4.2. Posters

P01 HEAT BALANCE OF BIOREACTOR

LIBOR BABÁK, EVA VÍTOVÁ
and MOJMÍR RYCHTERA

Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, babak@fch.vutbr.cz,

Introduction

It is possible to form a balance model for the bioreactor working in quasi-adiabatic configuration. The model describes both heat production and its losses to the surround.

Liquid fill volume of the bioreactor determines the area A, the area B indicates isolation space including fermentor vessel and the area C is allocated to the surround. The heat extends by convection and interphase conversion between outer areas, in addition heat can be supplied or dissipated by convective flows connected with aeration and the substrate passage. In the specific case it is necessary to take into account also the source item linked with the mechanical energy dissipation and heat production by biochemical reactions.

It is practicable to describe the heat dynamics with two balances – for the area A and the area B. The area C forms so large heat reservoir, its temperature is not affected by the heat transfer from the isolated bioreactor[1].

Balance of the area A:

$$m_A \cdot c_{pA} \cdot dT_A/dt = -K_{TA} \cdot (T_A - T_B) - q_A$$
$$T_A(0) = T_P \tag{1}$$

The left side presents the heat accumulation, first term on the right side represents heat transfer to the area B and q_A is the source item.

Balance of the area B:

$$m_B \cdot c_{pB} \cdot dT_B/dt = -K_{TA} \cdot (T_A - T_B) - K_{TB} \cdot (T_B - T_C)$$
$$T_B(0) = T_C \tag{2}$$

The left side presents the heat accumulation again, first term on the right side represents heat transfer from the area A and second term heat losses to the surround.

It is possible to convert the equations system (1, 2) into suitable form (3, 4) with utilize acceptable transformations (5):

$$dT_A/dt = -p_1 \cdot (T_A - T_B) - p_2$$
$$T_A(0) = T_P \tag{3}$$

$$dT_B/dt = p_3 \cdot (T_A - T_B) - p_4 \cdot (T_B - T_C)$$
$$T_B(0) = T_C \tag{4}$$

the transformations are:

$$p_1 = K_{TA}/(m_A \cdot c_{pA}) \quad p_2 = q_A/(m_A \cdot c_{pA})$$
$$p_3 = K_{TA}/(m_B \cdot c_{pB}) \quad p_4 = K_{TB}/(m_B \cdot c_{pB}) \tag{5}$$

Experimental

Experiments were practised in the bioreactor Biostat B. Braun with working volume 2 dm³. The system is fitted with temperature, pH, air flow and mechanical stirring regulation. Fermentor was filled with destilled water, or the cultivation medium (lactose 3 g dm⁻³, $MgSO_4 \cdot 7H_2O$ 1 g dm⁻³, $(NH_4)_2SO_4$ 0.4 g dm⁻³, KH_2PO_4 6 g dm⁻³, yeast extract 2 g dm⁻³, peptone 8 g dm⁻³).

Simple experiments, liquid in the fermentor was heated up significantly over the surround temperature 333.15 K (60 °C), then the temperature regulation was inactivated, but the system continue to run. The temperature decay was monitored during the time of twenty hours. It was observed the influence of isolation type, liquid type and aeration. A model optimization and identification is constructed on the basis of the measured data with the application software language PSI/c.

Results and discussion

Results of the model identification are summarized in the Table I.

The parameter p_1 has the similar value for all orderings. It is heat conversion from the medium to the mechanical part in principle and therefore this result is correct. The source item is the highest for the system with water such as medium and Mirelon isolation without the perceptible resolution of the aeration influence. The aeration influence is not distinct in other systems too, the parameter value p_2 decreases with degressive isolation quality. Fall of the discussed parameter is noted also at the system with the real medium in the comparison with water. It will be necessary with the convection term in the balance to calculate resolutely. It is interesting the aeration declines evidently heat transfer intensity by the wall to the isolation a meta. That is not in conflict with engineering estimations when gas hold-up lowers both density, viskosity
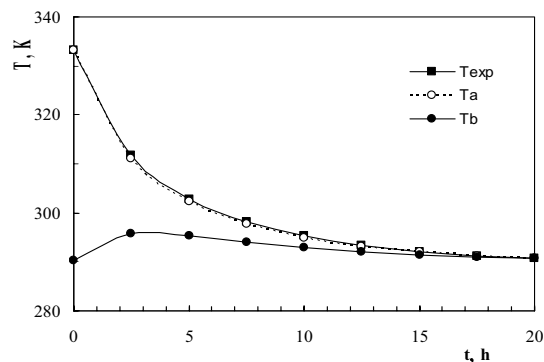

Fig. 1. **Optimized system. Model curves are T_a and T_b (equation 3, 4 after model identification). T_{exp} presents the experimental data for the bioreactor system with aeration, Mirelon isolation and water fill**

Table I
Experiments design and model optimization. Conditions in the bioreactor: volume of liquid 2 dm³, stirring 250 min⁻¹, aeration flow (for aerated systems) 10 dm³ min⁻¹, pH (for systems with medium fill) was adjusted to 6.5, fermentor isolation was (in isolated systems) either aluminium(Al) foil, or isolation material with the manufacturing name Mirelon

Experiment set	Optimized model parameters				
	p_1 [h⁻¹]	p_2 [h⁻¹]	p_3 [h⁻¹]	p_4 [h⁻¹]	T_C [K]
aeration, no isolation, water fill	0.4062	0.0101	0.0928	0.3207	296.05
aeration, Al isolation, water fill	0.3674	0.0791	0.1080	0.2532	292.30
aeration, Mirelon isolation, water fill	0.3266	0.1858	0.1166	0.2517	290.37
no aeration, no isolation, water fill	0.4098	0.0214	0.1800	0.2997	296.90
no aeration, Al isolation, water fill	0.3535	0.0880	0.2013	0.2301	297.01
no aeration, Mirelon isolation, water fill	0.3773	0.2242	0.2307	0.1805	298.65
aeration, Mirelon isolation, medium fill	0.4076	0.0457	0.0634	0.2994	288.80
no aeration, Mirelon isol., medium fill	0.3972	0.0377	0.1300	0.2257	294.94

and effective heat conductivity of liquid at the calculation of aeration influence on the basis of similarity theory. Conversion from isolation to the surround runs in all arrangements equally. That is not in the conflict with notorious knowledges too. But small isolation type influence is evident.

Heat dynamism optimized model of the cultivation set together with the relevant experimental data is perceptible from the exemplary graph for one of studied systems (Fig. 1.).

Notation

c_{pA}, c_{pB}	specific heat capacity of area A, or B [J kg⁻¹ K⁻¹]
K_{TA}, K_{TB}	heat-transfer coefficient from area A (or B) to B (or C) [J s⁻¹ K⁻¹]
m_A, m_B	weight of area A, or B [kg]
p_1, p_2, p_3, p_4	parameters [s⁻¹]
q_A	source item [J s⁻¹]
t	time [s]
T_A, T_B, T_C	temperature of area A, B, or C [K]
T_P	initial temperature of bioreactor fill [K]

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P02 POLYCYCLIC AROMATIC HYDROCARBONS IN THE CZECH FOODSTUFFS IN THE YEAR 2004

IVANA BORKOVCOVÁ, MARCELA DOFKOVÁ, IRENA ŘEHUŘKOVÁ and JIŘÍ RUPRICH
National Institute of Public Health – Centre for Hygiene of Food Chains, Palackeho 3a, 612 42 Brno, Czech Republic,
borkovcova@chpr.szu.cz

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are classified as persistent organic environmental contaminants. They constitute a large class of organic compounds containing two or more fused aromatic rings. Many of them have been identified as being carcinogens, with possible genotoxic properties. PAHs are formed during incomplete combustion of organic matter. Human exposure to them is unavoidable. The major routes of exposure to PAHs are from contaminated food and inhaled air.

In food, PAHs may be formed during processing and domestic food preparation, such as smoking, drying, roasting, baking, frying or grilling. Unprocessed food, namely vegetables, may be contaminated by the airborne deposition or by growth in contaminated soil.

Material and methods

The objective of our study was to provide data enabling to assess the health risk of our population related to the exposure to PAHs from foodstuffs consumed in the Czech Republic. PAHs have been determined within „The Project on Dietary Exposure to Selected Chemical Substances" in the National Institute of Public Health, Centre for Hygiene of Food Chains in Brno.

PAHs were determined in 100 composite food samples in the range of the so-called food basket of foodstuffs of the

Czech population. These samples represent food consumption in the Czech Republic[1]. The samples were analysed after culinary treatment, i. e. in the same condition as they are consumed.

The dietary exposure data were obtained by multiplying analytical data by the conversion factor for culinary treatment and by the average consumption of foodstuffs in the Czech Republic. Factor for culinary treatment describes the changes of weight of the food sample caused by the culinary treatment[2].

The analytical method consisted of the three consequent steps – solvent extraction of the sample, followed by the clean-up and finally the HPLC determination. Homogenous samples were first extracted either with a dichloromethane on a dispersant Polytron, and then in ultrasonic bath, or with the mixture of hot solvents – petroleum ether: aceton (2 : 1). The interfering coextracts (fat and pigments) were removed using gel permeation chromatography (Waters GPC Clean-up System). PAHs from liquid samples were isolated by means of liquid-liquid extraction. HPLC analysis was carried out using Agilent 1100 chromatograph with the gradient elution and fluorescence detection. The mobile phase was composed of acetonitrile and water. Excitation and emission wavelenghts were changed as programmed. PAH Waters column with the reverse phase was used.

The accuracy of the method was ensured via CRM 2977 Mussel Tissue analyses and participation in FAPAS (UK) prroficiency testing. Recovery of the method was determined on the matrixes of vegetable oil and chicken meat and ranged between 90–105 %. Repeability (n = 10) for most analytes, except of naphtalen (48 %), was 2–15 %.

Results and discussion

The concentration of 15 PAHs according to the US-EPA protocol (naphtalene, acenaphtene, fluorene, phenenthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(ghi)perylene, indeno(1,2,3-cd)pyrene) was determined.
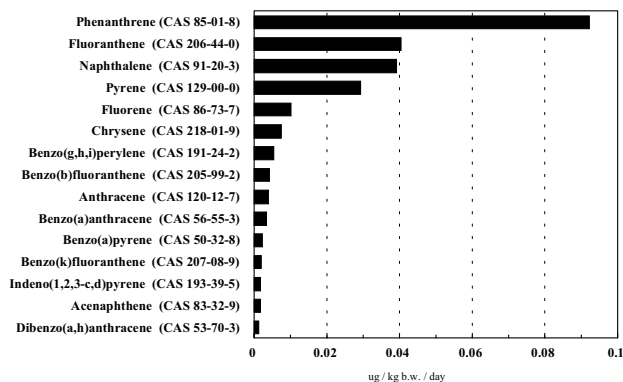
Those PAHs ranged in the food samples purchased on the market and culinary treated at the trace levels ($\mu g \ kg^{-1}$ and less).

The most abundant of all the measured PAHs were those containing three and four aromatic rings, e.g. fenanthrene, anthracene, fluoranthene and pyrene. The concentration individual PAHs ranged from 0,01–10 $\mu g \ kg^{-1}$.

The most contaminated commodities were vegetable oils, smoked fish and spices. Occurence of PAHs in these commodities is ascribed to processes of drying, frying and extractions at elevated temperatures. Considering the food consumption, the main sources of PAHs exposition in the Czech Republic are coffee, canned fish and bakery. Daily dietary exposure doses of PAHs in the Czech Republic ($\mu g \ kg^{-1}$ b.w./day) are shown in the Fig. 1.

Of the many hundreds of PAHs, the most studied is benzo(a)pyrene, which is often used as a marker for PAHs in food. According EU Scientific Commetee on Food it can be used as an indicator of occurence, concentration and effect of the carcinogenic high-molecular mass PAHs in food[3]. The foodstuffs with the highest content ($\mu g \ kg^{-1}$ of specified food) and the most important sources of dietary exposure ($\mu g \ kg^{-1}$ b.w./day) of benzo(a)pyrene in the Czech Republic are shown in the Fig. 2. and 3.
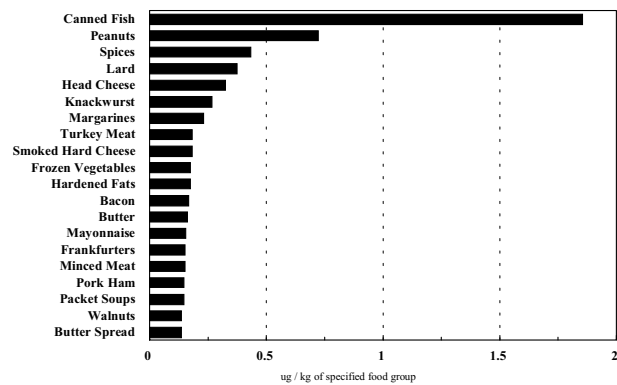
Fig. 2. Foodstuffs with the highest content of benzo(a)pyrene ($\mu g \ kg^{-1}$ of specified food group)

Fig. 1. Daily dietary exposure doses of PAHs in the Czech Republic ($\mu g \ kg^{-1}$ b.w./day)

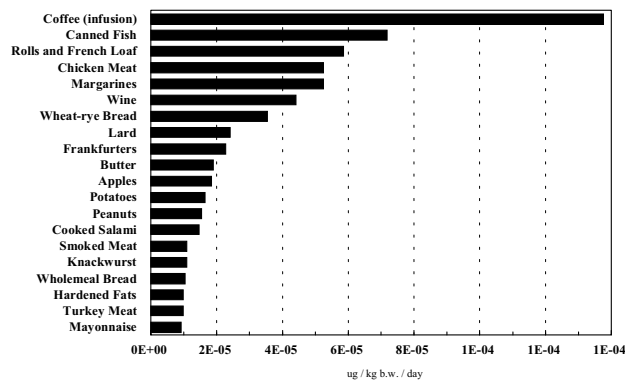**Fig. 3. The most important sources of dietary exposure of benzo(a)pyrene in the Czech Republic ($\mu g \ kg^{-1}$ b.w./day)**

Conclusions

The content of polycyclic aromatic hydrocarbons in the samples of the food basket of the Czech Republic was monitored in the year 2004. The individual PAHs included in the assesment comprised the 15 compounds, selected according to the US-EPA protocol. Their concentration was determined by means of HPLC with fluorescence detection after solvent extraction of the sample and clean up of the extract.

The concentration of monitored PAHs in the analysed foods was very low, ranging from 0.1 to 10 µg kg^{-1}.

On the basis of thus obtained results the exposition doses were estimated.

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P03   ENZYMATIC PREPARATION OF SUGAR-LIPID CONJUGATES

NINA BORODOVČÁKOVÁ[a],
DAGMAR BILANIČOVÁ[b], VLADIMÍR MASTIHUBA[b],
NAĎA PRÓNAYOVÁ[c], MÁRIA MASTIHUBOVÁ[d]
and MILAN ČERTÍK[a]

[a]*Department of Biochemical Technology,* [b]*Department of Food Sciences and Technology,* [c]*Central Laboratories, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava,* [d]*Institute of Chemistry, Slovak Academy of Sciences, Dúbravska cesta 9, 845 38 Bratislava, Slovak Republic,*
nina.borodovcakova@stuba.sk,
milan.certik@stuba.sk

Introduction

Surfactants are amphiphilic molecules, which are widely used in food application and many industries because of their low toxicity or environmental compatibility. Sugar fatty acid esters belong to group of nonionic surfactants, which are well-known as emulsifying, stabilizing and detergency agents. These molecules are utilizable in pharmaceutical, cosmetic, detergent and food industries[1].

Sugar fatty acid esters are produced from renewable and readily available substances. They are harmless to the environment due to their complete biodegradability under aerobic and anaerobic condition. These compounds are nontoxic, non-skin irritants, odorless and tasteless.

Chemical synthesis of sugar fatty acid esters is based on high temperature reaction between sugar and fatty acids in presence of an alkaline catalyst. Pyridine is usually used as organic solvent. Other technique is based on fermentation of selected microorganisms leading to formation of isomeric mixtures of biosurfactants. Finally, application of enzymes, particularly lipases, offers different methods for sugar ester production. Moreover, these methods can provide regio- and stereo-selective products[2].

A major problem in the production of sugar fatty esters using enzymes is low solubility of sugar in most of organic solvents. One possibility to overcome this difficulty is to employ alkylglycosides as substrates instead of sugar. Alkylglycosides are appropriatelly soluble in suitable organic solvents that might lead to higher reaction yields. Therefore, the aim of the work was to prepare sugar-lipid conjugates by enzymatic route from selected alkylglycosides and fatty acids.

Material and methods

Enzymes and chemicals. Lipase PS from *Burkholderia cepacia* was a gift from Amano Pharmaceutical Co. Butyl acetate used in column chromatography and ethyl acetate used in thin-layer chromatography was purchased from Mikrochem, acetonitril was obtained from Sigma-Aldrich. Linoleic acid (more than 99% purity) was prepared from sunflower oil by the urea fractionation method developed in our laboratory[3]. Alkylglykosides, n-penthyl-*O*-β-D-galactopyranoside and n-hexyl-*O*-β-D-galactopyranoside were prepared according to Bilaničová[4].

Esterification reaction. Reaction mixture for sugar fatty acid esters consisted of 1.11 ml linoleic acid, 300 mg n-pentyl-*O*-β-D-galactopyranoside or n-hexyl-*O*-β-D-galactopyranoside, 50 mg lipase PS and 30 ml acetonitril. The reaction was performed in 100 ml flasks on magnetic shaker at laboratory temperature. Product of the reaction was monitored during reaction time by TLC.

TLC analysis. Analyses were carried out on TLC plates (Silica gel 60, Merck) that were developed with ethylacetate. Spots were visualized with mixture H_2SO_4/methanol (5/95, v/v) followed by heating at 150 °C.

Isolation of the esterification product. Residues of alkylglycosides were removed from the reaction mixture by extraction with hexane and the product was detected in hexane fraction. Enzymes were inactivated in previous step by heating at 100 °C for 15 minutes. Isolation of the product was performed using column chromatography. A mixture of fatty acid esters, alkylglycoside and residual linoleic acid was applied on the silica gel column and eluted by butyl acetate. Fractions collected in selected time intervals were subsequently analyzed by TLC. Fractions with pure products were evaporated and analyzed by NMR.

