these varieties. In any case, weather conditions before harvest play a key role in levels of residues occurring in apples at the start of storage period. Only repeated experiments may give more general conclusions.

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P103 RELATIONSHIP BETWEEN XANTHOHUMOL, POLYPHENOLS, AND FLAVONOIDS CONTENT IN HOP LEAVES AND CONES WITH REGARD TO CULTIVAR AND VEGETATION PERIOD

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Introduction

Some secondary metabolites are present in plant cells in the form of precursors and are activated after pathogen attack[1]. Secondary metabolites having biocide effect include isoprenoids, phenol substances, organic sulphur compounds, saponins, alkaloids, proteins, and peptides[2].

In this perspective, increasing attention is being paid to the plant called common hop (*Humulus lupulus* L.). The plant is diecious. Wild-growing hop grows on wet soils, mainly in riparian zones, in shrubberies, on the edges of canals and in similar places[3,4]. All the main components are contained in lupuline glands[3,5], although some components, e.g. essential oils, were also identified in the leaves of the plant[6]. Concerning the chemical composition, hop resins are prenylated derivates of floroglucinol, comprising alpha-bitter acids and beta-bitter acids[5]. In recent years, antibiotic[7], antifungal[8], and antioxidant activities of humulone[9] have been identified. One of the big groups of substances contained in the hop plant is the group of polyphenol compounds[10], phenol acids, chalkones, flavanols, flavonols, antocyanides, catechines and proantocyanides. Flavonoids as a group of organic compounds display a wide range of biological and pharmacological properties[11], including antiviral, antimicrobial, antioxidant, and anticancer properties[12,13]. Stevens[14] identified three main groups of flavonoids in the hop plant including prenylflavonoids. The content of prenylated flavonoids in the hop plant fluctuates between 0.2–0.6 %[15]. The chemical composition of hop is not constant; it changes with time and depends on the conditions of post-harvest storage[4,15]. Regarding the chemical composition, an important role was played by such factors as the plant development stage, location, growing practices, weather, and pressure of pathogens. Langezaal[6] reports that in hop leaves there are essential oils in much lower concentrations than in cones. To our knowledge, the content of low-molecular polyphenyls in hop leaves has not been studied yet.

Experimental

Materials and Methods

We tested the samples of hop, cones and leaves taken from the Gene Bank of the Slovak Republic in the Institute of Plant Production, Piešťany (SCPV–VÚRV). The samples of different cultivars of hop were dried and homogenized. We used the following cultivars of hop: Osvald's clones 31, (K-31), and 72 (K-72), Bor, Sládek, Aromat, Zlatan, and Premiant. We collected the samples before flowering and at the end of vegetal period.

The content of phenol substances was determined by Singelton's method[16]. The amount of 0.2 ml of methanol extract was mixed with 1 ml of Folin-Ciocalteu's reagent and 0.8 ml of 7.5 % (w/v) Na_2CO_3. Absorbance was measured at 750 nm wave-length after 30 minutes of incubation in dark. The content of polyphenols was compared with the absorbance of galic acid.

Flavonoids were determined by Rakotoarison's method[17]. 1 ml of methanol extract was added to 1 ml of methanol solution of $AlCl_3 \cdot 6H_2O$, 2% (w/v), and absorbance was measured after 10 minutes at 394 nm. Quercetin was taken as the standard.

Xanthohumol was determined by HPLC. Dry material was homogenized with methanol in a sonificator (Bandelin Electronic, Germany) for about 10 minutes, and then purified by centrifugation and filtration on a column (Waters, USA). Eluate (5 μl) was injected into the HPLC instrument and detected at 370 nm. The mobile phase consisted of 900 ml of methanol with 100 ml of water. The analytical column was Nucleodur Sphinx RP EC 150/4.6 (5 μm). Xanthohumol was purchased form Sigma, Germany.

Results

The concentration ranges of phenol substances, flavonoids and xanthohumol in hop cultivated in the Slovak Republic are summarized. The content of polyphenols and flavonoids was monitored during the vegetal period of plant growth. All cultivars showed different contents of secondary metabolites during the vegetal period, their content in leaves decreased from the end of June to September (Table I).

Table I
Polyphenols content in hop leaves during the vegetal period

Cultivar	Polyphenols [mg g^{-1} DM] 06/07*	 09/07*
K-31	8.83	1.22
Bor	5.00	2.25
Sládek	5.76	2.04
K-72	7.46	0.99
Aromat	6.56	0.98
Premiant	5.52	0.90
Zlatan	6.23	1.82

*06/07 and 09/07 – date of sampling

Phenol substances were in the interval between 0.90–2.25 mg g^{-1} of dry matter in leaves, and from 4.0 to 21.1 mg g^{-1} of dry matter in cones (Table II) at the end of the vegetal period.

The content of polyphenols was dependent on the type of cultivar and on climatic conditions as well. Comparison of the contents of polyphenols in extracts from hop cones in 2006 and 2007 is shown in Fig. 1. The content of phenol substances in leaves did not basically relate to the content of phenol substances in cones. The highest content of polyphenols was detected in the aromatic cultivar Osvald's clone K-72.

Table II
Phenol substances content in hop cones at the end of the vegetal period

Cultivar	Polyphenols [mg g^{-1} DM]
K-31	16.2
Bor	17.4
Sládek	18.8
K-72	21.1
Aromat	11.2
Premiant	20.0
Zlatan	17.0

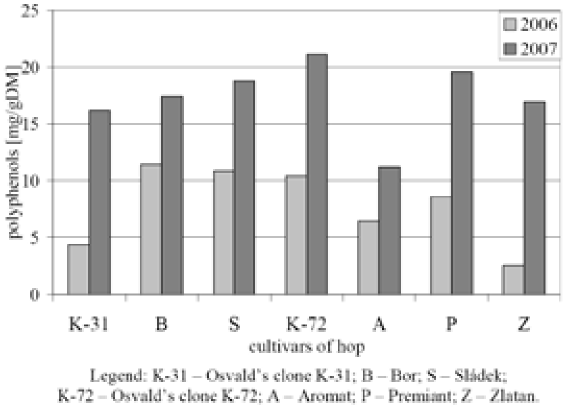

Fig. 1. **Comparison of the content of polyphenols in hop cones in 2006 and 2007**

Table III
Flavonoids content in hop leaves during the vegetal period

Cultivar	Flavonoids [mg g^{-1} DM]	
	06/07*	09/07*
K-31	6.37	0.65
Bor	3.45	0.71
Sládek	3.24	0.76
K-72	6.31	0.48
Aromat	5.89	0.50
Premiant	2.13	0.72
Zlatan	5.03	0.61

*06/07 and 09/07 – date of sampling

Table III and Table IV show the data on flavonoid content in leaves, compared with xanthohumol content. We noted a decline during the vegetal period in both cases, so as in phenol substances.

Table IV
Averaged content of xanthohumol in hop leaves during the vegetal period

Cultivar	Xanthohumol [% wt.]		
	9/2006	6/2007	9/2007
K-31	0.009	0.055	0.014
Bor	0.012	0.067	nd
Sládek	0.013	0.068	0.014
K-72	0.010	0.060	0.015
Aromat	0.011	0.041	0.013
Premiant	nd	0.047	0.009
Zlatan	0.011	0.039	0.016

nd – no data

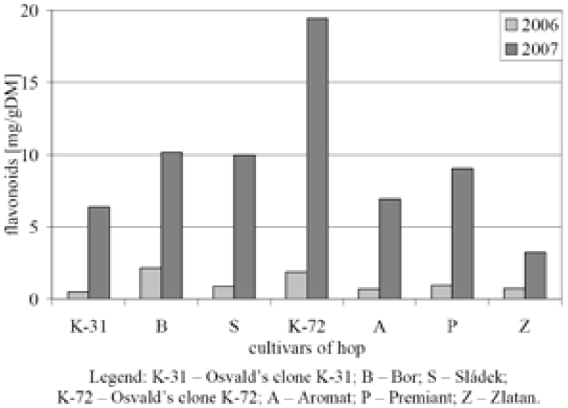

Fig. 2. **Flavonoids content in hop cones for 2006 and 2007**

Table V
Averaged content of xanthohumol in hop cones for 2006 and 2007 (cones sampled on September 22, 2006 and September 24, 2007)

Cultivar	Xanthohumol [mg g^{-1} DM]	
	2006	2007
K-31	0.39	1.14
Bor	1.98	3.11
Sládek	1.11	8.87
Aromat	0.49	0.62
Zlatan	0.29	2.32

Xanthohumol, prenylated flavonoid, was defined in extracts from hop cones. Its content, like the content of phenol substances and flavonoids, was the highest at the end of vegetal period in cones (Figure 2). The level of xanthohumol was influenced by the climatic conditions (Table V).

Conclusions

The concentrations of secondary metabolites, phenol substances, and flavonoids depended on the vegetal period at first.

The quantity of phenol substances was within the range of 5.00–8.83 mg g^{-1} of dry matter, for flavonoids 2.13–6.37 mg g^{-1} of dry matter in the leaves before flowering. The ranges decreased to 0.90–2.25 mg g^{-1} of dry matter of phenol substances, and to 0.48–0.76 mg g^{-1} of dry matter at the end of vegetation.

The climatic conditions influenced the content of secondary metabolites; the contents of xanthohumol were very different in identical cultivars, compared to the years 2006 and 2007.

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P105 SEA BUCKTHORN – SOURCE OF VITAMIN C

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Introduction

Sea buckthorn (*Hippophae rhamnoides*) is a bush giving small orange fruits. The bush comes from Asia but it has spread almost all over the world during last century because it can be grown even in harsher climate conditions without special agrotechnical technology. The largest producers of sea buckthorn are China, Russia, Finland and Germany at present.

Sea buckthorn has been used as a natural drug in Asia to treat heart diseases, digestive tract diseases, cough, etc. Today, sea buckthorn extract is added not only in homeopathic remedies and food supplements, but also into syrups, jams, sweets, oils, face creams, shampoos, soaps, teas and many other products all over the world. Sea buckthorn contains many substances beneficial to human health, particularly vitamin C in amount larger than in other kinds of fruit or vegetable. Among other substances, also carotenoids, flavonoids, essential unsatured fatty acids and phytosterols should be mentioned. Their content depends not only on individual genotypes and cultivars of this plant but also on the site at which the plant is growing. This, naturally, holds also for the vitamin C, which frequently serves as one of basic indicators of alimentary and economical attractivity of planted sea buckthorn cultivars.