Results and discussion

An increased interest in biosurfactants focused attention on new types of these compounds finding applications in food, pharmaceutical and cosmetic fields. One group of the demanded compounds is represented by bioactive saccharide-lipid surfactants. The work was aimed on the possible

enzymatic preparation of sugar-lipid conjugates. The present work describes the synthesis of unsaturated fatty acid with β-pentylgalactoside or β-hexylgalactoside esters by lipase-catalyzed (lipase PS) esterification in acetonitrile. Reaction conditions were optimized with the aim to increase efficiency of esterification and to prohibit undesirable hydrolysis.

Table I
Characteristic of 1-penthyl-6-O-linoleyl-β-D-galactopyranoside

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NMR characteristic

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^{1}H NMR (300 MHz, DMSO-d_6): δ 0.86 (t, 6H, 2CH$_3$), 1.10-1.48 (m, 18H, 9CH$_2$), 1.42-1.64 (m, 4H, CH$_2$-1', CH$_2$-3''), 1.94-2.22 (m, 4H, CH$_2$-8'',CH$_2$-14''), 2.27 (t, 2H, CH$_2$-2''), 2.64-2.82 (m, 2H, CH$_2$-11''), 3.24-3.31 (m, 2H, H-2, H-3), 3.53-3.60 (m, 2H, H-4, H-5), 3.54 (ddt, 2H, CH$_2$-1'), 4.03 (dd, 1H, $J_{5,6b}$ = 3.9 Hz, H-6b), 4.05 (m, 1H, H-1), 4.19 (dd, 1H, $J_{5,6a}$ = 7.8 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.62 (d, 1H, OH), 4.77 (d, 1H, OH), 4.87 (d, 1H, OH), 5.26-6.55 (m, 4H, H-9'', H-10'', H-12'', H-13'').

^{13}C NMR (75 MHz, DMSO-d_6): δ 13.9 (2$\underline{C}$H$_3$), 22.0 ($\underline{C}$H$_2$), 22.1 ($\underline{C}$H$_2$), 24.5 ($\underline{C}$H$_2$), 25.23 ($\underline{C}$H$_2$-11''), 26.6 ($\underline{C}$H$_2$-14''), 27.1 ($\underline{C}$H$_2$-8''), 27.7 ($\underline{C}$H$_2$), 28.4 ($\underline{C}$H$_2$), 28.5 ($\underline{C}$H$_2$), 28.6 ($\underline{C}$H$_2$), 28.7 ($\underline{C}$H$_2$), 28.8 ($\underline{C}$H$_2$), 28.9 ($\underline{C}$H$_2$), 29.1 ($\underline{C}$H$_2$), 33.5 ($\underline{C}$H$_2$-2''), 63.6 ($\underline{C}$H$_2$-6), 68.6 ($\underline{C}$H$_2$-1'), 68.53, 72.13 ($\underline{C}$H-4, $\underline{C}$H-5), 70.3, 73.1, ($\underline{C}$H-2, $\underline{C}$H-3), 103.4 ($\underline{C}$H-1), 127.7, 127.8, 129.7, 129.8 (C-9'', C-10'', C-12'', C-13''), 172.8 (COO).

Table II
Characteristic of 1-hexyl-6-O-linoleyl-β-D-galactopyranoside

NMR characteristic

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^{1}H NMR (300 MHz, DMSO-d_6): δ 0.86 (t, 6H, 2CH$_3$), 1.10-1.48 (m, 20H, 10CH$_2$), 1.42-1.64 (m, 4H, CH$_2$-1', CH$_2$-3''), 1.94-2.22 (m, 4H, CH$_2$-8'',CH$_2$-14''), 2.27 (t, 2H, CH$_2$-2''), 2.64-2.82 (m, 2H, CH$_2$-11''), 3.24-3.31 (m, 2H, H-2, H-3), 3.53-3.60 (m, 2H, H-4, H-5), 3.54 (ddt, 2H, CH$_2$-1'), 4.03 (dd, 1H, $J_{5,6b}$ = 3.9 Hz, H-6b), 4.05 (m, 1H, H-1), 4.19 (dd, 1H, $J_{5,6a}$ = 7.8 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.62 (d, 1H, OH), 4.77 (d, 1H, OH), 4.87 (d, 1H, OH), 5.26-6.55 (m, 4H, H-9'', H-10'', H-12'', H-13'').

^{13}C NMR (75 MHz, DMSO-d_6): δ 13.9 (2$\underline{C}$H$_3$), 22.0 ($\underline{C}$H$_2$), 22.1 ($\underline{C}$H$_2$), 24.5 ($\underline{C}$H$_2$), 25.23 ($\underline{C}$H$_2$-11''), 26.6 ($\underline{C}$H$_2$-14''), 27.1 ($\underline{C}$H$_2$-8''), 27.7 ($\underline{C}$H$_2$), 28.4 ($\underline{C}$H$_2$), 28.5 ($\underline{C}$H$_2$), 28.6 ($\underline{C}$H$_2$), 28.7 ($\underline{C}$H$_2$), 28.8 ($\underline{C}$H$_2$), 28.9 ($\underline{C}$H$_2$), 29.1 ($\underline{C}$H$_2$), 33.5 ($\underline{C}$H$_2$-2''), 63.6 ($\underline{C}$H$_2$-6), 68.6 ($\underline{C}$H$_2$-1'), 68.53, 72.13 ($\underline{C}$H-4, $\underline{C}$H-5), 70.3, 73.1, ($\underline{C}$H-2, $\underline{C}$H-3), 103.4 ($\underline{C}$H-1), 127.7, 127.8, 129.7, 129.8 (C-9'', C-10'', C-12'', C-13''), 172.8 (COO).

Esterification of galactosides and linoleic acid required relatively long time reaction. Products were detected on the fourth day of the reaction and the esterification continued further up to sixteen days. Products of the reactions were identified by NMR as 1-penthyl-6-*O*-linoleyl-β-D-galactopyranoside (weight 143 mg; yield 23,3 mol %) and 1-hexyl-6-*O*-linoleyl-β-D-galactopyranoside (weight 97.1 mg; yield 16,3 mol %), respectively. NMR characteristics of these two products are shown in Tables I and II.

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P04 STUDY OF COMPOSITION SPELT PROLAMIN COMPONENT

EVA BURÁKOVÁ, BERNADETTA KRKOŠKOVÁ, EVA MACOVÁ and DARINA SVETLÍKOVÁ
Food Research Institute, Priemyselná 4, 824 75 Bratislava, Slovak Republic, eva.macova@vup.sk

Introduction

Spelt is a distant cousin to modern wheat. Modern wheat varieties have been bred to be easier to grow and harvest, to increase yield, as well as to have a high gluten content for the production of high-volume commercial baked goods. Spelt, on the other hand, has retained much of its original character. It retains a sturdy husk or hull which remains with the kernel, as opposed to modern wheat varieties which have been bred to lose their husks when harvested. This hull protects the spelt grain from pollutants and insects. Furthermore, unlike other grains, spelt is not normally treated with pesticides or other chemicals. Spelt is stored and shipped with its protective hull intact; it is separated just before being milled into flour. Leaving the husk on the grain not only protects the kernel, but enhances the retention of the nutrients in the kernel and improves freshness[1].

Spelt's uniqueness is also derived from its genetic makeup and nutrition profile. Spelt has high water solubility, so the nutrients are easily absorbed by the body. Spelt contains special carbohydrates (mucopolysaccharides) which are an important factor in blood clotting and stimulating the body's immune system. It is also a superb fiber resource and has large amounts of B complex vitamins[2]. Total protein con-

tent is from 10 to 25 % greater than the common varieties of commercial wheat[3].

Gliadins and glutenins are the storage or gluten-forming proteins in wheat and spelt. Gluten is of greatest technological significance. The gross composition of gluten is extremely heterogeneous since it is a complex mixture of many highly polymorphic proteins. Gliadins exhibit physical and chemical properties that are useful in food manufacturing. They are therefore used extensively in the food industry. Gluten ranks among those proteins to which some persons are intolerant. Celiac disease is a disorder of the small intestine. It is triggered in susceptible individuals by ingestion of prolamins found in wheat, barley, rye and triticale[4]. One the most beneficial differences between spelt and wheat is the fact that many wheat and gluten-sensitive individuals have been able to include spelt based foods in their diets. Spelt can be substituted for whole wheat in breads, cookies, crackers, and cakes as well as muffins, pancakes and waffles[5,6].

Experimental part

One sample of spelt, species Bauländer (*Triticum spelta L.*) and products of this spelt, namely spelt flour and pasta, spelt flakes, as well as one common winter wheat species Petrana (*Triticum aestivum L.*) were observed.

In all samples the content of gliadins was determined. Gliadin extracts prepared by extractive fractionation were tested on Sephacryle S-200 HR column (fi Amersham Biosciences). Eluted fractions were analysed by SDS-PAGE according to the modified method of Laemmli using the Mighty Small Electrophoresis Unit (Hoefer Pharmacia Biotech).

Presence of immunological active gliadin fractions was detected by IMMUNOLAB Gluten-EIA (Immunolab GmbH). Determined part is the gliadin fraction of the gluten.

Results and discussion

Gliadins comprise a heterogeneous group of proteins with respect to their molecular weights (usually they are divided in to 4 groups – alfa, beta, gama and omega gliadins), but the molecular weights of most gliadin species fall within the range 30 000 to 45 000. Only omega gliadins have much higher molecular weights (75 000–79 000).

SDS-PAGE pattern of common wheat gliadin fraction exhibited zones for several protein groups, namely:
–   fraction with relative molecular weight (MW) 60 000–61 000
–   fraction for gama gliadins with relative MW 43 000–41 000, typical for common wheat species
–   subunits with relative MW 38 000–36 000
–   subunits with relative MW 31 000, 25 000 and 22 000–21 000
–   polypeptides with relative MW in range 19 000–14 000.

In spelt gliadin fraction were found bands for following protein groups:
–   fraction with relative MW 58 000

–   fraction for gama gliadins with relative MW 44 000, in range for durum wheat gama gliadins
–   subunits with relative MW 38 000–36 000 like as in case of common wheat
–   subunits with relative MW 27 000–26 000
–   polypeptides with relative MW 16 000–15 000.

Based on comparison of protein spectrum for gliadin fraction from common wheat with protein spectrum of gliadin fraction from spelt it is evident that spelt doesn't contain neither alfa gliadins in range of MW 25 000–21 000 nor polypeptides with relative MW 19 000–18 000. Absence of this fraction can represent cause for tolerance of spelt and spelt products for some gluten-sensitive individuals.

Following Table I contain results of gliadin determination obtained by immunochemical method. Presence of celiac active proteins was tested also in industrial spelt products. Content of immunological active gliadin components in monitored products achieved only 20–35% from gliadin content in common wheat. Codex Alimentarius recommends "…gluten-free means that the total nitrogen content of the gluten – containing cereal grains used in the product does not exceed 0.05 g per 100 g of these grains on a dry matter basis". International references standards permit for gluten free foods total gluten content no more than 200 mg kg^{-1} on a dry matter basis. This limit was exceeding in all spelt products, but in different extent. In general, it can be concluded that the examined products can be suitable for celiac patients only in limited amounts witch must be determined individual, based on determination of immunological active gliadins in particular product.

Table I
Gliadin determination results by immunochemical method[a]

Gliadin content [b] [mg kg^{-1}]				
Wheat Petrana	Spelt groats Bauländer	Spelt flour Bauländer	Spelt flakes Bauländer	Spelt pasta Bauländer
28 000	6000	9800	5200	6400
Gluten content [mg kg^{-1}]				
56 000	12 000	19 600	10 400	12 800
Gluten content in dry mater [mg kg^{-1}]				
61 700	13 500	21 700	11 340	14 000

[a] – IMMUNOLAB Gluten-EIA. Detection limit: 0.0004% gliadin. Extended uncertainty of measurement: 5 %
[b] – average value of 2 determination

In spelt and spelt products was followed also composition of protein part by fractional extraction. Proportions of main protein groups in spelt and some spelt products demonstrate Fig. 1. Spelt groats and flour showed similar composition of protein part. Albumins represented about 13 %, globulins 3 %, prolamins 40 % and glutelins 45 % of

total protein content. Composition of proteins from pasta was characterised by lower albumins and prolamins content.
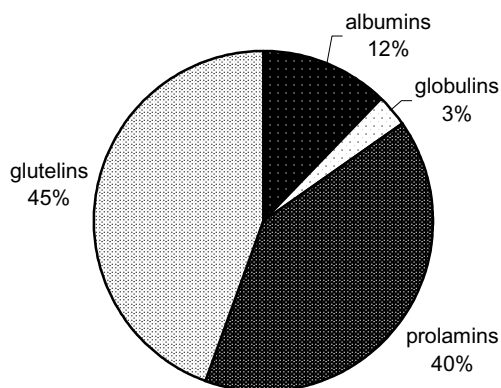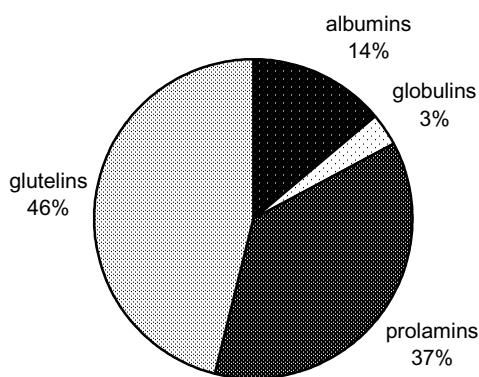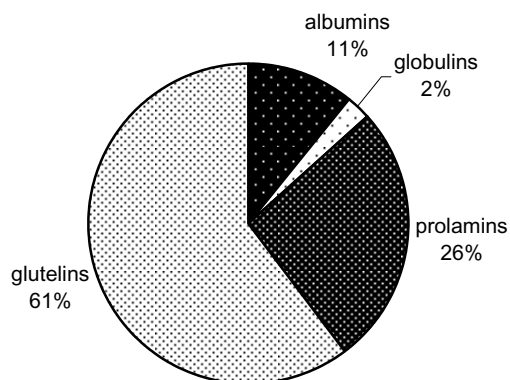

Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1. Proportions of fractions in protein extract. A – spelt groats from distribution chain, B – spelt flour from distribution chain, C – spelt pasta from distribution chain

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P05   FRACTIONATION OF IODINE IN IODINE-ENRICHED ALGAE *CHLORELLA*

IVETA BURIANOVÁ[a], JIŘÍ MACHÁT[a], EVA NIEDOBOVÁ[a], JIŘÍ DOUCHA[b] and VIKTOR KANICKÝ[a]

[a]*Laboratory of Atomic Spectrochemistry, Faculty of Science, Masaryk University Brno, Kotlarska 2, 611 37 Brno, Czech Republic, iveta.burianova@email.cz; machat@chemi.minu.cz*
[b]*Laboratory of Microalgal Biotechnology, Institute of Microbiology, Czech Academy of Sciences, Opatovický mlýn, 379 81 Trebon, Czech Republic*

Abstrakt

Iodine as an essential element has a great importance in human and animal health. Concerning animal husbandry, feed fortified by iodine provides adequate supply of this element and influences the health of animals and the quality of production. The bioavailability of iodine depends on the form of iodine. This work deals with determination of iodine forms in iodine-enriched algae Chlorella, which is cultivated in Institute of Microbiology (Czech Academy of Science) and consecutively applied in in-vivo experiments at University of Veterinary and Pharmaceutical Sciences. Fractionation protocol including sequential extraction by chloroform, water and sodium dodecylsulphate solution was designed. Inductively coupled plasma optical emission spectrometry was employed for determination of iodine in fractions solubilized with TMAH. Iodides were quantified in aqueous extract using vapour-generation ICP-OES after separation of iodides on anion exchange resin. Fractionation gives information on the content of iodine in non-polar, protein, polar water-soluble and non-soluble organic fractions and iodides. Methodology was applied in analysis of twelve algae samples that were cultivated with different cultivating conditions (hetero- or autotrophic). Results of this research are used in optimization of cultivation conditions to get desired composition of iodine-enriched Chlorella.

Introduction

Iodine is an essential element to human. It is found in highest concentration in the thyroid gland, muscles, and va-

rious endocrine tissues. The primary function of iodine in the body is as a constituent of the hormones thyroxin and triiodotyrosine, both of which are secreted by the thyroid gland and affect growth, nervous system, development, and the metabolic rate of the body. Iodine deficiency is the primary cause of simple goiter and can lead to cretinism[1,2].

The recommended daily intake of iodine for adults is ranging from 150–200 μg. This amount is hardly achievable in inland country, because sea fish are the main source of iodine in nutrition. There is iodine added to table salt or to other foodstuff in inland country.

The bioavailability of iodine for vital organisms depends on the chemical form of iodine (inorganic/organic)[3]. The natural resource of organic form of iodine is seaweed, alternative the iodine-enriched algae Chlorella for inland country. Chlorella is cultivated in Microbiological Institute (Czech Academy of Science) in Třeboň[4,5].

The cultivation (photo-autotrophic or heterotrophic) is provided in the presence of iodide, when iodine is incorporated into cell structure. Chlorella is a single-celled alga with rapid reproduction. Disintegrated cell wall provides the amazing source of concentrated nutrition, vitamins and minerals.

Any work dealing with determination of chemical species of iodine in algae Chlorella is not known so far. Chemical species of iodine were studied in marine algae by using neutron activation analysis combined with chemical separation[6]. There were determined the content of total iodine, water soluble iodine, I^-, IO_3^-, and organic iodine in water leachate. In the aqueous leachate, iodine is found as I^-. Content of iodides is 61–93 % of total water soluble iodine and percentages of organic iodine are 5.5–37.4 % and the content of IO_3^- is about 5 %. The algae *Sargassum kjellmanianum*[7] was studied by the same authors. They investigated the fractionation of organic forms of iodine. The maximum content of iodine was found in protein (65.5 %), polyphenols (3.09 %) and pigments (1.57 %), the minimum content of iodine was found in polysaccharides.