The aim of the presented work is therefore the comparison on the vitamin C content in the sea buckthorn cultivars and genotypes growed by Faculty of Horticulture, Mendel University of Agriculture and Forestry Brno. Two methods have been applied for the determination of the vitamin C content in the sea buckthorn juice: HPLC with photometric detection and the direct coulometric determination of the vitamin C.

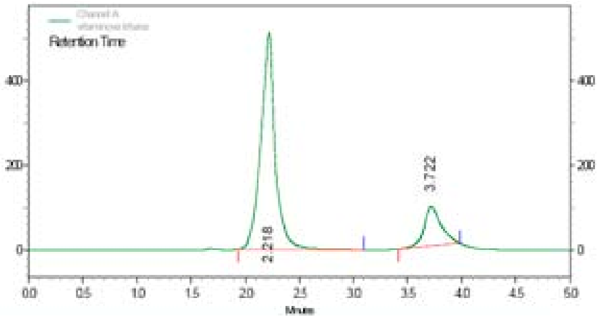

Fig. 1. **Analysis of the Vitaminová cultivar**

Experimental

The HPLC Determination

The Liquid chromatograph Spectra System equipped with the UV 6000 LP detector and the Chromquest software (Thermo Separation Products, USA) were used. for analyses of sea buckthorn juices prepared from the sea buckthorn cultivars as described below. The juices were injected on the TESSEK Separon SGX RPS Column (TESSEK, Praha, Czech Republic). The separation bed of 5 μm particles was of 5 mm in diameter and of 250 mm in length. 0.01M oxalic acid was the mobile phase. The column effluent was monitored at 254 nm.

Direct coulometry

The coulometric analyser PCA/C-Vit (ISTRAN, Bratislava, Slovak Republik) equipped with porous working electrode served for direct coulometric determination of vitamin C without preliminary separation of the compound from sea buckthorn juices. The determination utilizes oxidation of ascorbic acid to dehydroascorbic acid

$$C_6H_8O_6 - 2e \rightarrow C_6H_6O_6 + 2H^+ \tag{1}$$

The Preparation of Juices

We analysed 12 cultivars of sea buckthorn listed in Table I. The cultivars were planted in the breeding station of the Horticulture Faculty, Mendel University of Agriculture and Forestry Brno in Žabčice. Fruits harvested October 4, 2006 were immediately freezed to –18 °C and stored at this temperature in the freezing box till the analyses started April 2007. Approximately 5 grams of dry fruits freely warmed to room temperature was weighed to five digits and homogenized in the mortar with small amount oxalic acid, quantitatively transfered to the 100 ml volumetric flask by 2 % (w/v) solution of oxalic acid in water and than filled with the acid solution to the division line. For chromatographic analyses, aliquot part of solution was filtered using the Büchner slit-sieve funnel and by cellulose 3-μm microfilter. The filtered sample was enriched by the special reaction solution R-020T (ISTRAN, Bratislava, Slovak Republic) for coulometric analyses. The analysed sample were prepared daily just before use and stored in dark during analyses.

Results and Discussion

A typical chromatogram of C vitamin analysis at conditions specified in Experimental is given on at Fig. 1. Vitamin C is in second peak, first peak is a mixture of unindentified impurities.

Main validation parameters of successively repeated chromatographic analyses are as follows: LOD and LOQ of ascorbic acid is 0.2 and 0.7 mg dm^{-3}, respectively. Correlation coefficient of ascorbic acid is $R^2 = 0.9942$ for the concentration range of vitamin C from 0.0007 to 500 mg dm^{-3}. Repeatability of analyses in concentration ranges 151–200 mg dm^{-3}, 51–150 mg dm^{-3} and 10–50 mg dm^{-3} given by RSD vaules is 4 %, 6 % and 10 %, respectively. Mean recovery of used analysis is 89.5 %. However, repeatability

Table I
Content of C vitamin in sea buckthorn cultivars and genotypes determined by HPLC

Cultivar or genotype	Content of C vitamin [mg 100 g^{-1}]			
	1st run**	2nd run**	3rd run**	Mean content
Aromat*	18.2	25.7	22.4	22.1
Buchlovice I*	69.7	62.7	79.3	70.6
Buchlovice III*	41.5	46.1	44.6	44.1
Hergo	32.0	24.1	27.7	27.9
Kyjevský	105.2	92.9	94.0	97.2
Leicora	158.0	137.0	136.1	143.7
Olejová	56.0	57.4	56.3	56.6
Trofimovskaja	22.4	15.2	17.3	18.3
Velkoosecký I	183.4	187.3	168.9	179.9
Velkoosecký II	167.9	166.3	168.5	167.6
Velkoosecký III	161.3	165.0	152.1	159.5
Vitaminová	98.0	91.0	103.5	97.5

*genotype
**mean value from three subsequent analyses

Table II
Content of C vitamin in selected sea buckthorn cultivars and genotypes determined by direct coulometry

Cultivar or genotype	Mean content [mg 100 g^{-1}]	Total measurement error [%]
Buchlovice I*	60.2	21
Hergo	20.9	29
Leicora	120.4	18
Olejová	40.5	26
Trofimovskaja*	10.3	35

of the whole method including the juice preparation was lower as expected. This show columns 2, 3 and 4 in Table I. The yield variation in the preparation of the filtered juice is the reason for differences in paralel determinations that exceed repeatability of chromatographic analyses. Data summarized in Table I evidence that the vitamin C content differes by one order of magnitude in individual cultivar. Coulometic measurements summarized in Table II prove this principal finding of liquid chromatgraphy analyses Total error of analyses with the coulometric determination of the vitamin C content is rougly one order of magnitude higher than total error of analyses with the vitamin C content determined chromatographically. Moreover, the error of the coulometric determination increases with the decreasing vitamin C content in the filtered juice as indicate data in Table II. This evidences that columetry without vitamin C separation may be utilized as the final analytical step in need however only if semiquantitative precision of the whole method is acceptable, e.g., in preliminary analyses. For precise analyses, liquid chromatography has to be used as the final step.