Fractionation was used for determination of iodine and its forms in algae Chlorella. Fractionation consists in extraction of sample of algae Chlorella. For extraction three reagents were used: water, chloroform and 2 % solution of SDS (sodium dodecylsulphate). Iodine in individual fractions was determined by the method inductively coupled plasma optical emission spectrometry (ICP-OES) and iodides were determined in aqueous leachate by vapour-generation ICP-OES after separation on anion-exchanger column.

Experimental
C h e m i c a l s
KI solution is prepared freshly before measurement. Tetramethylammonium hydroxide (TMAH, pure, 10%) was obtained from Lach-Ner (Neratovice, ČR).

A p p a r a t u s
Iodine content in sample of algae and in individual fractions was determined by optical emission spectrometry with inductively-coupled plasma source Jobin-Yvon 170 Ultrace (Jobin-Yvon Horiba, Longjumeau, France). The system was operated under standard conditions listed in Table I. Specific determination of iodide in aqueous leachate of algae was realized using vapour generator in connection with spectrometer mentioned above. Glass column filled by strong-basic anion exchanger (Ostion AT, Spolchemie, Ústí nad Labem, ČR) of 5×70 mm of resin column was used for separation.

Table I
Operating conditions of spectrometer Jobin Yvon 170 Ultrace

Conditions	Nebulization	Vapour generation
Introduction of the sample to ICP	Meinhard nebulizer, Cyclonic spray chamber	Vapour generator
Forwarded power, W	1200	
Plasma Ar flow rate, l min^{-1}	15	
Carrier gas Ar flow rate, l min^{-1}	0.8	0.5
Sheath Ar flow rate, l min^{-1}	0.2	0.5
Sample flow rate, ml min^{-1}	1.0	2.5
Reagent flow rate, ml min^{-1}	–	1.0
Analytical line	II 182.980 nm	II 172.218 nm

The fractions were obtained and processed using current laboratory equipment (angle centrifuge, horizontal agitator, drying oven).

S a m p l e   p r e p a r a t i o n
The sample of algae was obtained from Mikrobiological institute (Czech Academy of Science) in Třeboň. An alga was cultivated in presence of sodium iodide or potassium iodide under the various conditions (autotrophic or heterotrophic, different concentration of iodide…)[4,5].

Two techniques of extraction (sequential and non-sequential extraction) were carried out in this work. In sequential extraction sample was extracted by chloroform, water and 2 % SDS solution. For non-sequential extraction new portion of sample was used for each extraction. The results of both extraction techniques were comparable; hence non-
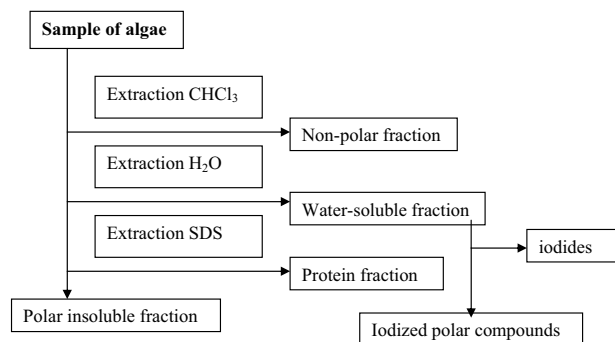

Fig. 1.   **Scheme of fractionation of algae *Chlorella***

-sequential extraction was used in further experiments for its easier practicability. The expected composition in individual fractions is summarized in Table II. The scheme of sequential extraction is shows in Fig. 1.

Table II
The results fractionation of iodine by sequential and non-sequential extraction

Fraction	Expected composition	Sequential extraction	Non-sequential extraction
Non-polar (extract $CHCl_3$)	Non-polar iodized compounds (alkanes, ketones, isoprenoides, pigments, …), (I_2)	22 ± 2	23 ± 1
Water-soluble (extract H_2O)	Polar organic compounds (aminoacids, peptides, proteins), iodides (iodates)	28 ± 1	30 ± 2
Protein (extract SDS)	Proteins etc.	41 ± 3	40 ± 3
Polar insoluble (residue)	Polysaccharides etc.	9 ± 1	7 ± 4

A portion (200 mg) of sample was weighed into a test-tube and than 7 ml extraction reagent was added (water, $CHCl_3$ or 2 % solution of SDS). The influence of extraction time on iodine content was monitored at intervals 15–120 minutes. For extraction 15 minutes agitation with reagent was chosen as sufficient. After extraction by $CHCl_3$, the insoluble residue was caught on paper filter. After extraction by water or 2 % SDS, the extraction was repeated once more after 10 minutes centrifugation. Extracts and insoluble residues were solubilized by tetramethylammonium-hydroxide (TMAH) at 90 °C for 3 hours, filled up to defined volume and centrifuged (chloroform evaporated in the presence of TMAH). Iodides in aqueous leachate were determined by vapour-generation ICP-OES. The aqueous leachate of sample (2 ml) was passed through a strong-basic anion exchanger column (Ostion AT)The column was washed with 50 ml distilled water (elimination of chemical interference of organic matrix). Iodides were eluted by 2 mol l^{-1} NaNO$_3$ and iodine vapour generated to ICP (1 mol l^{-1} H_2O_2 in 5 mol l^{-1} H_2SO_4).$2I^- + H_2O_2 + 2H^+ \rightarrow 2H_2O + I_2$.

Results

Extraction by water

Water-soluble and water-insoluble fractions are determinated by this extraction. In water-insoluble fraction organic-bounded iodine is assumed[1]. In water-soluble fraction inorganic forms of iodine (iodides – from cultivation, iodates – photooxidation of iodides) and water-soluble polar oragnic compouds (iodizated aminoacids, peptides and eventually water-soluble proteins) are expected[7].

Content of total iodine in various algae samples is 130–5400 mg kg^{-1}.

Content of iodides is 10–100 % of total iodine and 50–100 % of water-soluble fraction for majority of samples. The results are listed in Table III. The samples of high insoluble iodine are interesting for further investigation of organic forms (extraction by SDS and $CHCl_3$).

Extraction by SDS

Proteins and less-polar substances are solubilized and all water-soluble substances pass to solution. Protein fraction is calculated as a difference of SDS and water extractable fraction.

Extraction by $CHCl_3$

Iodine content in chloroform extract is determined after evaporation of $CHCl_3$ in presence TMAH, nevertheless in some sample the losses of volatile compounds of iodine are 85–100 %. In this sample iodine is probably present in non-polar volatile compounds, which volatilize together with chloroform vapour. Non-polar fraction is therefore calculated as a difference of total iodine and insoluble fraction. Presence of volatile iodizated alkanes C1–C4 was proved in seaweed[8]. Presence of elemental iodine is improbable in this fraction. The results are shown in Table IV.

Determination of iodide

Iodides, which are added to nutrient solution as sodium or potassium iodide, are determined in aqueous leachate by the method of vapour-generation ICP-OES after separation on anion-exchanger column. Iodides are oxidized by H_2O_2 to elemental iodine and iodine vapour are led to plasma discharge. Liquid phase is removed in phase-separator, hence spectral line 178.218 nm can be used for measurement (elimination of spectral interferences of phosporous).

Discussion

Content of total iodine in various algae is 130–5400 mg kg^{-1}. Some samples have minimum iodine in water-insoluble fraction that means organic bounded iodine (below 10 %). Iodine is present in cell as iodide from 76 to 100 % in this alga. 78–100 % of water-soluble fraction is found as iodide.

The alga sample A/2/28 is remarkable. This alga contains low amount of iodide (11 %), but very high amount of iodine in water-insoluble fraction (70 %). Iodide is here evidently metabolized to organic compounds, where 40 % is bounded in protein fraction and 23 % is in non-polar fraction. Sample of algae was used for in-vivo experiments at Veterinary and Pharmaceutical University in Brno. Influence of organic bounded iodine in nutrition of sows on iodine content in milk was investigated here[9].

Conclusion

The chemical species of iodine in algae were studied using ICP-OES and iodides were determined by the me-

Table III
Content of total iodine, fractionation of iodine species by water extraction and determination of iodides

Sample of algae	Content of total iodine [mg/kg]	Water extraction		In soluble fraction		
		Insoluble fraction	Soluble fraction [%][1]	Iodized polar	Iodides	Iodides [%][2]
H/1/1	170	52	45	35	10	22
H/1/2	120	50	50	18	32	64
H/1/3	510	36	61	23	38	63
H/1/4	700	26	74	17	57	76
H/2/1	5400	2	99	0	100	100
H/2/2	1200	8	90	4	86	95
H/2/3	280	45	53	28	25	48
H/2/4	1200	5	92	10	82	90
H/2/5	750	8	91	15	76	84
A/2/28	1200	70	30	19	11	37
H/3/1	2300	2	98	22	76	78
A/4/1	860	10	90	31	59	66

[1] Percentages of total iodine content, range of RSD is 5–10 % rel.; [2] percentages of iodides in water- soluble fraction; [H]heterotrophic algae; [A]autotrophic algae

Table IV
Fractionation of iodine species by SDS and CHCl₃ extractions (sample selection based on results of aqueous extraction)

Sample of algae	Water extraction		In insoluble fraction	
	Insoluble fraction	Protein	Non-polar	Polar insoluble
		[%][1]		
H/01/1	52	15	18	19
H/01/2	57	14	12	31
H/01/3	36	11	12	12
H/01/4	30	6	16	8
H/02/3	41	8	17	17

[1] Percentages of total iodine content range of RSD: 5–10% rel

thod of vapour-generation. In this work a methodology for fractionation of iodine in iodine–enriched algae Chlorella is elaborated. For fractionation water, SDS and CHCl₃ extractions were used. Fractionation is efficient to separate iodine into several fractions (non-polar, protein, polar water-soluble and organic insoluble fraction). The results will be used for optimization of cultivation conditions to obtain the algae with maximum content of desirable components. Fractionation is a starting point for further speciation (non-polar, volatile, polar compounds, macromolecules...). Composition of iodized Chlorella was comparable to various marine algae (iodides 61–93 %, organic forms 5–37 % of water-soluble fraction)[6,7].

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P06 COMPARISON OF SOURCES OF ROSMARINIC ACID

VIERA ČULÁKOVÁ, MAGDA MÁRIÁSSYOVÁ and ĽUDMILA HEILEROVÁ

Food Research Institute Biocentre Modra, Kostolná 7, 900 01 Modra, Slovak Republic,
vup-bc.modra@ba.telecom.sk,

Introduction

Higher plants are very important sources of secondary metabolities, which have therapeutic and pharmaceutical applications. One group of the secondary metabolites are phenolic acids. Rosmarinic acid with another phenolic acids (e. g. caffeic acid, chlorogenic acid) are mainly found in plants of *Lamiaceae* family. The aim of these work was screening mostly *Lamiacae* plants for rosmarinic acid and other phenolic acids, because they have antioxidant activity, antiinflamatory activity, antimicrobial activity, antiviral activity and other activities (e. g. treatment of hyperthyreosis, cholera).

Materials and Method

Materials

Rosmarinic acid (Aldrich), Chlorogenic acid (Sigma), Caffeic acid (Sigma), Ferulic acid (Aldrich), Protocatechuic acid (Sigma), Gallic acid (Fluka), Trifluoracetic acid (Aldrich), Acetonitrile Chromasolv HPLC gradient grade (Riedel de Haën).

Method

HPLC, UV/VIS detection wavelength 325 and 294 nm LC 10 ADVP pump with gradient FCV 10 ALVP, detector UV-VIS SP 10 AVVP, (Shimadzu, Japan); Conditions: column Reprosil 100 C18, 5 μm, 250 × 4 mm (Watrex), flow rate 0.5 ml min^{-1}; mobil phase A: 0.1 % TFA, B: acetonitril; gradient: 0–5 min 10 % B; 5–20 min 35 % B; 20–40 min 70 % B; 40–41 min 90 % B; 41–42 min 50 % B; 42–43 min 25 % B; 43–44 min 5 % B; 45 min 100 % A; stop analyses 46 min.

Calibration curves

Calibration curves of standardes phenolic acids were in determined ranges linary and their equations by MMS are:
Chlorogenic acid: $y = 17.99 \times - 57.71$; $R^2 = 0.9945$
Caffeic acid: $y = 27.42 \times - 20.37$; $R^2 = 1.0$
Ferulic acid: $y = 20.57 \times - 33.82$; $R^2 = 0.9998$
Rosmarinic acid: $y = 9.67 \times - 3.11$; $R^2 = 0.9950$

Results and discussion

Analysis of phenolic acids were made from ethanolic extracts of dryied plants. Extacts were prepared with the 50% ethanol in the ratio 1:20 (g dryied plant/extraction solution). Extracts were prepared from lemon balm (*Melissa officinalis L*), rosemary (*Rosmarinus officinalis L*), mint (*Mentha spicata L*), lavender (*Lavandula L.*), agrimony (*Agrimonia eupatoria L*), sage (*Salvia officinalis L*), linden (*Tilia F*). and hyssop (*Hyssopus officinalis L*). Prunella (*Prunella vulgaris*) was analysed in different solvents in the same ratio. From analysis sumarized in Table I is evident, that the highest content of rosmarinic acid was extracted from lemon balm, 0.455 mg ml^{-1} and Table II showed, that from prunella was

Table II

Rosmarinic acid in Prunella vulgaris extracted in different solvents at different temperatures

Solvent	Rosmarinic acid [mg ml^{-1}]
isopropanol conc., 20 °C	0.07 ± 0.01
isopropanol conc., 40 °C	0.09 ± 0.01
acetone conc., 20 °C	0.05 ± 0.01
ethanol conc., 20 °C	0.53 ± 0.04
ethanol conc., 40 °C	0.80 ± 0.04
ethanol 40 %, 40 °C	0.43 ± 0.01
methanol conc., 20 °C	0.95 ± 0.09
methanol 40 %, 40 °C	0.65 ± 0.06
methanol 40 %, 60 °C	0.61 ± 0.06
water, 40 °C	*
water, 60 °C	*

Table I

Content of phenolic acids in ethanolic plant extracts

Plant	Chlorogenic acid [mg ml^{-1}]	Caffeic acid [mg ml^{-1}]	Ferulic acid [mg ml^{-1}]	Rosmarinic acid [mg ml^{-1}]
Lemon balm	0.006 ± 0.001	0.021 ± 0.001	0.04 ± 0.01	0.46 ± 0.05
Rosemary	*	*	0.040 ± 0.007	0.22 ± 0.05
Mint	0.018 ± 0.001	0.009 ± 0.002	*	0.150 ± 0.002
Lavender	0.043 ± 0.001	0.025 ± 0.005	0.013 ± 0.001	0.070 ± 0.007
Agrimony	0.043 ± 0.002	0.022 ± 0.002	0.105 ± 0.001	0.222 ± 0.003
Sage.	0	*	0.03 ± 0.01	0.27 ± 0.04
Linden	0.049 ± 0.009	0.006 ± 0.002	0.025 ± 0.006	*
Hyssop	0.350 ± 0.002	0.05 ± 0.01	0.041 ± 0.003	0.38 ± 0.02

*traces

the highest content of rosmarinic acid ectracted by concentraced methanol 0.947 mg ml^{-1}. Protocatechuic acid and gallic acid were identified only in traces in list of plants.

In some plant sources (e. g. lemon balm, prunella) in our climate is enough rosmarinic acid to isolation. It is worthy to continue in the work with the aim to develop the proces of isolation of rosmarinic acid, because of it has antioxidant activity, antiinflamatory activity, antimicrobial activity, antiviral activity and other activities (e. g. treatment of hyperthyreosis, cholera).

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P07　CHANGES OF CAROTENOID CONTENT IN SUB-CELLULAR FRACTIONS OF RED YEASTS GROWN UNDER EXTERNAL STRESS

MICHAELA DRÁBKOVÁ[a], RADKA KOČÍ[a], JITKA KUBEŠOVÁ[a], VOLKMAR PASSOTH[b] and IVANA MÁROVÁ[a]

[a]*Department of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00, Czech Republic, drabkova@fch.vutbr.cz,* [b]*Department of Microbiology, Uppsala Genetic Centre, Swedish University of Agricultural Sciences, Box 7025 S-750 07, Uppsala, Sweden, volmar.passoth@mikrob.slu.se*

Introduction

Qualitative and quantitative changes in a cell metabolite complement can be induced by environment, stress and other factors. Thus, identification of metabolic markers characteristic for certain events provides important insight into the mechanisms of pathways occurring in the organism and can also lead to the regulation of production of industrially significant metabolites. Especially in microorganisms is production of metabolites strongly influenced by series of external factors. Environmental stress surrounding yeast cells evokes various changes in their behaviour in order to survive under unfavourable conditions. Under stress, various specific compounds including lipidic substances are overproduced (e. g. glycerol, phospholipids, carotenoids, ergosterol etc.). However, more information is needed about regulation of production of these substances.

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. It is not clear, whether carotenoids are present in plasma membrane only or in other inner membrane systems as well as in cell wall. Also distribution of individual carotenoid derivatives in individual sub--cellular fractions was not studied yet. Moreover, significant changes of these parameters under exogenous stress could occur, which could influence potential biotechnological use of red yeasts to industrial production of carotenoids.

In this work some techniques to isolation and separation of sub-cellular fractions (cell wall, membrane fraction, cytosol) of red yeast cells grown in optimal conditions and under osmotic and oxidative stress were tested. Further, analysis of carotenoids in these fractions as well as in whole cells was done. Results of antioxidant properties of sub-cellular fractions were compared with carotenoid composition and antioxidant activity of some standard carotenoids. The aim is to find, what is the distribution and trafficking of carotenoids in the cell and which carotenoids are the main contributors to antioxidant activity in individual cell compartments.

Methods

Industrial yeast *Rhodotorula glutinis* CCY 20-2-26 was used as tested strain. Yeasts were cultivated on glucose medium aerobically at 28 °C. Exogenous stress was induced by 2–5 % NaCl and 2–5 mM H_2O_2.