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P106 ARSENIC SPECIATION IN FISH USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH HYDRIDE GENERATION ATOMIC FLUORESCENCE SPECTROMETRY

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Introduction

Investigations of the chemical constituensts of aquatic organisms can provide useful informations about the environment as well as toxicologically relevant data about the composition of biological species consumed by humans[1]. Arsenic causes a variety of adverse health effects to humans after acute and chronic exposures[2]. Toxicity, environmental mobility and accumulation in living organisms usually depend on the form in which the element is present. Information on the chemical forms is important for understanding the role of the element present as well as revealing its environmental cycle[3]. In the environment arsenic can be found as several compounds ranging from inorganic arsenite and arsenate to methylated compounds like monomethylarsonic acid, dimethylarsinic acid, trimethylarsine oxide, arsenobetaine, tetramethylarsonium ion and arsenocholine. In biologic samples several arsenosugars and arsenolipids can be present as well[4].

The determination of arsenic compounds is mostly carried out by hyphenated techniques, the main parts being liquid chromatographic separation and element specific detection. By far the most used systems are liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS) and liquid chromatography hydride generation atomic fluorescence spectrometry (LC-HG-AFS)[5]. Atomic fluorescence spectrometry (AFS) can be a good alternative to inductively coupled plasma mass spectrometry (ICP-MS) detector, with the advantage of a lower cost of investment and handling[5,6].

Experimental

Standards and Reagents

All solutions were prepared with deionized water (18.2 MΩcm). Stock solutions of 1,000 mg(As)dm^{-3} were prepared by dissolving $NaAsO_2$ (Fluka, Switzerland), $Na_2HAsO_4 . 7H_2O$ (Fluka, Switzerland), $CH_4AsNaO_3 \cdot 1.5H_2O$ (Chem. Service, USA), $(CH_3)_2AsHO_2$ (Fluka, Switzerland) and arsenobetaine (Fluka, Switzerland). All arsenic solutions were stored in the dark at + 4 °C. These stock solutions were diluted with deionized water to the desired concentrations before use.

For the analytical procedures the following chemicals were used: potassium dihydrogen phosphate (Fluka, Switzerland), potassium monohydrogen phosphate (Fluka, Switzerland), hydrochloric acid (Analytika, Czech Republic), potassium persulfate (Fluka, Switzerland), sodium borohydride (Riedel-de Haën, Germany), sodium hydroxide (Riedel-de Haën, Germany), nitric acid (Analytika, Czech Republic), hydrogen peroxide (Lach-Ner, Czech Republic), ascorbic acid (Riedel-de Haën, Germany), potassium iodide (Analytika, Czech Republic), magnesium nitrate (Merck, Germany). All chemicals were at least of analytical grade.

Instrumentation

A Gynkotek P508 (Gynkotek, Germany) HPLC pump and six-port injection valve (Ecom, Czech Republic) were used in conjunction with a strong anion-exchange column Hamilton PRP X-100 (Hamilton, USA). An Excalibur atomic fluorescence detector (PS Analytical, UK) equipped with a boosted discharge hollow cathode lamp (Photron, Australia) was used for detection. The measurements are carried out on the resonance line of As 193.76 nm. For data collection and evaluation was used CSW software – version 1.7 (Data Apex, Czech Republic).

Sample Preparation

The muscle of commercially available fish samples were homogenized and freeze-dried.

For the determination of total arsenic by AFS the microwave digestion (using HNO_3 and H_2O_2) was employed; followed by the addition of $Mg(NO_3)_2$ to complete the destruction.

Triplicate of samples were extracted with 10 ml of deionized water using ultrasonic bath at 50 °C for 2 hours. The extraction vessels were shaken every 15 min to ensure dispersion of the sample. After extraction the samples were centrifuged and extract was filtered through a 0.45 µm membrane filter and kept at 4 °C till speciation analysis.

Conditions of Total Arsenic Determination

A KI/ascorbic acid reducing solution was employed to reduce arsenic species to As (III). The reducing solution was added at least 30 minuts before analysis. Conditions for hydride generation: 1 % $NaBH_4$ in 1 % NaOH at flow rate the 4.5 ml min^{-1} and 4.9 mol dm^{-3} HCl at flow rate 9 ml min^{-1}.

Speciation analysis conditions

The mobile phase was 10 mM K_2HPO_4/KH_2PO_4 adjusted to pH 6.1 at flow rate 1 ml min^{-1}. The hydride generation of the arsines was carried out by adding 6 mol dm^{-3} HCl and 1.4 % $NaBH_4$ in 1 % NaOH, both at the 3 ml min^{-1} flow rate. 1 % $K_2S_2O_8$ in 1 % NaOH at the flow rate 0.3 ml min^{-1} was used as oxidation solution, oxidation was supported by UV.