Sub-cellular fractions of *R. glutinis* cells were obtained by gradually separation using combination of enzymes and detergents. Cell wall fraction (surface layer) was obtained using sonification followed by ethanol precipitation. Spheroplasts were prepared using incubation of yeast cells with lyticase and glucuronidase. Plasmatic membrane was disrupted by detergents and osmotic lysis. Membrane fraction and cytosol were separated by ultracentrifugation.

In selected fraction lipid profiles were analyzed using TLC. Levels of carotenoids – lycopene, alpha-carotene, beta-carotene, torulen and phytoene were analyzed using HPLC/MS. Ergosterol was analyzed by RP-HPLC (280 nm) and glycerol using Boehringer kit. Antioxidant activity of individual sub-cellular fractions was tested using ABTS Metod (Randox kit). Protein profiles under stress conditions were compared too.

Results and discussion

Preparation of individual sub-cellular fractions from *R. glutinis* cells was strongly complicated by lipotrophic character of this strain. Especially preparation of spheroplasts was very difficult and yields of membrane fractions were very low. As surprising finding can be noted high content of carotenoids found in upper cell wall fraction. Presence of carotenoids, mainly beta-carotene, was detected in plasma membrane as well as in inner membrane fraction. The highest antioxidant activity was found in surface structures.

Under stress conditions, about 100 proteins were overproduced. Expecting shock proteins, it could be some enzy-

mes that catalyze overproduction of stress metabolites including carotenoids. Under both oxidative and osmotic stress pigments were overproduced, higher amount was detected mainly in surface cell structures (cell wall and plasma membrane). Ergosterol was found both in cytosol and membrane fractions and its production under stress changed simultaneously with carotenoid formation. Glycerol was detected above all in cytosol fraction and its production under stress was inversely to carotenoid and ergosterol production.

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P08   ACRYLAMIDE LEVELS IN FOODSTUFFS FROM CZECH MARKET

LENKA DUNOVSKÁ, TOMÁŠ ČAJKA, and JANA HAJŠLOVÁ

Department of Food Chemistry and Analysis, Institute of Chemical Technology, Prague, Czech Republic,
lenka.dunovska@vscht.cz

Introduction

Acrylamide represents processing contaminant presence of which in heat processed foodstuffs has been proven only recently. This chemical is classified as human carcinogen by the International Agency for research on Cancer (IARC). Increased amounts of acrylamide have been found mainly in starch enriched food such as potato chips, French fries, roast potatoes, breakfast cereals and crisp bread.

Several analytical methods have been implemented for a determination of acrylamide in various foodstuffs. Most of procedures employ LC/MS-MS systems with triplequadrupole analyzer for obtaining of low detection limits. SPE cartridges are often used to remove interfering impurities from crude extract prior to determination step. In most of methods using GC/MS bromination of acrylamide is carried out in the first step to improve detection parameters (higher m/z values of target compound)[1].

In presented study simple GC procedure enabling direct determination of acrylamide has been developed. GCT high-resolution time-of-flight mass spectrometer (HRTOF-MS) was used for highly selective determination of acrylamide. Isotope dilution technique employing D_3-acrylamide was used for compensation of taget analyte looses and matrix-induced chromatographic response enhancement.

Using the new method, various foodstuffs from Czech market were examined for occurrence of acrylamide. The resuls are discussed in this paper.

Experimentlal

M a t e r i a l s

Acrylamide (99.8%) and acrylamide(2,3,3-D_3) (98%) were purchased from Sigma-Aldrich (Germany) and Cambridge Isotope Laboratories (USA), respectively. Samples for analysis of acrylamide (potato chips, French fries, crackers, breads, breakfast cereals and oat flakes) were purchased at Czech retail market.

M e t h o d s

Samples were homogenized using laboratory blender. 3 g of representative sample were weighed into a 45 ml centrifuge flask with a screw cap. After addition of 30 µl (50 µg ml^{-1}) of D_3-acrylamide (internal standard) and 4.5 ml of demineralised water sample was allowed to swell 30 min in ultrasonic bath held at 70 °C. Homogenate was mixed 5 min with 24 ml of *n*-propanol by Ultra Turrax. Centrifugation (10 min at 11 000 rpm) followed. 10 ml of supernatant were transferred into a 50 ml flask. 5 drops (about 60 mg) of an olive oil were added and the water/propanol azeotropic mixture was removed by rotary evaporator. The residue left on the wall of flask was re-extracted with 2 ml of acetonitrile and defatted by shaking with *n*-hexane (10 ml and 5 ml). 1 ml of the acetonitrile (bottom) phase was transferred into a 14 ml cetrifuge tube containing 60 mg of PSA sorbent. This mixture was mixed on a Vortex mixer for 30 s and centrifuged at 11 000 rpm for 1 min. Supernatant was placed into an autosampler vial for GC–HRTOF-MS analysis.

I d e n t i f i c a t i o n   a n d   q u a n t i f i c a t i o n

Analyses were performed by a gas chromatograph GC System 6890 Series (Agilent Technologies, Palo Alto, CA, USA) coupled to a GCT high-resolution time-of-flight mass spectrometer (Micromass, Manchester, UK). The GC system was equipped with an electronic pressure control (EPC), a split/splitless injector and a PAL Combi autosampler (CTC Analytics, Zwingen, Switzerland). An Innowax (30 m × 0.25 mm × 0.25 µm) capillary column used for separation was operated under following conditions: oven temperature program: 70 °C for 1.0 min, 20 °C min^{-1} to 200 °C (8.50 min); helium flow rate: 1.0 ml min^{-1}; injection mode: pulsed splitless 1.0 min, 4 ml min^{-1}; injection temperature: 250 °C; injection volume: 1 µl. Acquisition rate: 2 Hz; pusher interval: 33 µs (30.303 raw spectra/second); inhibit push value: 14; time-to-digital converter: 3.6 GHz; mass range: *m/z* 45–500; ion source temperature: 220 °C; transfer line temperature: 200 °C; detector voltage: 2200 V. The limit of quantification (LOQ) was 10–15 µg kg^{-1} according to the matrix and the repeatability of measurements expressed as a relative standard deviation (RSD) was 8.0 %.

Table I
Examples of acrylamide level variability in tested food groups.

Type of sample	Number of samples	Content of acrylamide [μg kg^{-1}]				
		Maximum	Minimum	Median	Mean	RSD [%]
Crackers	14	726	50	215	257	73
Bread	11	160	<15	64	63	84
Oat flakes	3	71	<15	64	50	79
Breakfast cereals	2	175	35	105	105	67
French fries (frozen)	14	154	<10	41	55	91
Potato chips	23	2713	67	942	1135	60

Results and discussion

To our knowledge this is the first report on application of GC-HRTOF-MS in direct (no derivatization) analysis of acrylamide in food samples. In our monitoring study 67 starch-rich food samples including potato chips, French fries, crackers, breads, breakfast cereals and oat flakes were analysed. The data reported in Table I are average of two parallel determinations. The variability of acrylamide levels occurring in various food groups is documented.

The largest variation of acrylamide levels were recognized in crackers and potato chips. This phenomenon could be explained by fluctuation of acrylamide precursors (asparagine and sugars) in respective raw materials (potatoes and cereals). The content of these compounds is different not only among plant varieties[3] but also changes during storage of crop occur depending on storage temperature and time[2].

The relatively low levels of acrylamide in some samples in particular product category indicate choice of raw materials with low levels of precursors. In addition modification of technological parameters should be considered as the way to achieve reduction of acrylamide levels in final food products. Close colaboration with industrial partners is needed.

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P09 PURIFICATION OF OLIGO-D-GALACTOSIDURONATE HYDROLASE FROM CARROT ROOTS

MÁRIA DZÚROVÁ[a], JIŘINA OMELKOVÁ[b] and EVA STRATILOVÁ[a]
[a]Institute of Chemistry of Slovak Academy of Sciences, Dúbravská cesta 9, SK-845 38 Bratislava, chemevi@savba.sk, [b]Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Technical University Brno, Purkyňova 118, CZ-612 00 Brno

Introduction

Exopolygalacturonases [poly(1,4-α-D-galacturonate) galacturonohydrolase, EC 3.2.1.67] are exo-hydrolases catalyzing the hydrolytic cleavage of glycosidic α-1,4-bonds of D-galacturonan at its nonreducing end under releasing D-galactopyranuronic acid as the sole reaction product. The particullar enzymes differ from each other by the range and rate of the effects on substrate in relation to the chain length. It was supposed that the most suitable substrates for exopolygalacturonases of plant origin are polymeric D-galacturonan or a partly degraded D-galacturonan of DP about 20 (ref.[1]). In 1996 the multiple forms of exopolygalacturonase from carrot roots were identified[2] and later the partial separation and characterization of four forms of this enzyme was described[3]. One of them, with pH optimum 3.8, preffered clearly oligomeric substrates. Purification of this enzyme was the aim of our work.

Methods

Extraction of exopolygalacturonases from carrot roots: juice extractor – juice precipitated with ammonium sulfate
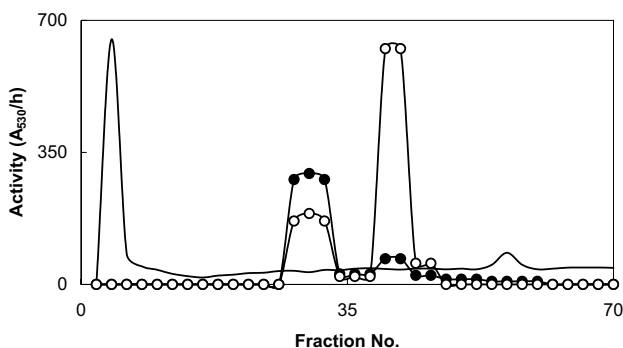
Fig. 1. **The separation of exopolygalacturonase and oligoga-lacturonate hydrolase from carrot roots on Phenyl Superose column (FPLC). Activity expressed as A_{530}, (●−●) − 0.5% sodium pectate, pH 5.0 and (○−○) − 1 mM pentagalacturonate, pH 3.8 were used. Proteins expressed as A_{280} (−). Conditions: linear gradient of 0.05 M phosphate buffer, pH 7.0 with 1.7 M ammonium sulfate to 0.05 M phosphate buffer, pH 7.0, flow rate 0.5 ml min^{-1}**

and with 96% ethanol, desalted on a Sephadex G-25 Medium column. Purification of oligogalacturonate hydrolase: ion--exchange, affinity, gel-permeation[2] and hydrophobic interaction based chromatographies. Identification: activity assay according to Somogyi[4], SDS-PAGE, preparative IEF.

Results and discussion

Two fractions with exopolygalacturonase activity were obtained by the separation of protein precipitate from carrot juice on CM-Sephadex C-50 column[2]. The pH optima determination on pectate of the first fraction showed the presence of two enzymes; exopolygalacturonase with pH optimum 5.0, described and relatively well characterized earlier[1] and an apparent minor enzyme form with pH optimum 3.8. The determination of pH optimum on penta-D-(galactosiduronic) acid compared with previous one shows clearly the preference of substrates with lower degree of polymerization for this enzyme. The attempt to separate these two enzyme forms on Concanavalin A – bead cellulose, on Superose 12 or Mono P columns was not successful. A huge amount of contaminants was removed but the affinity of both enzymes to Concanavalin A was the same, the molecular masses as well as the isoelectric points were very similar. The preparative isoelectric focusing with activity assay in both substrates of difference brought the evidence that really two enzyme forms were present in this fraction[3].

Separation of oligogalacturonate hydrolase and exo-polygalacturonase was reached utilizing hydrophobic interactions on Phenyl Superose column (Fig. 1.). Single band of glycosylated enzyme was obtained (Fig. 2.). Purified enzyme was further characterized.

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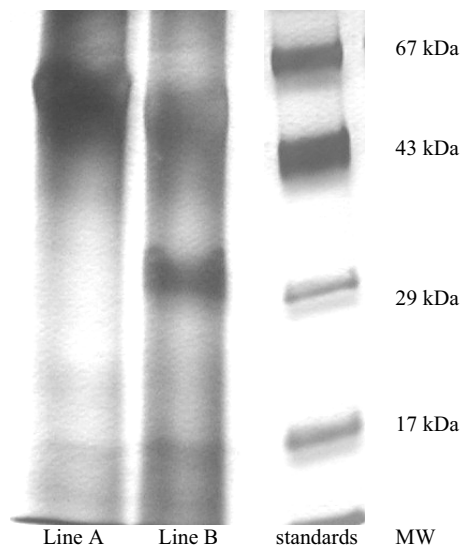1. Heinrichová K.: Collect. Czech. Chem. Commun. *42*, 3214 (1977).

Fig. 2. **SDS-PAGE of purified oligogalacturonate hydrolase from carrot roots. Line A – natural enzyme, line B – enzyme deglycosylated by N-glycosidase F. The silver–staining method used for band visualization. The standard calibration proteins in the range 14–94 KDa (Serva) were utilized: Phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa)**

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P10   OCCURRENCE OF *FUSARIUM* TOXINS IN CEREALS, CZECH REPUBLIC 2002–2004

MONIKA GOCIEKOVÁ[a], KATEŘINA LANCOVÁ[a], MICHAELA NEVRKLOVÁ[a], JANA HAJŠLOVÁ[a] and MARIE VAŇOVÁ[b]

[a]*Department of Food Chemistry and Analysis, Institute of Chemical Technology in Prague, Czech Republic, monika.gociekova@vscht.cz,* [b]*Agricultural Research Institute Kroměříž, Ltd., Czech Republic*

Introduction

Trichothecenes, secondary metabolites of a number of microscopic filamentous fungi genera, most notably *Fusarium*, are important natural toxins that can contaminate a wide spectrum of agricultural commodities. *Fusarium* toxins have been shown to cause a variety of toxic effects

in humans and animals. In addition to a health risk, fungal infection may cause considerable economic losses in agriculture. Preliminary studies have shown that not only climatic but also agrotechnological conditions affect the occurrence of mycotoxins. In the years 2002–2004, pilot study concerned with these problems was carried out by the Institute of Chemical Technology in Prague in co-operation with Agricultural Research Institute Kroměříž. The purpose of this study was to evaluate trichothecene content in wheat and barley from different regions of the Czech Republic.

Materials and methods
Materials

In the years 2002–2004, sampling of winter wheat and spring barley grains was carried out. In total 271 cereal samples representing different cultivars were collected by the Agricultural Research Institute, Kroměříž in five localities in Czech Republic see Table I. Sub-samples (200 g of milled grains) were supplied for determination of selected *Fusarium* mycotoxins to the Institute of Chemical Technology in Prague. Various soil treatments (minimum tillage, tillage, without tillage, various amount of fertilizers) and type of front crops (rape, wheat, maize, pea) during the process of cultivation of barley and wheat were used. The applied agriculture practices did not change in particular localities during the study.

Table I
Overview of examined samples

Locality	Type of crop	Number of samples		
		2002	2003	2004
Kroměříž	winter wheat	–	–	63
	spring barley	11	26	–
Ivanovice	winter wheat	15	12	12
	spring barley	12	12	12
Žabčice	winter wheat	–	3	24
	spring barley	–	–	–
Branišovice	winter wheat	9	3	3
	spring barley	–	3	3
Troubsko	winter wheat	18	18	–
	spring barley	–	–	12
	Sum	65	77	129

Chemical analysis

Slightly modified method described by Radova *et al.*[1] was employed for determination of seven trichothecene mycotoxins - deoxynivalenol (DON), nivalenol (NIV), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), fusarenon-X (FUS-X), HT-2 toxin (HT-2 tox.) and T-2 toxin (T-2 tox.). The analytical procedure consisted of following steps: (i) 10 g milled cereals was extracted with 100 ml acetonitrile-water mixture (84:16, v/v); (ii)

the crude extract was purified using dedicated solid phase extraction cartridge (MycoSep[TM] #225 column); (iii) volatilization of analytes was accomplished by trifluoroacetic acid anhydride; (iv) trichothecene trifluoroacetyl derivatives were separated and quantified by high resolution gas chromatography with electron capture detection. The detection limits for target analytes ranged from 5 to 200 μg kg^{-1}, depending on the chemical structure of particular *Fusarium* toxin. The accuracy of acquired data for DON content was demonstrated through participation in Food Analysis Performance Assessment Scheme (FAPAS) organized by Central Science Laboratory (York, UK). The value of z-scores below 1 was obtained in several proficiency testing rounds.

Results and discussion

Currently, DON is the only regulated mycotoxin representing *Fusarium* toxins group. On this account (DON as trichothecenes contamination marker) our experiments were focused mainly on this mycotoxin. The average levels of DON in cereals are documented in Fig. 1. In Tables II and III, more detailed information on the experimental data set is shown.
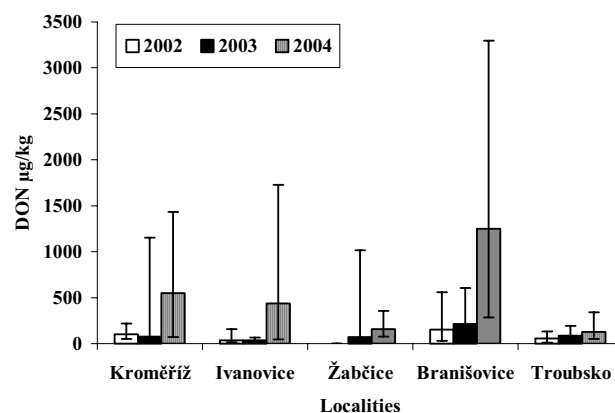

Fig. 1.　**Average content of DON (μg/kg of milled cereal grains) in sampling localities, harvest years 2002–2004 (vertical bars show range of minimum and maximum concentration)**

Table II
Content of DON (μg kg^{-1} of milled cereal grains) in experimental years – aggregated data

	Content of DON in μg kg^{-1} of cereals		
	2002	2003	2004
average	72.1	72.6	431.5
min	8.6	10.6	32.0
max	560.2	1153.1	3294.7
median	53.4	50.4	269.5

Table III
Content of DON (µg kg^{-1}) in wheat and barley, harvests 2002–2004

	2002		2003		2004	
	Spring barley	Winter wheat	Spring barley	Winter wheat	Spring barley	Winter wheat
average	100.9	63.7	42.7	95.6	185.8	466.6
max	560.2	219.6	82.6	604.7	1085.2	3294.7
min	17.6	8.6	18.8	10.6	44.7	77.7

Table IV
Occurrence of mycotoxins above limit of quantification (LOQ) in % of total number of analysed samples

Occurrence of mycotoxins exceeding LOQ in % of total number of analysed samples				LOQ [µg kg^{-1}]
	2002	2003	2004	
DON	100	91	100	15
NIV	57	31	42	10
15-DON	38	25	47	20
HT-2 tox.	16	21	6	40
T-2 tox.	8	0	0	400

Ubiquitous occurrence of *Fusarium* toxins in cereals was shown in our study with detectable DON in almost all examined samples. In the years 2002, 2003 and 2004, 2 %, 3 % and 26 %, respectively, of total number of analysed samples exceeded the DON hygienic limit (0.5 µg kg^{-1} of milled cereal grains). Many of the analysed samples also contained other trichothecene mycotoxins above the limit of quantification (LOQ), see Table IV.