When analysing a samples containing arsenobetaine (AsB) it is necessary to analyse the sample twice, once with the UV lamp switched off and once with it switched on. This is due to the fact that AsB coelutes with arsenite. If the UV lamp is off AsB don't react with $NaBH_4$ and don't give a signal.

Results and Discussion

Total Arsenic Concentrations

The total arsenic concentrations in fish muscle are summarized in Table I. These results are lower than limits given by legislative of Czech Republic. All samples were also measured by atomic absorption spectrometry with hydride generation to ensure quality results. Results obtained by HG-AAS were comparable to HG-AFS results.

Table I
Total arsenic concentrations in the investigated samples determined by AFS

Sample	Total arsenic [$\mu g\,kg^{-1}$]	RSD [%]
Salmon 1	186	2
Salmon 2	741	4
Trout 1	968	0
Trout 2	966	1
Trout 3	618	4
Tunny	2059	1

Speciation Analysis of Arsenic

The influence of pH of mobile phase on the separation efficiency has been investigated. The pH values were in the range of pH 4–8. The best results were obtained with pH 6.1. Separation of the five arsenic standards is shown in the Fig. 1.

Typical chromatogram of fish extract *(Thunnus atlanticus)* is presented in Fig. 2. All analysed samples of fish contained only non-toxic arsenobetaine.

The results of the speciation analysis are shown in the Table II. Comparision with total arsenic content reveals that extraction efficiencies ranged from 66 to 106 %.

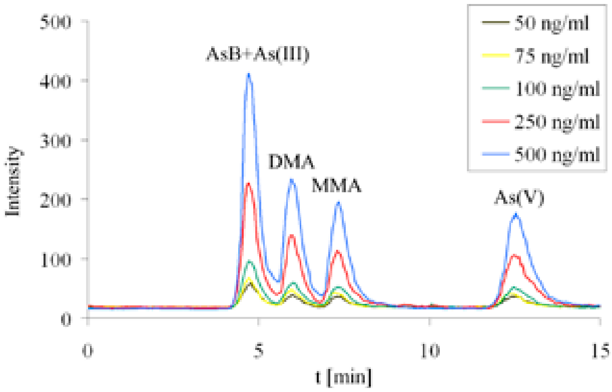

Fig. 1. **Standard chromatogram of five arsenic species**

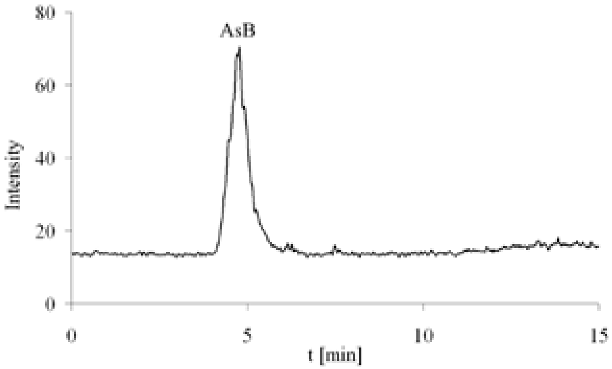

Fig. 2. **Peak of arsenobetain in the chromatogram of tunny**

Table III
Arsenobetaine concentrations in the investigated samples determined by HPLC-UV-HG-AFS

Sample	Extractable arsenic [$\mu g\,kg^{-1}$]	RSD [%]
Salmon 1	170	9
Salmon 2	783	4
Trout 1	714	0
Trout 2	644	1
Trout 3	407	4
Tunny	1760	2

Conclusions

Amount of total arsenic in the samples was lower than limits for arsenic given by legislative of Czech Republic. Arsenic was present in the form of nontoxic arsenobetaine in all analysed samples of fish.

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P107 COMPARISON OF AROMA PROFILES OF SEVERAL TYPES OF DARK CHOCOLATE

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Introduction

Chocolate (CHC) aroma has been the subject of extensive research. Most of the research work deals with cocoa beans, powder, liquor and CHC. CHC aroma is very complex and is determined by the cocoa plant variety and the fermentation and roasting process. Unfermented cocoa beans are nearly without odour and have a bitter and astringent flavour. Cocoa specific aroma precursors are formed during fermentation by acid induced proteolytic processes[1]. Correct fermentation is essential to produce a good flavour in the final CHC. The different ways of fermenting will give rise to different flavour, unfermented beans do not develop any CHC flavour and are excessively astringent and bitter[2].

The following roasting decreases water content and a full CHC flavour is developed. The high temperatures remove many of the volatile acids and other aroma compounds are formed, in particular as a result of caramelization of sugars, Maillard reaction, protein degradation and sulphur compounds synthesis. The sources of hundreds of volatiles found in roasted beans are the reducing sugars, free amino acids and oligopeptides[3,4]. Several hundreds compounds have been found in roasted cocoa, the most significant are N- and O-containing heterocyclic compounds – alkylpyrazines, furans and pyrroles. Roasting is the most important step for flavour development, it is possible that cocoa subjected to an improper roasting generates undesirable aroma[5].

Finally, conching ends flavour development. The conching is the mixing and shear of the CHC and results in the removal of some undesirable volatiles[2].

Current European legislation allows the addition of vegetable fats to CHC up to a level of 5 % of the product weight, provided that the addition is indicated on the label[6]. However, this addition can influence flavour of CHC. The aim of this work was to compare aroma profiles of three types of dark CHC, produced with or without addition of vegetable fat.

Experimental

Samples

Three types of plain CHC: Figaro plain CHC (min. 48 % cocoa), Figaro for cooking (min. 37 % cocoa) and Kaumy CHC glaze (min. 35 % cocoa) were tested in this work.

Methods

Aroma compounds were determined by the SPME-GC. The SPME fibre CAR™/PDMS 85 μm, extraction 20 min 35 °C. GC conditions: gas chromatograph TRACE™ GC (ThermoQuest Italia) equipped with FID and split/splitless injection port, DB-WAX capillary column (30 m × 0.32 mm × 0.5 μm). The injector 250 °C, splitless mode, the desorption time 5 min, linear purge closed for 5 min. The detector 220 °C. The carrier gas (N_2) 0.9 ml min^{-1}. The oven temperature program: 40 °C, 1 min, 5 °C min^{-1} to 200 °C, 7 min.

Results

SPME-GC was used for the analysis of volatile aroma compounds in three types of dark CHC with different content of vegetable fat. This method is simple, rapid and very mild, so it is suitable for the characterization of the food aroma. The reproducibility, linearity and detection limits of the method were determined in previous work[7]. Aroma active compounds were identified and quantified using standards, in total 56 compounds were found in CHC samples: 14 acids, 9 aldehydes, 8 ketones, 17 alcohols, 7 esters and 1 nitrogen compound. According to the literature, the main cocoa flavour components are alcohols, ethers, hydrocarbons, furans, thiazoles, pyridines, acids, esters, aldehydes, imines, amines, oxazoles, pyrazines and pyrroles[5,8].

The total contents of aroma compounds of CHC tested were compared. This comparison is presented in Fig. 1. The comparison of single groups of compounds is in Fig. 2.

Figaro plain CHC is classic CHC, produced without addition of vegetable fat. In total 50 aroma compounds were identified in this type of CHC, the most abundant were ethanol, propan-1-ol, butan-2,3-diol and phenylacetaldehyde. These compounds arise during fermentation of cocoa beans by the activity of present microflora[3].

Figaro for cooking is plain CHC, intended for making of various CHC sweets, glazes etc. It contains certain amount of vegetable fat up to 5 %. In total 47 compounds were identified here, the most abundant were methanol, ethanol, propan-1-ol, butan-2,3-diol, acetic acid, acetone and phenylacetaldehyde.

Kaumy is CHC imitation, in which cocoa butter is completely replaced by vegetable fat. Although its appearance is very similar to CHC, different fat influences taste, it is not so delicious thus is not suitable for direct consumption. Kaumy is best for making of CHC sweets, where its unpleasant off flavour is not so obvious. In total 46 aroma compounds were found here, the most abundant were methanol, ethanol, propan-1-ol, butan-2,3-diol and acetic acid.

All types of CHC contained the significantly high concentrations of alcohols and fatty acids (Fig. 2.). The highest content of alcohols and also quite high content of fatty acids was in Kaumy glaze, so several these compounds probably contribute to its unpleasant off flavour. Kaumy surprisingly had also the highest total content of aroma compounds (Fig. 1.), plain CHC, which is supposed to have the best cocoa flavour, the lowest.

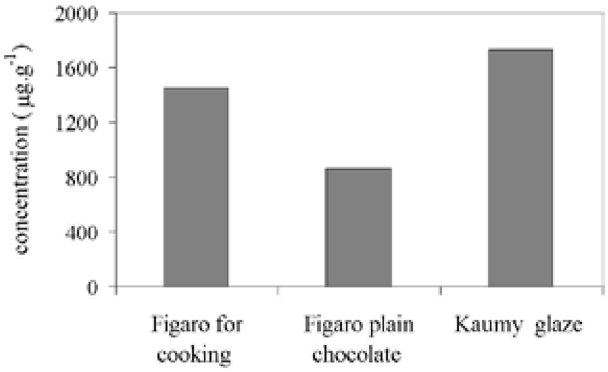

Fig. 1. **The comparison of total contents of aroma compounds of three types of dark chocolate**

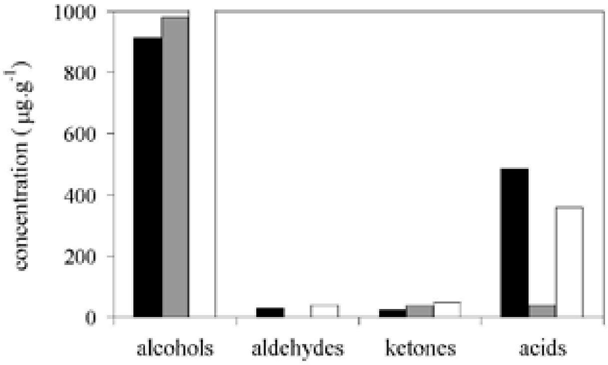

Fig. 2. **The comparison of single groups of aroma compounds of three types of dark chocolate. Figaro for cooking ■, Figaro plain chocolate ▨, Kaumy glaze ☐**

Conclusions

Some important aroma compounds were found in three types of dark CHC. CHCs have similar aroma profiles, but owing to their different composition (cocoa butter, vegetable fat, cocoa dry matter) concentration of several aroma compounds is different. Fatty acids and alcohols were quantitatively the most important compounds in all types of CHC. The highest total content of all aroma compounds was in Kaumy glaze, so several these compounds probably contribute to its unpleasant off flavour.