The trichothecene levels in cereals varied largely from year to year. Also some differences among localities were recognized; within all crop years the highest levels of DON were found in cereals from locality Branišovice. In the second and the third experimental year, the levels of DON in wheat were higher than in barley, see Table III, probably wheat is more resistant against the infection of cereals caused by pathogens causing *Fusarium* Head Blight (FHB).

Several factors may play a role in the extent of DON and related mycotoxin occurrence. Not only weather conditions (regional and seasonal differences in trichothecenes production were observed), but also differences in virulence of various *Fusarium spp*. Another factors playing a role in this context are the genetic resistance of wheat/barley varieties and – last but not least – the applied agriculture practice. As shown in our previous studies (Váňová et al.[2]), maize as a front crop and the use of minimum tillage may have a negative effect in terms of contamination of cereals harvested in the subsequent crop year. In conclusion, the presented study has clearly documented the need to monitor occurrence of *Fusarium* toxins in cereals since these are ubiquitous and undoubtedly represent an important food safety issue especially in terms of chronic dietary exposure. Based on the deep knowledge of major factors influencing contamination of food crops, relevant measures such as selection of resistant varieties, employing agrotechnical practices reducing *Fusarium* Head Blight (FHB) etc. might be adopted.

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P11 ANTIMICROBIAL AND TECHNOLOGICAL ASPECTS OF SELECTED LACTIC ACID BACTERIA

MÁRIA GREIFOVÁ[a], MARCELA KONTOVÁ[b], GABRIEL GREIF[a] and VLADIMÍR MASTIHUBA[a]
[a]Department of Food and Science Technology, Faculty of Chemical and Food Technology STU, Radlinského 9, 812 37 Bratislava, Slovak Republic, maria.greifova@stuba.sk, [b]Dairy Research Institute Žilina, Dlhá C-95, 041 00 Žilina, Slovak Republic, kontova@vumza.sk

Introduction

An important factor in application of non-starter adjuncts lactic acid mesophilics lactobacilli (NSLAB) is not only in their defense against undesired microorganisms, but also preservation of demanded typical technological and sensory properties of cheeses. Aims of the work were: 1) testing of inhibition activity of cell-free supernatant from viable cultures (*L. plantarum* ALC01 and *L. rhamnosus* LC 705) against *L. monocytogenes, Listeria* spp, *B. cereus, E. coli, Ent. aerogenes, Ps. fluorescens, S. aureus, E. faecium, A. pullulans*; 2) study of the influence of non-starter adjuncts lactic acid mesophilics lactobacilli (*L. rhamnosus* LC 705 and *L. plantarum* ALC01) in combinations with starters mesophilics lactococci (M53, DL Mix, LC Mix) on the souring, ripening and organoleptic properties of semi-hard Edamer-type cheeses.

Material and methods

a) *Non-starter adjuncts lactic acid mesophilics lactobacilli (NSLAB): L. rhamnosus* LC 705; *L. plantarum* ALC01 (Danisco-Cultor Niebüll, SRN)

b) *Starter mesophilics lactococci (SML):* MT 53, DSM, Netherlads, Lc Mix, DL Mix, (Danisco-Cultor Niebüll, Germany)

c) *Preparation of LAB supernatants:* Procedure for preparation of partially purified supernatants arose from modification of several published methods[1–6].

e) *Determination of inhibitory effect:* activity of particular supernatant fractions against selected microorganisms was tested using diffusion assay[7].

f) *Cheese preparation:* Cheeses were made from pasteurized milk. In a model experiment, pasteurized milk was distributed into six 10 dm³ vats, the second trial was realized directly in cheese plant in six 12 000 dm³ vats. All vats were inoculated with lyophilized commercial starter lactococci culture; the controlled modes of production were produced only with starter cultures and experimental modes of production with combined cultures (starters and adjuncts).

g) *Chemical analyses:* 1. pH was routinely measured by laboratory pH meter. 2. Evaluation of proteolysis – proteolysis in cheese was monitored after 1, 4 and 8 months by photometric method with OPA (o-phthaldialdehyde) in which ethandiol was substituted by 2-sodium salt of mercaptoethanol sulphonic acid according to Tschager[8].

h) *Sensory evaluation:* Cheeses were evaluated after 1, 4, 8 months after manufacture as described by Lorenzen at all[9]. Cheeses were graded for flavor and aroma as well as body and texture; maximum score of 10, minimum of 3 respectively; therefore each cheese could achieve maximum 20 and minimum 6 points.

Results and discussion

Antibacterial activity assays

Using purified supernatant of the *L. plantarum* ALC01 strain, the inhibition effect was observed by diffuse method towards *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, E. aerogenes, S. aureus, E. faecium, A. pullulans*. The purified supernatant *L. rhamnosus* LC 705 inhibited growth of *L. monocytogenes, L. innocua, L. welhsimeri, B. cereus, E faecium, A. pullulans*.

Assessment of potential growth and activity of acidification of individual types of starter cultures

For growth assessment and calculation of growth characteristics, a stationary cultivation of SML alone or in combination with NSLAB was affected in sterile milk during 24 h. Results from these experiments had been already presented[10]. At the same time acidification activity of tested starters was monitored. SML alone acidified the cheese more rapidly comparing to their combination with NSLAB. However, the final acidity used to be either the same in both cases, or lower in case of LC mix with NSLAB.

Comparison of proteolytic changes in mutual combination of cultures

Proteolysis is one of the majority results of biochemical reactions that are running during the ripening of cheeses. Cheeses manufactured with adjunct cultures of lactobacilli proved more significant changes. The higher was the GA, the higher was also the depth of proteolysis. The observed results are stated in Table I.

Sensory profile of cheeses

After 21 days of ripening, minimal differences in taste and consistence of cheeses were detected in pilot operations. Cheeses in working operations after 1 month of ripening produced significant differences, however cheeses proved substantial difference mainly in consistence and taste after 4–8 months of ripening. Cheeses produced with NSLAB were softer and more elastic and the cheese taste was more full. The results stated in organoleptic properties were acceptable and contributed to better quality of Edamer-type of cheeses.

Table I
Content of glutamine acid (GA) (g/kg) in cheeses

Cultures used	Content of glutamine acid [g/kg]						
	Cheeses from pilot operation			Operating cheeses			
	7 days	14 days	21 days	7 days	1 month	4 months	8 months
MT-53	3.16	3.40	3.70	2.97	8.88	9.51	10.56
MT-53+LC705	3.21	3.62	4.28				
Lc-Mix	3.27	4.18	4.11	3.87	9.89	10.51	11.96
Lc-Mix+LC705	3.48	4.25	4.22	3.87	10.98	12.08	16.43
DL-Mix	3.75	4.29	4.65	4.71	10.89	15.99	19.85
DL-Mix+LC705	3.77	4.76	4.91	4.04	19.21	21.05	25.88

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P12 DIURNAL REGULATION OF RUBISCO ACTIVITY IN NORWAY SPRUCE

MIROSLAV HRSTKA and EVA PETRŮ

Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, hrstka@fch.vutbr.cz

Introduction

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) enzyme is the most widespread and perhaps the most remarkable protein on the Earth because it provides the only quantitatively significant link between the pools of inorganic and organic carbon in the biosphere. 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 activity may be modulated by the amount of this enzyme and by the carbamylation of their active site. The extent of this carbamylation depends on: (1) the concentrations of CO_2 and Mg^{2+}, (2) the concentrations of RuBP, 2-carboxy-D-arabinitol-1-phosphate (CA1P) or some other phosphorylated sugars which can block the active site, and (3) the activity of an enzyme called Rubisco activase. The activity of this latter enzyme is controlled by the ratio ATP/ADP, in effect by light intensity. The relative contribution of each mechanism of regulation varies with time and species.[1]

Rubisco active site, more exactly the side chain Lys201, exists at least in these forms: E ($E–NH_2$), ER ($E–NH_2–RuBP$), EC ($E–NH–CO_2$), ECM ($E–NH–CO_2Mg^{2+}$), ECMR ($E–NH–CO_2Mg^{2+}–RuBP$), ECMI ($E–NH–CO_2Mg^{2+}–inhibitor$). Catalytic competent are only ECM and ECMR forms. The binding of inhibitors to carbamylated Rubisco prevents catalysis, whilst binding of the substrate RuBP to the non-carbamylated enzyme prevents carbamylation of the lysine residue that is essential for activity.[2]

Whereas measurement of Rubisco activity immediately upon extraction (initial activity) reflects activity *in vivo*, i. e. number of active sites that are in the state ECM and ECMR, the carboxylation potential (total activity) can be determined by incubating extracts with saturated concentrations of CO_2 and Mg^{2+} prior to assay and expresses the number of active sites that are in the states E, ER, EC, ECM and ECMR. Therefore, total activity does not involve the active sites blocked by tight binding inhibitors (e. g. CA1P). The maximal carboxylation potential (maximal activity) is only revealed if steps are first taken to remove any inhibitors bound to the active sites.[3]

In this work we measured diurnal changes of Rubisco initial and total activities as well as Rubisco amount to find the mechanism of Rubisco activity regulation in Norway spruce.

Material

The experiment was conducted the 19th October 2004 on the full-grown solitaire Norway spruce (*Picea abies* [L.] Karst.). Needles were sampled at 6:00 (prior to sunrise) and then every unpaired hour till 19:00. Samples were weighed, their leaf areas were measured and put into liquid nitrogen.

Methods

Extraction of Rubisco for activity assay was conducted according to Hrstka et al.[4]. Rubisco initial and total activity was assayed spectrophotometrically by the continuous measurement of 3-phosphoglycerate-dependent NADH oxidation in a coupled enzyme system based on the method of Lilley and Walker[5], modified by Heringová[6]. Rubisco amount was determined by SDS-PAGE, according to Petrů[7]. Number of repetition n = 5 both for activity assay and for Rubisco amount determination.

Results and discussion

On the 19th October the sky was clear and when the early vapour disappeared, the irradiance sharply increased and at 11:00 it reached its daily maximum (730 μmol m^{-2} s^{-2}). At 13:00 the irradiance was 600 μmol m^{-2} s^{-2} and then it decreased sharply so at 15:00 it was only 100 μmol m^{-2} s^{-2} (Fig. 1A). The initial activity was extremely low at 6:00 prior to sunrise. With increasing irradiance both initial and total activities increased up to 9:00 and than decreased up to 13:00 (midday depression[8,9]). The second maximum of activities we observed at 17:00 (Fig. 1B). The activation state of Rubisco (measured by comparison of initial and total activity)
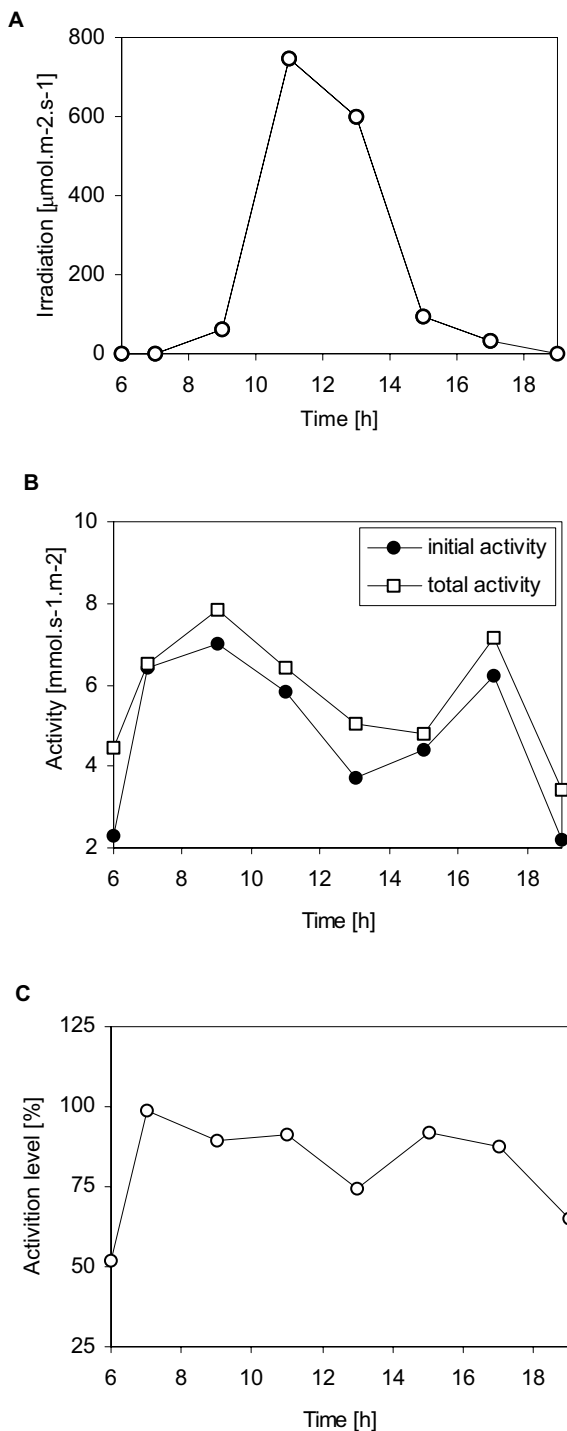
A

B

C

Fig. 1. **Diurnal changes of irradiation (A), Rubisco initial and total activity (B) and Rubisco activation level (C)**

at 15:00 and it is probable that this decrease of translation rate may be a response to the high irradiation and high activation state of Rubisco.

Total activity does not include active sites blocked by tight binding inhibitors, which are not removed by incubation with CO_2 and Mg^{2+}. Consistent with this is the observation that total activity changed significantly during the day. Whilst the difference between initial and total activities of Rubisco is a measure of the regulation by carbamylation, changes in total Rubisco activity can be attributed to regulation by: (1) Rubisco amount, (2) CA1P and similar inhibitors[3]. Relative low morning and evening values of total activities indicate that in Norway spruce plays an important role nocturnal inhibitor CA1P. However, the midday depression of total activities shows that besides CA1P there are present some other inhibitors of Rubisco[10]. These are: (1) D-xylulose-1,5-bisphosphate (XuBP) and 3-keto-D-arabinitol-1,5-bisphosphate (3-KABP)[11] which are formed during the catalytic reaction and for that reason this inhibition is called catalytic inactivation, (2) some another, little characterized daily inhibitor[10].

We concluded that Rubisco activity in Norway spruce is regulated to match the capacity of the leaf to regenerate RuBP, being modulated in vivo either by carbamylation which depends on irradiance, or by the binding of inhibitors within the active site.

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was 52 % at 6:00, but it was nearly 97 % at very low irradiance at 7:00. From 7:00 to 11:00 the activation state remained on the high level (about 90 %), at 13:00 we observed a midday depression and at 15:00 it was the second maximum (Fig. 1C). Rubisco amount was 3.1 g m^{-2} at 7:00 and 1.4 g m^{-2}

P13　INTERACTION OF PLANT POLYPHENOLS WITH IRON IONS

KATEŘINA CHVÁTALOVÁ and JIŘÍ SLANINA

Department of Biochemistry, Faculty of Medicine, Masaryk University, Komenského nám. 2, 662 43 Brno,
jslanina@med.muni.cz

Introduction

Polyphenols are secondary plant metabolites, which are a common part of the human diet. Their daily intake is estimated to be about 1 g a day[1]. Some epidemiological studies expect a relationship between the consumption of polyphenol-rich foods or beverages and the prevention of cardiovascular diseases and cancer. These pathological conditions are connected with the excessive free radical production. A number of studies demonstrated the ability of plant polyphenols to scavenge free radicals and their potential role as chain-breaking antioxidants[2]. There is a general view that the transient metals, especially iron and copper, can cause the formation of free radicals *in vivo*. The transient metal chelators are able to decrease the production of free radicals generated by the transient metals. Several reports have been made to elucidate the iron or copper ion chelating ability of polyphenols[3], although the interaction of plant polyphenols with the transient metal ions has not been fully resolved. In this study we focused on the influence of plant polyphenols, especially the phenolic acids, on the redox state of iron.

Results

Formation of complexes of the phenolic acids with iron

The formation of complexes of the plant polyphenols with the iron ions at pH 7.4 was studied. The spectroscopic studies indicated that only those polyphenols, which contain the *ortho*-3′,4′-dihydroxy (catechol) or 3′,4′,5′-trihydroxy substitution on the aromatic ring, formed complexes with ferric ions. We further investigated the interactions of phenolic acids possessing the catechol moiety with ferrous ions. Surprisingly, the spectra of catechols obtained with ferrous ions were closely related to those found after the addition of ferric ions. With regard to the similarities of both spectra, these observations suggest, that catechols bind either ferrous or ferric ions to form complexes (Fig. 1.). Therefore we investigated the influence of phenolic acids on the redox state of iron.

Influence of phenolic acids on redox state of iron

We determined the rate of Fe^{2+} autooxidation in the presence of polyphenols. In order to further assess the effect of polyphenols on the redox state of iron, we examined the ability of 13 polyphenols, ascorbic acid and cysteine to reduce Fe^{3+} to Fe^{2+}.