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P108 MONITORING OF CHANGES IN POPULATION OF YEASTS DURING FERMENTATION OF GRAPE MUST

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Introduction

Traditionally, wine fermentation has been carried out in spontaneous way by indigenous yeasts present on the grapes when harvested, or introduced from the equipment and cellar during the fermentation process. At present most wine producers carry out the fermentation process by adding a pure Saccharomyces strains to the must to improve final wine quality[1,23].

The strains at the species level can be identified using conventional methods based on biochemical and physiological characteristic or molecular techniques. Over the last decade molecular techniques for the identification of yeast strains have been widely studied[4,5,8]. The applicability of techniques as the RAPD method and restriction enzyme analysis of PCR amplified DNA fragments (PCR-RFLP) has been demonstrated as a tool for taxonomic purposes[11,16,19].

The fermentation of grape must into wine is a complex microbiological process characterized by the presence of a large number of different microorganisms. The composition of yeasts population on grape berries and leaves plays an important role in wine fermentations, as different genera, species and strains with their metabolic activity influence the sensory quality and organoleptic characteristics of wine.[3,7,8,13,15,16,18] Yeasts associated with grape or wine ecosystem are usually classified in 15 different genera[21]: *Brettanomyces/Dekkera, Candida, Cryptococcus, Debaryomyces, Hanseniaspora/Kloeckera. Kluyveromyces, Pichia, Metschnikowia, Rhodotorula, Saccharomyces, Saccharomycodes, Schizosaccharomyces, Zygosaccharomyses*.

The aim of this study was monitoring of the changes in yeast population during spontaneous alcoholic fermentation of cider of *Veltlin green* cultivar. We isolated yeasts in different period of grape cider fermentation. Identification of the individual strains was carried out using PCR-RFLP analysis of 5.8S-ITC region.[6,7,11,12]

Experimental

Sample Preparation

The samples analyzed in this study were isolated from white wine cider ("*Veltlin green*") obtained from local producer from wine region Velke Pavlovice. Control yeast strains used in this study were from the Culture Collection of Yeasts, Bratislava, Slovakia (CCY).

Sampling at different stages of fermentations was performed at days: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 Aliquots of each sample (several dilutions) were spred on to plates of wort agar. Cells were directly collected from a fresh yeast colony using a microbiological loop.

DNA was extracted and purified by Ultra Clean Microbial DNA Isolation Kit – obtained from Elizabeth Pharmacon spol. s.r.o., CR. 3–10 ng of DNA in 1–2 μl of TBE buffrer were used in a 50 μl amplification reaction.

PCR Amplification of the Yeast DNA

The amplification reaction of the rDNA region spannding the 5.8 rDNA gene and the ITS regions were carried our under the following conditions: 50 μl of reaction mixture contained 5–20 ng template DNA, polymerase buffer, 0.2 mM of dNTP (N = A, T, G, C) 0.5 μM of each primer and 1 U Taq DNA polymerase. The amplification protocol included the following steps: Initial denaturation at 94 °C for 4 min, 25 cycles of amplification: denaturation at 94 °C for 1 min, annealing at 48 °C for 30 sec, and extension at 72 °C for 1 min. The final extension was at 72 °C for 10 min. Amplification products were analysed on 0.7% (w/v) agarose gel in TBE buffer. PCR amplification was carried out in a PTC-100™ thermocycler (MJ.Research,Inc.). Upper primer: (ITS1) 5'-TCC GTA GGT GAA CCT GCG G-3',Lower primer: (ITS4) 5'-TCC TCC GCT TAT TGA TAT GC-3' (VBC BIOTECH, GmbH)[6]. With these two primers a different products (900, 800, 700, 500 and 450 bp) were obtained after amplification with DNA template from species of genus *Saccharomyces* (Fig. 1.).

Restriction Analysis and Gel Electrophoresis

PCR products (20 μl) were digested in 20 μl of reaction volume with 1U of restriction endonuclease using the manufacturers conditions.

The restriction enzymes used: Alu I, Hae III, Hinf I and Mse I. (Fermentas, Lithuania)

The producst of digestion were analysed by horizontal gel electroforesis system (OWL, USA) in 2% (w/v) agarose gels and comparison with yeasts from CCY. After electroforesis the gels were stained with EtBr and visualized under UV light (Fig. 2.).

Results

The restriction fragments of amplified part of DNA from 27 yeasts isolated from wine must were compared with those of 40 yeasts from the Collection of yeasts of Chemical institute of Slovak Academy of Sciences in Bratislava.

Based on the identity of length and number of restriction fragments the taxonomic assignment of yeasts was performed. In some cases no species, but only genus assignment was carried out. Samples No. 83, 107, 70, 9, 30, 62 and 102 were identified as Pichia fermentans, the sample No. 6 and 106 were identified as Hanseniaspora uvarum.