The autooxidation of half of the initial Fe^{2+} concentration without the presence of ligands took about 10 min at pH 7.4. In general, most of the phenolic acids significantly enhanced the rate of autooxidation. The most potent ligand, caffeic acid, was efficient from the concentration that corresponded to 0.4 % of the initial amount of Fe^{2+}. The stimulatory effect of ligands on the process of autooxidation decreases in this order caffeic acid ≥ protocatechuic acid > catechol > gallic acid > quercetin > chlorogenic acid > methylgallate. In order to determine the impact of the carboxyl group of phenolic acids we compared the potency of gallic acid with that of its methyl ester and with that of its decarboxylation product, pyrogallol. Pyrogallol autooxidized under this condition. Methylgallate was substantially less effective in increasing the rate of Fe^{2+} autooxidation in comparison to the gallic acid. Similarly, protocatechuic acid was more effective in increasing the rate of autooxidation than catechol, its decarboxylation product. This is probably because phenolic acids unlike their esters or simple phenols carry the negative charge at pH 7.4.

To explore the effect of partial methylation of the phenolic hydroxyls on the rate of autooxidation, we further investigated the effect of vanilic and syringic acid. Surprisingly, vanilic acid inhibited the rate of Fe^{2+} autooxidation even in low concentrations, the molar ratio of vanilic acid : Fe^{2+} being 4 : 1. Similarly, syringic acid reduced the rate of autooxidation, but the effect was evident from the concentration of syringic acid approximately tenfold over the Fe^{2+} concentration. Both ascorbic acid and cysteine reduced the rate of Fe^{2+} autooxidation and showed a potent Fe^{3+} reducing ability. On the
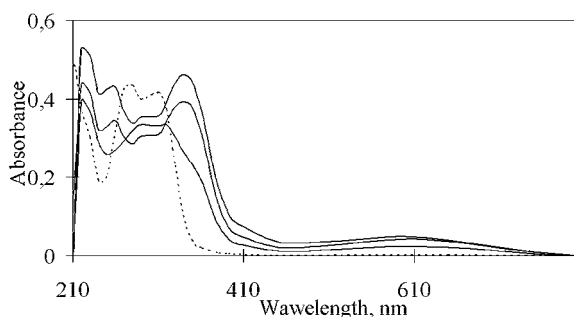

Fig. 1.　**Absorbance spectra of caffeic acid (25 μmol l⁻¹) in HEPES buffer (10 mmol l⁻¹) pH 7.4 without Fe^{3+} addition (dotted line), with Fe^{3+} addition – caffeic acid to Fe^{3+} molar ratio 2 : 1, 1 : 1, 1 : 2 (solid line)**
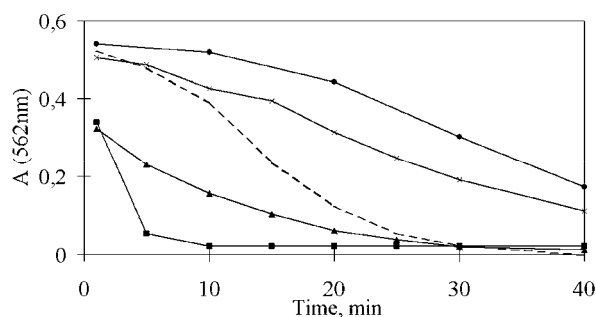

Fig. 2.　**The influence of various compounds on the autooxidation of Fe^{2+}, HEPES pH 7.4 (- - - - autooxidation, ■ – caffeic acid to Fe^{2+} molar ratio (4 : 1), ▲ – pyrogalol (4 : 1), x – p-hydroxybenoic acid (1 : 10), ● – cystein (1 : 10))**

contrary, all polyphenols tested with exception of pyrogallol were not able to reduce Fe^{3+} (Fig. 2.).

Discussion

Iron is an essential element for a human life. Although the iron deficiency can cause anemia, the iron overload may have harmful consequences by inducing pro-oxidant conditions through the interaction of iron with O_2 and H_2O_2 in the body.

We have studied the formation of complexes of the plant phenolic acids with ferrous and ferric ions. The spectroscopic studies indicate that only those phenolic acids with catechol moiety were capable of chelating iron ions at physiological pH 7.4. The rate of Fe^{2+} autooxidation was significantly influenced by the ligands used in the experiments. Polyphenolic acids with the 3,4-dihydroxyphenyl (catechol) moiety significantly increase the rate of autooxidation. The negative charge of the phenolic acid anions significantly promoted the rate of autooxidation. The results obtained corresponded with the theory, that the chelators with oxygen ligands, which would stabilize Fe^{3+}, would also increase the rate of autooxidation. Conversely, polyphenols with only one phenolic hydroxyl stabilized Fe^{2+} and inhibited the rate of autooxidation, although not as significantly as the catecholic chelators had a stimulatory effect.

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P14　THE MONITORING OF DIETARY EXPOSURE OF CZECH POPULATION TO INDICATOR POLYCHLORINATED BIPHENYLS IN 1994–2003

EVA JANOUŠKOVÁ, MIROSLAVA KRBŮŠKOVÁ, SYLVIE BĚLÁKOVA, IRENA ŘEHŮŘKOVÁ, IVANA BORKOVCOVÁ and JIŘÍ RUPRICH
National Institute of Public Health – Centre for the Hygiene of Food Chains, Palackého 3a, Brno, 612 42, Czech Republic, janouskova@chpr.szu.cz

Introduction

Polychlorinated biphenyls (PCBs) are a class of non-polar, non-flammable, industrial fluids with good thermal and chemical stability and electrical insulating properties. They are highly persistent, lipophilic and bioaccumulative industrial contaminants. PCBs have been produced as mixtures; individual congeners are hardly synthetised[1].

PCB mixtures were used in a wide scale of applications. Its major uses were in electronic appliances (dielectric fluids in transformers and capacitors), heat transfer systems and hydraulic fluids, flame retardants and plasticizers in paints, copying papers, adhesives, sealants and plastics and also in formulation of lubricating and cutting oils.

Since most PCBs congeners are very lipophilic and persistent, PCBs tend to accumulate in soils, sediments and lipid-rich tissues and magnify up a food chain.

PCBs accumulate in organisms. PCBs are toxic for both man and animals: maternal exposure may cause intellectual impairment in newborns, PCBs may play a role in the etiology of lymphatic/hematological malignancies and the role in breast cancer has been suggested. Some congeners may have significant estrogenic activity[2]. In addition, they show high persistence in the environment.

Therefore, for many years PCBs have been very closely controlled substances as contaminates of our environment[3]. Also, their occurrence in the environment has been monitored.

Since the foodstuffs are recognized as a significant source of the exposure to PCBs, the monitoring of their content in foods is a prerequisite for the assessment of the risk these substances present to human health. The data from such monitoring must be coupled with the knowledge of the consumption of individual foods.

The Centre for the Hygiene of Food Chains in Brno participates in "The Project on Dietary Exposure to Selected Chemical Substances" the objective of which is to describe the dietary exposure of the population of the Czech Republic to chemical substances[3]. In the framework of this project PCBs have been monitored since 1994. The seven most significant indicator PCBs (28, 52, 101, 118, 138, 153, 180) were determined in this project.

Material and methods

PCBs are determined in food samples in the range of the so-called food basket of foodstuffs of the Czech population[4]. The selection is based on the conception of monitoring the dietary exposure[3].

The food samples (46 or 108 types of matrixes from 1994 to 1998, respectively from 1999 to 2003) were first of all subjected to culinary treatment so that they could be analysed in the same condition as they are consumed. The result of the preanalytical treatment is a homogenous sample, which is then analysed.[3]

After culinary treatment and pre-homogenisation the amount of 50–200 g of the samples was homogenized and extracted with mixture of petroleum ether/acetone (ratio 2:1) as a solvent at high frequency of revolutions on a dispersant or using hot solvent. All of the sample extracts were cleaned using gel permeation chromatography and column chromatography using Florisil as the stationary phase. Thus cleaned samples were analysed by gas chromatograph (GC – Hewlett-Packard 5890) with two-column system and electron capture detection. Two columns with different sta-

tionary phases (J&W DB-5 and DB-17, 30 m i. d. 0.25 mm, thickness of film 0.25 μm) were used. Internal standards were used to determine the extent of recovery of the analytical procedure. The accuracy of the method was confirmed by analysing CRMs. Limits of quantification, depending on the type of the matrix, range between 0.002 and 0.05 μg kg^{-1}. The linearity of response of detector is tested by correlation coefficient (critical value 0.99) and QC coefficient (critical value 5.0). All estimated coefficients are acceptable in term of linearity. The repeatability of injection is found to range from 1.5 to 3.9 %. The proficiency testing of the method was carried out (FAPAS – UK) and the method was accredited according EN ISO/IEC 17025.

The detected concentrations of PCBs in foodstuffs are used to estimate the dietary exposure to these substances.

The dietary exposure is determined not only by the content of analytes in the given foodstuffs, but also by the food consumption. To calculate the dietary exposure it is necessary to take into account the changes caused by the culinary treatment of given food which are described by the so called culinary factor[5].

Results and discussion

Every year from 1994 to 2003 the content of 7 indicator PCB congeners was determined in the samples of the food basket of the population of the Czech Republic. As an example the content of the sum of 7 PCB congeners in fresh water fish, butter and cooked salami samples is shown in Fig. 1. for the period from 1994 till 2003.
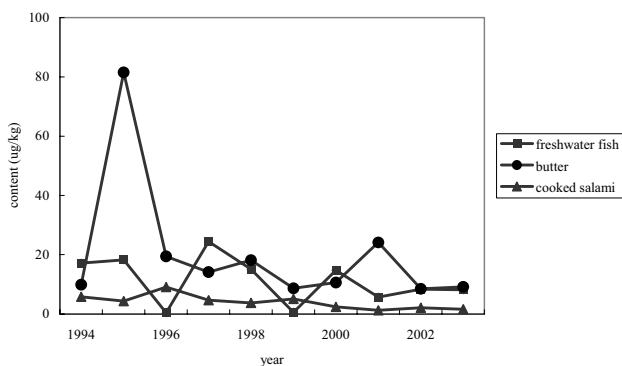

Fig. 1. **The content of the sum of 7 PCB for the period from 1994 till 2003 in selected samples**

The dietary exposure was estimated using the information on the content of PCBs in food samples. Exposure data acquired in the course of one year represent the estimation of exposure for an average person in the Czech Republic.

In Fig. 2. an example comprising fifteen food samples of the highest values of exposure doses are shown in 2003. In Fig. 3. an example comprising fifteen food samples of the highest values of concentration is shown in 2003.

The Fig. 4. shows the trend of exposure of the specific population groups from 1994 to 2003.
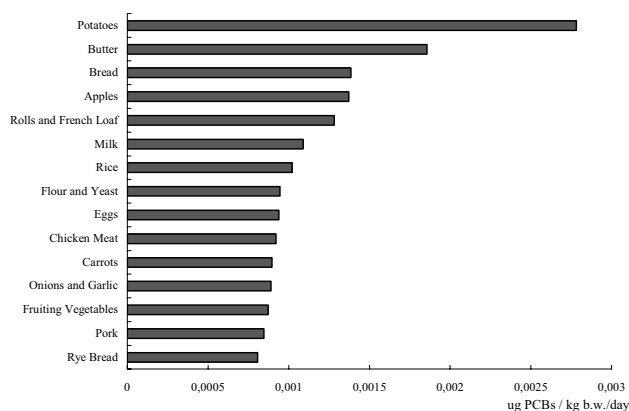

Fig. 2. **Dietary exposure sources of sum PCBs in the Czech Republic in 2003 (contribution of doses from individual food sources)**
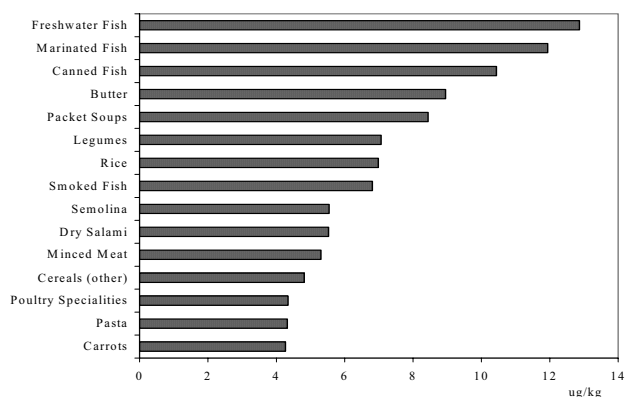

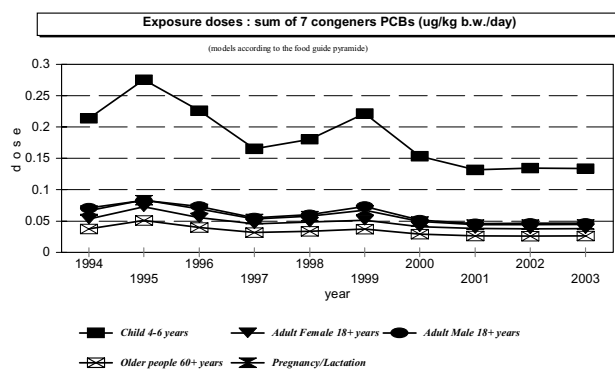Fig. 3. **The content of the sum of 7 PCB in selected samples in 2003**

Fig. 4. **The trend of exposure to sum 7 PCBs from 1994 to 2003**

In the period from 1994–2003, the estimates of exposure doses to the sum of 7 congeners tend to decrease slightly. The first temporary increase was observed in the years 1998 and 1999 and could be hypothetically a consequence of the floods

of 1997. The second very slight increase of exposure observed in 2002 and 2003 could be due to the floods of 2002. The evaluation was carried out using the recommended food doses model[5].

Conclusion

As the PCBs can accumulate in the animal tissues, the foods of animal origin are one of the most significant sources of the exposure. Higher exposure doses can be particularly expected in persons with high intake of animal fats. Hence, the decrease in consumption of animal fats can significantly contribute to lowering exposure doses[5].

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P15 THE MONITORING OF DIETARY EXPOSURE OF CZECH POPULATION TO ORGANOCHLORINATED PESTICIDES IN 1994–2003

EVA JANOUŠKOVÁ, MIROSLAVA KRBŮŠKOVÁ, SYLVIE BĚLÁKOVÁ, JOSEF SLAVÍK, IRENA ŘEHŮŘKOVÁ and JIŘÍ RUPRICH

National Institute of Public Health – Centre for Hygiene of Food Chains, Palackého 3a, 612 42, Brno, Czech Republic, janouskova@chpr.szu.cz

Introduction

The Centre for the Hygiene of Food Chains in Brno participates in "The Project on Dietary Exposure to Selected Chemical Substances" the objective of which is to describe the dietary exposure of the population of the Czech Republic to chemical substances. 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, methoxy-chlor, heptachlor and its metabolites heptachloroepoxide (A+B), HCB, alpha and gamma chlordane, oxychlordane and mirex – have been monitored in this project since 1994 (ref.[1]).

Some of these pesticides come under the group of persistent organic pollutants, which, according to the Stockholm Convention, should be completely prohibited or widely limited[2,3].

The most of OCPs are persistent, almost insoluble in water and soluble in organic solvents; it is highly stable and semi-volatile. It is easily bound to water sediments and its bio-concentration takes place in the fat of organisms[4]. Some of OCPs have been classified as possible human carcinogens and some have adverse effect on the hormonal system.

Material and methods

PCBs are determined in food samples in the range of the so-called food basket of the Czech population (46 or 108 types of matrixes from 1994 to 1998, respectively from 1999 to 2003)[1]. The selection is based on the conception of monitoring the dietary exposure[5].

The food samples collected in the market of the Czech Republic were first of all subjected to culinary treatment so that they could be analysed in the same condition as they are consumed. The result of the preanalytical treatment is a homogenous sample, which is then analysed[1].

After culinary treatment and pre-homogenisation the amount of 50–200 g of the samples was homogenized and extracted with mixture of petroleum ether/acetone (ratio 2:1) as a solvent at high frequency of revolutions on a dispersant or using hot solvent. All of the sample extracts were cleaned using gel permeation chromatography (Waters GPC Clean-up System) and column chromatography using Florisil as the stationary phase. Thus cleaned samples were analyzed by gas chromatograph (GC – Hewlett-Packard 5890) with two-column system and electron capture detection. Two columns with different stationary phases (J & W DB-5 and DB-17, 30 m i. d. 0.25 mm, thickness of film 0.25 μm) were used. Internal standards were used to determine the extent of recovery of the analytical procedure. The accuracy of the method was confirmed by analyzing CRMs. Limits of quantification, depending on the type of the matrix, range between 0.002 and 0.05 μg kg^{-1}. The proficiency testing of the method was carried out (FAPAS – UK) and the method was accredited according EN ISO/IEC 17025.

The detected concentrations of PCBs in foodstuffs are used to estimate the dietary exposure to these substances.

The dietary exposure is determined not only by the content of analytes in the given foodstuffs, but also by the food consumption. To calculate the dietary exposure it is necessary to take into account the changes caused by the culinary treatment of given food which are described by the so called culinary factor[6].

Results and discussion

At the Table I the monitored analytes, the count of samples, the count of positive capture of samples and the ranges of content are shown.

The dietary exposure was estimated using the information on the content of OCPs in food samples. Exposure data acquired in the course of one year represent the estimation of exposure for an average person in the Czech Republic.

Table I
Results of the monitoring of OCPs in 1994–2003

	1994–1998				1999–2003		
Analyt	Count of samples	% positive	Range [µg kg^{-1}]	Analyt	Count of samples	% positive	Range [µg kg^{-1}]
Aldrin	2897	2.45	11–0.01	Aldrin	540	10.93	0.8–0.02
alpha–HCH	2897	7.42	12–0.01	alpha–HCH	2700	22.96	6–0.01
beta–HCH	2897	10.56	12–0.01	beta–HCH	2700	27.30	9–0.01
delta–HCH	2897	8.46	15–0.01	delta–HCH	2700	12.19	12–0.01
gamma–HCH	2897	20.37	20–0.01	gamma–HCH	2700	33.37	6–0.01
HCB	2897	42.53	22–0.01	HCB	2700	29.22	16–0.01
o,p'DDD	2897	4.97	16–0.01	o,p'DDD	2700	31.52	11–0.01
p,p'DDD	2897	11.87	30–0.01	p,p'DDD	2700	21.96	11–0.01
				o,p'DDE	2700	25.93	8–0.01
p,p'DDE	2897	59.37	15–0.01	p,p'DDE	2700	58.11	70–0.01
o,p'DDT	2897	4.25	24–0.01	o,p'DDT	2700	16.19	20–0.01
p,p'DDT	2897	7.87	27–0.01	p,p'DDT	2700	25.56	42–0.01
Dieldrin	2897	4.63	8–0.01	Dieldrin	540	27.04	5–0.01
Endrin	2897	3.04	9–0.01	Endrin	540	20.93	2–0.01
				Endrinketone	483	17.18	3–0.01
Endosulfan	2897	6.18	10–0.01	Endosulfan I	540	40.19	5–0.01
				Endosulfan II	540	33.70	2–0.01
				Endosulfan sulfate	483	7.04	2–0.01
Heptachlor poxide	2897	4.31	7–0.01	Heptachlor epoxide A	540	27.41	4–0.01
				Heptachlor epoxide B	540	24.07	2–0.01
				Heptachlor	483	15.11	1–0.01
Methoxychlor	2897	2.07	9–0.01	Methoxychlor	540	9.44	4–0.01
				alfa–chlordane	216	19.44	1–0.02
				gamma–chlordane	216	31.94	1–0.02
				oxychlordane	216	23.15	0.9–0.02
				Mirex	216	7.87	0.3–0.01

None of the exposure standards for each of the 26 OCPs was exceeded throughout the above monitoring period. Exposure doses for all the analytes for the population of the Czech Republic usually amounted to less than 0.5 % of the exposure standards, the highest values of the former never exceeding 3 % of the exposure standards (ADI or RfD). As examples the figures 1–4 show the trend of exposure of the specific population groups from 1994 to 2003.

The observable decline in exposure values of the sum of DDTs has slowed down in the past few years. In fact, a slight increase of the exposure was observed in 2002.

The exposure dose of HCB for population decreased from 1996 to 2003. In the last years the decline is more moderate. The dose of alpha HCH isomer slightly fluctuates throughout the above mentioned period.

Whereas at the beginning of the period 1994–2003 the exposure doses of lindane decreased significantly, they remained approximately constant from 1995 on[6].
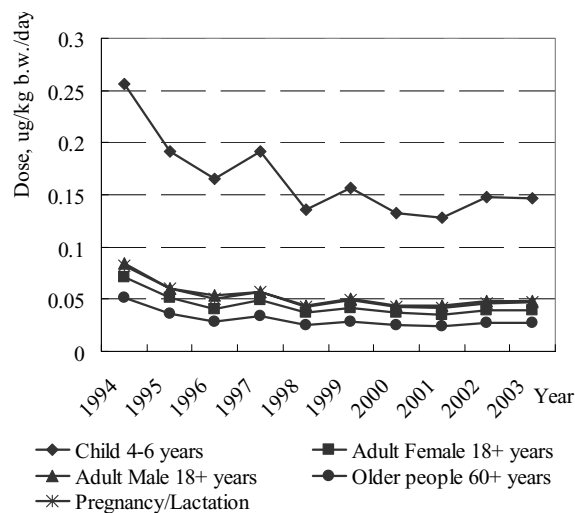

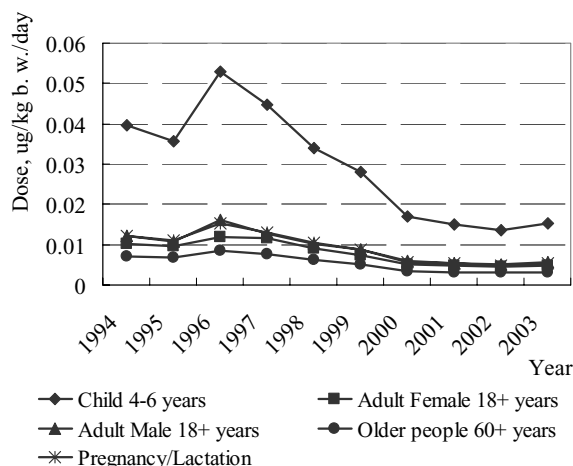Fig. 1. **The trend of exposure to sum of DDT from 1994 to 2003**
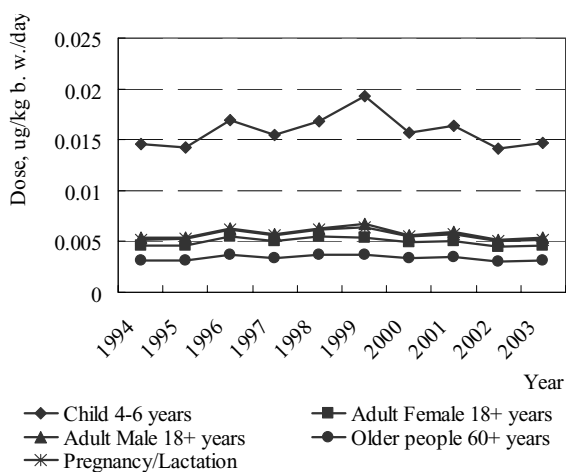
Fig. 2. **The trend of exposure to HCB from 1994 to 2003**

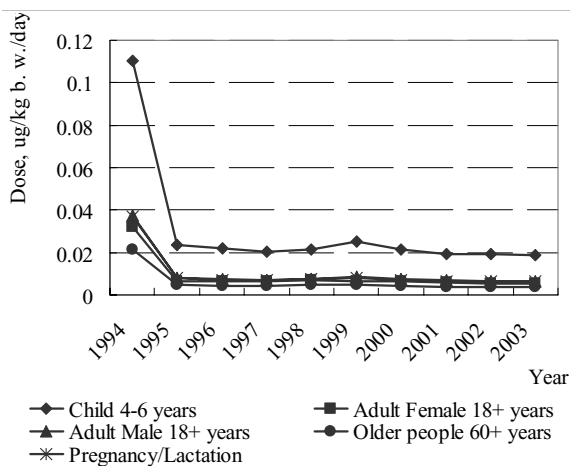Fig. 3. **The trend of exposure from to alpha-HCH 1994 to 2003**

Fig. 4. **The trend of exposure to lindane from 1994 to 2003**

Conclusion

The trends in population exposure were monitored. 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 OPC. The found exposition doses of individual OCP probably do not present a serious health risk for the average population of the Czech Republic. It is necessary, however, to continue the monitoring of their content in foods due to their tendency to accumulate in human fat, changing of the dietary habit and last but not least due to open food market.

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P16 APPLICATION OF IMAGE ANALYSIS METHODS IN YEAST CELL STUDY

PETRA JEŘÁBKOVÁ[a], OLDŘICH ZMEŠKAL[a] and MÁRIA VESELÁ[b]

[a]*Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, jerabkova@fch.vutbr.cz,* [b]*Institute of Chemistry of Foodstuffs and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno*

Introduction

Recently, a great development has taken place in the field of light microscopy, which enables many applications in the microbiology research. New fluorescent dyes, better cameras and new microscopy techniques have been developed. The combinations of recent imaging cameras and computer image processing permit, for example, identification and calculation of objects in the visual field, measurement of their size or categorization into selected classes according to predefined conditions, or observation of continuous processes such as reproduction of microorganisms.

A significant role in the image analysis is played by the integral transformation, namely periodic (e. g. Fourier transformation) and wavelet (e. g. Haar transformation). The Haar transformation can effectively be used for the fractal analysis

by the box counting method. The described method is a part of the HarFA software used for the analysis[1].

With the assistance of the box counting method, it is possible to investigate black and white fractal structures, which can be obtained from color pictures by a process called *thresholding*. The box counting method is based on laying the graticule on a black and white picture and finding the number of black N_B, white N_W and black and white N_{BW} squares. Based on the dependence of the number of black N_B, white N_W and black and white N_{BW} squares on their size n, it is possible to determine fractal dimension of white and black areas and their interface (D_{BBW}, D_{WBW}, D_{BW}).

The wavelet transformation (or the Haar one) makes the calculation of squares of different sizes of a laid mesh more effective with the box counting method provided that a square area is being analyzed. This transformation comes out from the system of orthogonal Haar functions which acquire values +1, 0, –1 multiplied by the power of number $2^{i/2}$, where i = 0, 1, 2, … The first two Haar rectangular functions are identical with the Walsh ones, the higher Haar functions are obtained from the lower (i. e. previous) ones by changing the measure and shift. Based on the coefficients of the Haar transformation it is easy to determinate for black and white pictures the numbers of black N_B, partially black N_{BW} and white N_W squares for different mesh sizes n (1×1, 2×2, 3×3, … pixels). From their power dependence on the measure size it is again possible to determine the basic structure parameters, a so-called fractal dimension D and fractal measure K of black and white areas and their interface. These parameters can be used for picture ordering evaluation, but also e. g. for specifying the number of defined objects without having to count them[2].

Experimental part

The fractal analysis was used when studying different species of yeast (*Saccharomyces cerevisiae, Saccharomyces fragilis, Candida vini, Kloeckera apiculata, Geotrichum candidum, Dipodascus magnesii*). Growth and reproduction of yeast in single cultivation under aerobic conditions was observed. Two culture media were used, namely wash enriched with vitamins and glucose-peptone medium containing the yeast extract. To record these processes, the combination of the light microscope and digital camera, or video camera, was used. A digital camera Nikon Coolpix 990 with resolution of 2048×1536 and CCD camera PixeLink with resolution of 1280×1024 were used, the Lucia Net 1.16.5 software being used in the process of using the video camera for records of pictures. The monitoring was employed in different ways of reproduction, primarily concerning multipolar and bipolar budding.

Fig. 1. shows a record of budding of yeast *Candida* vini in wash using microscope Nikon Eclipse E200 with the phase contrast. Yeast was cultivated on an inclined agar by the room temperature for 17–24 hours and, then, was inoculated into a liquid culture medium where it was cultivated for a minimum of one hour prior to making the measurements so that it

could adapt to the new culture environment. The thresholding of pictures was made by intensity ranging 0–88. A first bud appeared after two hours and 30 minutes after the inoculation into the liquid culture medium. The picture shows that the bud expanding into space also influences the value of the fractal dimension.
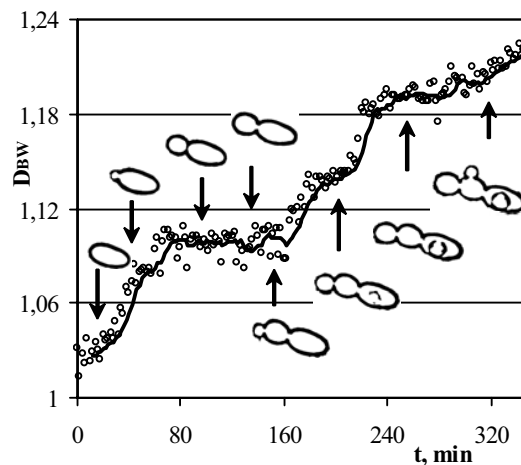

Fig. 1. D_{BW} **fractal dimension dependence on changing the structure of yeast Candida vini. Total magnification of 2400×**

Furthermore, the fractal analysis was used for specifying the number of live and dead cells of yeast *Saccharomyces cerevisiae* in the picture. Usually, the direct microscopic counting that can be performed in counting chambers (by Thoma and Bürker) is used to detect the number of cells in a specimen. Different fluorescent dyes, e. g. acridine orange, can be used to distinguish the living and dead cells. The monomeric form of the dye is yellowgreen in living cells and the aggregated dye form is red in dead cells. With the assistance of fluorescent labeling it is possible to threshold either dead or living cells on a black color in one picture.

In Fig. 2., the total number of cells is thresholded by intensity ranging 50–255, the living and dead cells being thresholded by coloured components of the RGB space (living cells: R = 0–254, G = 90–255; dead cells: R = 130–255, R = 0–255). To detect the convenient thresholding, it is possible to use the fractal spectrum, i. e. fractal dimension dependence on intensity or selected RGB component, which is accessible in the HarFA software as a tool referred to as Fractal *Analysis – Range*.

Fig. 2. **Thresholded pictures of yeast cells; total number (left), living cells (in the middle), dead cells (right)**

The number of cells was determined provided that cells are of spherical shape, similar in size and distinguishable on the background. For the calculation of cell numbers the following equation was used:

$$x = \frac{N_{BW}^2}{4\pi(N_B + N_{BW})}$$

where N_B is the number of black boxes, N_{BW} is the number of black and white boxes. The resulting cell number x is derived from value x that is the maximum from the calculated values for different sizes of the mesh. The maximum value is select because the fractal structure is bordered most conveniently for the given mesh size. With a smaller mesh size, the picture border composed of pixels would not be continuous and, with a greater mesh size, the border would not be rather smooth[3].
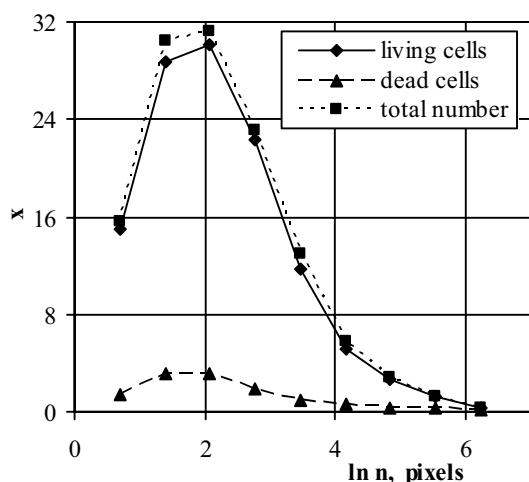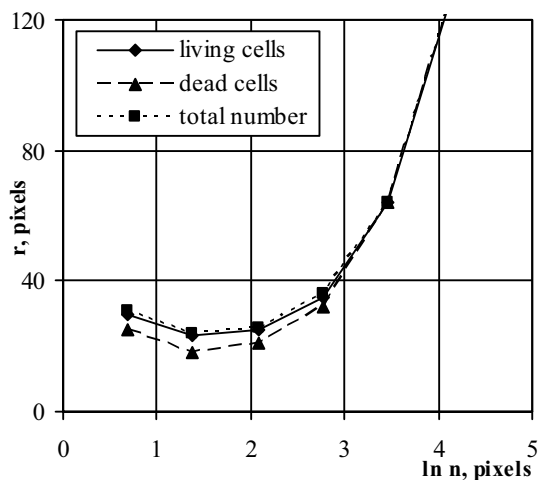

Fig. 3.　**Determining number of cells x**

Fig. 4.　**Determining average cell radius r**

Results and discussion

By appropriate setting of optical system parameters (optical filters, brightness, contrast), it is possible to facilitate the actual image analysis. When monitoring the growth and reproduction, it was discovered that after the appearance of a bud, the fractal dimension increases either continuously or in "fits and starts". A relaxation stage when that fractal dimension does not change was found between the individual leaps (Fig. 1.). During the relaxation stage, a cell complements its energy and substance reserves for the continuation of the division. The steepness of the fractal dimension increase is also determined by the number of buds developing simultaneously on the mother cell. The separation of a bud from the mother cell and subsequent division of the daughter cells was also recorded.

When calculating the living and dead cells, it was found out that the error of determining the number of cells depends on the size and number of cells. Error 18 % corresponds to the picture with 38 cells (total number), error 14 % corresponds to the picture with 35 living cells, and error 2 % corresponds to the picture with 3 dead cells. An error can also be caused by unequal size of cells and shapes, differences in coloring of the individual cells, quality of the recorded picture, etc. The picture resolution plays a significant role. If the number of cells is known, it is also possible to determine the average cell radius, which was 23 pixels again with an error of approximately 17 %.

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P17 DETERMINATION OF SELECTED AUTHENTICATION PARAMETERS IN THE ORANGE JUICES

JOLANA KAROVIČOVÁ, ZLATICA KOHAJDOVÁ, KRISTÍNA KUKUROVÁ and JARMILA LEHKOŽIVOVÁ
Department of Food Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic,
jolana.karovicova@stuba.sk

Introduction

Generally, adulterated foodstuff are products with changed appearance, taste, composition or another signs in the way of devalue and which are presented as genuine with accustomed appellation to the consumer[1].

Orange juices and nectars represent the food commodity that is very often subjected to adulteration. The main deviations are: lowering of fruit content (addition of sugars, acids, artificial mixtures), unlabelled sugar addition (usually without lowering of fruit content), pulp wash addition (including pure pulp wash juices), lower refractive index, low quality of water used for reconstitution and others[2].

Aim of this study was to authenticate of commercial orange juices by determination of selected authenticity markers (titrable acids, volatile acids, glucose, chlorides, ammonia, L-ascorbic acid, lactic acid, isocitric acid, ratio of citric and isocitric acid), to compare measured results with RSK and standards from Code of Practice[3,4].

Experimental part

Characterisation of Samples of Orange Juices. Orange juices analysed in experiment were randomly purchased from retail chain in spring 2003. Samples A – K represented 100% orange juices from Slovak producers, sample L 100% orange juice from Czech producer, sample M 100% orange juice from Austria producer, sample N 60% orange nectar from Slovak producer and sample P 12% orange juice from Czech producer.

Analytical Methods. The measurement of pH was performed by pH meter type OK-104 (Radelkis, Budapest, Hungary). The titrable acidity was determined by the visual titration with NaOH. The determination of volatile acids was performed by titration with NaOH using phenolphthalein indicator after destilation with steam. Chlorides were performed according to Mohr. Glucose was determined by visual titration with $Na_2S_2O_3$ with solution of starch as indicator after oxidation with iodine, ammonia was determined by microdiffusion. Measurements of organic acids was realised on the isotachophoretic analyser ZKI 01, Villa Labeco Spišská Nová Ves with conductivity detector[5,6].

Results and discussion

Results of evaluation of 100% orange juices were compared with reference values, that represent criteria for authentication of fruit juices valid in countries of EU. Selected au-

thentication parameters were determined in the commercial samples of orange juices.