Table I
Yeasts identification during fermentation of wine cider

Different stages of fermentation [days]	Number of samples	Identification
1	83, 107	*Pichia fermentans*
	106	*Hanseniaspora uvarum*
3	9, 70	*Pichia fermentans*
	6	*Hanseniaspora uvarum*
5	104	*Rhodosporium sp.*
	30	*Pichia fermentans*
	40	*Saccharomyces sp.*
7	7	*Saccharomyces mangini*
	62, 102	*Pichia fermentans*
9	95A	*Saccharomyces cerevisiae*
	95B, 96	*Pichia sp.*
	22, 87	*Saccharomyces mangini*
11	67, 58	*Saccharomyces mangini*
13	90, 72	*Saccharomyces mangini*
15	73, 88	*Saccharomyces mangini*
17	63	*Saccharomyces mangini*
19	18, 97	*Saccharomyces mangini*
	46	*Metschnikowia pulcherrima*

After restriction analysis with enzyme HinfI the sample No. 106 was identified as Pichia fermentans.

Nevertheless, the detailed analysis of restriction fragments with enzyme AluI and microscopic analysis led to the attribution to Hanseniaspora uvarum. (TableI)

After the restriction analysis of the sample No. 40 was assigned to the genus *Saccharomyces* without the determination of species. The sample No. 7 was identified as *Saccharomyces mangini*.Using the restriction analysis with enzymes HinfI, HaeIII and AluI the adherence to the genus *Saccharomyces* was proved by the samples 18, 22, 58, 63, 67, 72, 73, 87, 88, 90 and 97. The analysis of these samples with enzyme MseI enabled the species identification. The similarity with the collection species *Saccharomyces mangini* was evidenced.

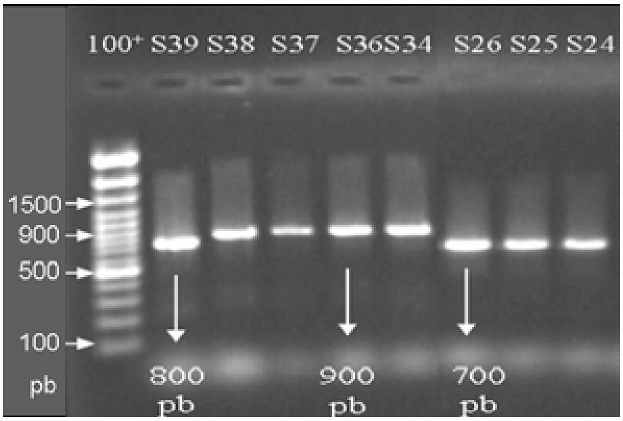

Fig. 1. **Example of agarose gel with PCR products of an amplification of the 5.8S-ITS rDNA (S24–S39 = CCY yeasts, 100^+ – size marker**

The restriction analysis using enzyme HinfI proved the similarity with the genus of Pichia in the case of the sample No. 96. Applying endonuclease HinfI the probe No. 96 was consequently assigned as *Pichia fermentans*. The submission to the genus of *Pichia* was determined also by the sample No. 95B using the enzyme HaeIII. By the sample No.95A the similarity with the collection species S*accharomyces cerevisiae* was proved. The result of restriction analysis using the enzymes HinfI, MseI, HaeIII and AluI documented the identity with the collection species *Metschnikowia pulcherrima* by the sample No. 46.

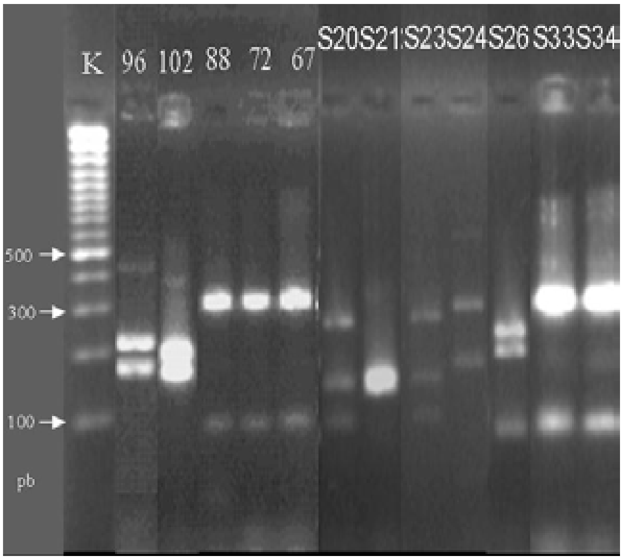

Fig. 2. **Example of digestion products of the amplificates obtained using the restriction enzyme HinfI (S20–S34 = CCY yeasts, 67, 72, 88, 96, 102 – wine yeasts,K – size marker 100pb)**

Conclusions

The result of the analysis is the identification of yeasts of genus *Pichia*, *Hanseniaspora* and *Rhodotorula* in the initiation stages of fermentation and that of Saccharomyces in the further stages of fermentation. The species assignment is *Pichia fermentans* and *Hanseniaspora uvarum*. The unambiguous taxonomic assignment can be performed with *Saccharomyces mangini*. By other yeasts of genus *Saccharomyces* the determination of species was not possible.

It was proved that in the stage of late time of must fermentation and of the formation of young wine the yeasts of genus *Saccharomyces* prevail. The presence of *Saccharomyces cerevisiae* was proved. The interesting result was identification of yeasts species *Metschnikowia pulcherrima*. This yeasts appears in the first stages of fermentation.

The method PCR-RFLP is suitable for taxonomic assignment of yeasts of different genus. Nevertheless, for the exact assignment the selection of primers and restriction enzymes plays the important role.

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