In 15 orange juices, the content of chlorides, total acidity, volatile acids and ammonia did not exceed values in Code of Practice. It was found that samples F and K (66.30 g dm^{-3} and 57.65 g dm^{-3}) contained more glucose than is allowed by Code of Practice. Juices G and K did not exceed RSK--values for the isocitric acid content (103.68 g dm^{-3} and 109.96 g dm^{-3}). Criterion according to citric and isocitric acid ratio corresponded to the normal values in 100% orange juice in samples G, K, L and also in 12% orange nectar P (69.3; 118.6; 51.4 and 72.3).

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P18 THE COMPARISON OF THE ACRYLAMIDE FORMATION IN THE MODEL SYSTEM WITH TWO STARCH MATRICES

EUGEN KISS, ZUZANA CIESAROVÁ, EMIL KOLEK and MILAN KOVÁČ
Food Research Institute, Priemyselná 4, 824 75 Bratislava, Slovak Republic, kiss@vup.sk

Introduction

The examination of acrylamide formation in model system under defined conditions can contribute to the explanation of the acrylamide mechanism and to the estimation of potential content of acrylamide in starch reached foods. Undesirable acrylamide concentration in heat treated foods was observed firstly by Swedish scientists three years ago[1] and since then the efforts to the acrylamide minimization in foods are on the front burner of food safety authorities[2,3]. The reason consists in the toxic effects of acrylamide on the ner-

vous system in humans and in its genotoxic and carcinogenic properties documented in animals studies[4,5].

It is known that acrylamide is preferentially formed from the amino acid asparagine and presented reduced sugars (e. g. glucose and fructose). Although the current literature suggests the Amadori compound formed from asparagine and glucose as the key intermediate, which is directly cleaved thus forming acrylamide[6,7], it was recently discovered that 3-aminopropionamide (3-APA) is the most potent among the acrylamide precursors reported up to now[8]. Acrylamide formation from asparagine during food processing is connected with Maillard-type reactions which are dependent apart from other parameters on the moisture in system[9].

In our study we have ascertained the influence of added water into the model system comprising dry starch (potato or wheat) and equimolar mixture of asparagine and glucose on the acrylamide content.

Materials and methods

Chemicals. Starch from potato and wheat, glucose, and asparagine were obtained from Fluka Chemie AG (Switzerland), 2,3,3-D3 acrylamide from Cambridge Isotope Laboratories Inc. (Andover, USA). All other solvents and chemicals used were of analytical grade.

Experimental Design. Starches from potato and wheat were dried at 105 °C to final moisture of 2 %. 1 g of starch was homogenized mechanically with 0.2 g of equimolar mixture consisting of glucose and asparagine in closed vessel. After adding of water (0.1–4.0 ml) solutions were kept in Thermochem Metal-block Thermostat (Liebisch Labortechnik, Bielefeld, Germany) at 180 °C. Heat treatment was stopped after 20 min and acrylamide was analysed after hot water extraction in ultrasonic bath, next extraction with ethyl acetate, clean-up through silica gel column, and washing with methanol/acetonitrile 15 : 85.

Method. Acrylamide was determined by GC-MS method in the NCI mode (Agilent 6890/MSD 5793 inert) under following conditions: split/splitless inlet 250 °C, 2 µl pulsed splitless, single tapered liner with glass wool, oven: 60 °C (1.0 min), 10 °C min⁻¹ to 190 °C (0 min), 50 °C min⁻¹ to 240 °C (2 min), column: 30 m × 0.25 mm × 0.25 µm DB-FFAP, 0.8 ml min⁻¹ constant flow, Negative Chemical Ionisation, SIM mode, internal standard: 2,3,3-D3 acrylamide, Interface/Source/Quad: 250 °C/150 °C/150 °C, tune: $NCICH_4.U$, reagent gas: methane 2 ml min⁻¹, EM offset: 400 above tune, resolution: low, dwell time 150 ms. All experiments were run in duplicates.

Results and discussion

In the present investigation different factors affecting the content of acrylamide during heat treatment were examined. It is obvious that the content of acrylamide increased not only with increasing temperature, but also with the time of heat treatment. While the amount of acrylamide was relatively low at temperatures between 120 and 140 °C, there was a drastically increase when the temperature reached

160 or 180 °C. The time dependent increase of acrylamide at constant temperatures during first ten minutes followed a linear function with enhanced slope at higher temperatures and with the maximum in 10th min (Fig. 1.). After 20 min heat treatment weak decreases of acrylamide concentration were observed in the whole range of examined temperatures (120–180 °C). For the present investigation the temperature of 180 °C and reaction time of 20 min were chosen. Model systems were performed under conditions with increased initial level of moisture. When potato starch was used as matrix for reaction of asparagine and glucose, it was found out that the minimum of acrylamide content (approximately 10 µg acrylamide/100 mg asparagine) was located between 20 and 35 % of moisture (Fig. 2.). Amount of acrylamide away from this range was higher (55–70 µg acrylamide/100 mg asparagine). In the case of wheat starch used as a matrix the course of moisture/acrylamide dependence was similar to potato starch one. The minimum of acrylamide concentration 10 µg/ 100 mg asparagine was between 30 and 40 % of moisture, the amount of formed acrylamide at higher moisture was lower compared to potato starch (Fig. 2.). Absence of starch resulted in 2.5-times higher amount of formed acrylamide (results not shown).
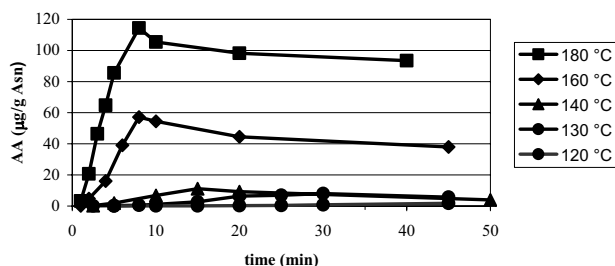

Fig. 1. **Time dependent acrylamide formation from equimolar mixture of glucose and asparagine at different temperatures**
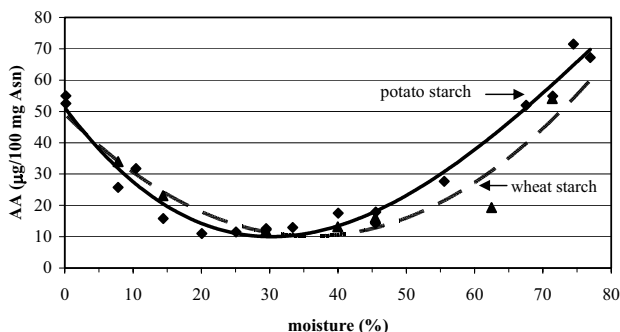

Fig. 2. **Acrylamide formation from equimolar mixture of glucose and asparagine in the presence of potato (continuous curve) and wheat (discontinuous curve) starch in dependence on initial moisture of mixture in closed vessels**

Conclusion

In spite of different physical and chemical properties of starch genetically dependent on origin (size and shape of

starch granules, amylose/amylopectin ratio etc.) the influence of two types of starch (potato and wheat) on the formation of acrylamide from asparagine and glucose in the presence of water is similar. The presence of starch reduced the amount of formed acrylamide. Moisture of mixture is important factor in formation of acrylamide. The minimum of acrylamide was observed at the moisture in the range between 25 and 40 %, away from this range acrylamide concentration was higher.

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P19 PRODUCTION OF INDUSTRIAL METABOLITES BY RED YEASTS IN STRESS CONDITIONS

RADKA KOČÍ, MICHAELA DRÁBKOVÁ, and IVANA MÁROVÁ

Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, koci-r@fch.vutbr.cz

Introduction

Different types of environmental and physiological stress conditions constantly challenge all living organisms. To cope with the deleterious effects of stress, cells have developed rapid molecular responses to repair the damage and protect against further exposure to the same and/or other forms of stress[1]. Lipidic substances are naturally accumulated by some yeast species (*Rhodotorula, Rhodosporidium*). In stress conditions, especially in media with higher concentrations of salt, different changes in production of lipidic substances can be observed. For example, glycerol is naturally formed by many yeasts as by-product during ethanol fermentation[2]. Osmotolerant yeasts are able to grow in presence of high concentration of sugar and salt, achieving up to 50 % of glycerol yield[3]. Other lipidic compounds, carotenoids, are produced by different organisms from algae to higher plants, as well as by red yeasts and many species of fungi as protective metabolites agaínts deleterious effects of UV-irradiation and oxidative stress[4,5].

Carotenoids and other lipidic compounds such as glycerol and/or ergosterol are currently used as food colorants, nutritional supplements and as well for cosmetic and pharmaceutical purposes. Therefore, the ability of red yeasts to adapt under stress conditions by means of overproduction of industrially significant lipidic metabolites could be of increasing interest.

The aim of this work was to study production of carotenoids and other industrial metabolites by several strains of red yeasts cultivated under stress conditions. Some types of chemical and/or physical exogenous stresses were used as factors of biosynthesis stimulation.

Methods

Yeast strains: For cultivation have been used carotenogenic yeasts *Rhodotorula glutinis* CCY 20-2-26 and *Sporidiobolus salmonicolor* CCY 19-4-8.

Cultivation: Yeast strains were cultivated in Erlenmayer's flasks on synthetic glucose medium under 28 °C and permanent aeration. Production of metabolites was induced by chemical (H_2O_2 2–5 mmol l^{-1}; NaCl 2–5 %; $NiCl_2$ 0.6 mmol l^{-1}) and/or physical stress factors (limitation by oxygene ensured by 50–250 ml of medium volume in 500 ml Erlenmayer´s falsks; temperature 28–45 °C; yeast cells were influenced by UV-irradiation of 253 nm from 5 to 55 min).

Isolation and identification of metabolites: Carotenoids and ergosterol were obtained from yeast cells using acetone extraction and saponification by ethanolic KOH solution. The sample was repeatedly extracted by diethylether, evaporated and dissolved into ethanol for HPLC. Carotenoids were identified and quantified by RP-HPLC with spectrofotometric detection (450 nm – carotenes, torulene, lutein, lycopene; 285 nm – phytoene, ergosterol). Glycerol was obtained from cells using extraction by acetone in homogenizer. The sample was then repeatedly extracted by diethylether, evaporated and dissolved in distilled water. Glycerol was quantified spectrofotometrically using Boehringer diagnostic kit.

Results and discussion

Influence of exogenous stress led to individual response which was dependent on properties and sensitivity of different yeast strains. In stress conditions *R. glutinis* produced

Table I
Optimal doses of physical stresses (maximum of metabolite productions) in *R. glutinis*

Type of stress Metabolite	Aeration of medium [ml]	Temperature [°C]	UV-irradiation time of exposition [min]
β-carotene (μg g^{-1})	248.73 μg g^{-1} in 200 ml	422.82 μg g^{-1} at 28 °C	73.86 μg g^{-1} in 35 min
Ergosterol (mg g^{-1})	0.12 mg g^{-1} in 150 $\mu\lambda$	0.24 mg g^{-1} at 28 °C	0.31 mg g^{-1} in 35 min
Glycerol (mg ml^{-1})	–	385.05 mg ml^{-1} at 28 °C	1.68 mg ml^{-1} in 5 min

Table II
Optimal doses of physical stresses (maximum of metabolite productions) in *S. salmonicolor*

Type of stress Metabolite	Aeration of medium [ml]	Temperature [°C]	UV-irradiation time of exposition [min]
β-carotene (μg g^{-1})	31.10 μg g^{-1} in 150 ml	34.30 μg g^{-1} at 35 °C	7.58 μg g^{-1} in 5 min
Ergosterol (mg g^{-1})	0.44 mg g^{-1} in 150 ml	0.26 mg g^{-1} at 35 °C	0.05 mg g^{-1} in 5 min
Glycerol (mg ml^{-1})	–	45.02 mg ml^{-1} at 35 °C	0.36 mg ml^{-1} in 20 min

higher quantity of studied metabolites while *S. salmonicolor* exhibited higher increase of biomass.

Production of metabolites was stimulated in both strains by physical stress factors in limited range only. In yeasts, the optimal levels of physical stress factors were observed, which induced maximum production of individual metabolites (Tables I and II).

In presence of chemical stress the stimulation of carotenoid biosythesis was higher in both studied strains. In *R. glutinis* carotenoids were overproduced mainly under combination of oxidative and salt stress. Addition of low amount of NaCl (2 mmol l^{-1}) into inoculum medium followed by addition of H_2O_2 (5 mmol l^{-1}) into production medium led to 5–6 times higher production of carotenoids and ergosterol. In *S. salmonicolor* the increase of pigment production was obtained in presence of H_2O_2 added in both cultivation media (2 mmol l^{-1} H_2O_2 in inoculum, 5 mmol l^{-1} H_2O_2 in production medium) (Fig. 1.). In most of chemical stress experiments production of ergosterol increased simultaneously with formation of carotenoids in both strains, while glycerol production was completely inverse.

Higher production of specific lipidic substances can act as an adaptive mechanism. Cross-protection and formation of similar metabolites in various types of stress conditions suggest the existence of an integrating mechanism that senses and responds to different forms of stress. Moreover, biotechnological use of yeasts takes advantage of utilization the whole biomass efficiently enriched not only with carotenoids but also with other significant components as ergosterol.

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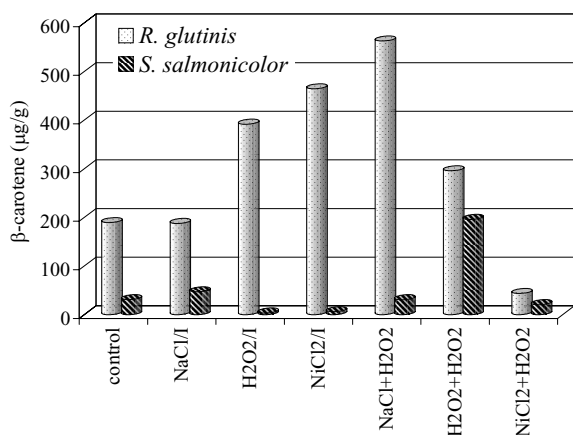
Fig. 1. **Production of β-carotene by red yeasts under chemical stress**

P20 CHEMICAL AND SENSORY EVALUATION OF LACTIC ACID FERMENTED CUCUMBER-ONION JUICES

ZLATICA KOHAJDOVÁ, JOLANA KAROVIČOVÁ and DRAHOMÍRA LUKÁČOVÁ

Department of Food Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic,
zlatica.kohajdova@stuba.sk

Introduction

Modern food processing is dependent on a range of preservative technologies to ensure that food is maintained at an acceptable level of quality from the time of manufacture through to the time of consumption. One of the oldest of these technologies is fermentation, a process dependent on the biological activity of microorganisms for production of a range of metabolites which can suppress the growth and survival of undesirable microflora in foodstuffs[1]. The lactic acid fermentation of vegetable products, applied as a preservation method for the production of finished and half-finished products, is again being ranked as an important technology and it is being further investigated because of the growing amount of raw materials processed in this way in the food industry[2].

The purpose of this study was to compare fermentation of cabbage juices with various additions of onion juice, to select suitable addition of onion juice for preparation of cabbage-onion juice and to determine optimal time of fermentation.

Experimental part

Preparation of vegetable juices: The fresh vegetables (cucumber and onion) were purchased in a local market in Slovakia. The cucumber was peeled and chopped to small slices. The onion was skinned and cutted to slices. The juices were obtained by pressing and filtration of crushed vegetables. The cucumber-onion juices were prepared by different addition of onion juice into cucumber juice: 0.5 %, 1 % and 2 % of onion juice was added into cucumber juices. After addition of onion juice, D-glucose and NaCl (to concentration 2 % and 0.5 % of these compounds in the juices) were added and juices were inoculated by *Lactobacillus plantarum* CCM 7039 (Faculty of Natural Science Brno, Czech collection of microorganisms, Czech Republic) at concentration 10^6 CFU ml^{-1}. The adjusted juices were placed into 250 ml sterile flasks (volume of juice in every flask was 150 ml).

Analytical methods. The measurement of pH was performed using a LABOR-pH-meter CG-834 SCHOTT, Germany. The total acidity was determined by the visual titration with a 0.1 M-NaOH using phenolphtalein indicator and expressed as lactic acid. Determination of reducing sugars was performed according to Schoorl. The non-reacted Cu^{2+} was determined after formation of Cu_2O. The KI was oxidized by $CuSO_4$ to I_2 that was determined by titration with $Na_2S_2O_3$.

Measurement of organic acids (lactic, acetic, citric and L-ascorbic acid)[3], nitrites and nitrates[4] was realized on the isotachophoretic analyser ZKI 01 Villa Labeco (Spišská N. Ves) with double-line recorder and conductivity detector.

Statistical method. For evaluation of analytical and sensory results, the multivariate statistic methods: principal component analysis was applied (SGWIN, Statgraphic for Windows, Version 1.4)

Sensory Evaluation. The samples were evaluated by 10 assessors. Turbidity and appearance were evaluated by a 5-point intensity scale. For evaluation of odour, taste, appearance of odour, appearance of taste and flavour, 100 mm graphical non-structured abscissas with the description of extreme points were. Results of sensory evaluations represent average values from evaluation of 10 assessors. The assessors were before sensory analysis checked for primary sensory tests.

Results and discussion

The cucumber juice with 0.5 % addition of onion juices was selected by assessors as the most suitable for preparation of cucumber-onion juices.

We recommended the stop of fermentation process all cucumber-onion juices in the 48 h of fermentation because in this hour, the juices had the highest intensity of harmonic taste, acceptance of odour, acceptance of taste and flavour and the pH (3.5–3.55) of juices was sufficiently low to prevent the growth hazardous food microorganisms.

It was found that *Lactobacillus plantarum* CCM 7039 reduced concentration of nitrates in the cucumber-onion juices.

Principal component analysis selected that for evaluation of cucumber-onion juices are the most important variables pH, lactic acid, odour descriptors: and taste descriptors: cucumber, cucumber-onion, sharp and sweet.

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P21 DETERMINATION OF PESTICIDES IN HOP SAMPLES

JANA KOHOUTKOVÁ, JANA TICHÁ, and JANA HAJŠLOVÁ

Department of Food Chemistry and Analysis, Institute of Chemical Technology, Technická 3, 166 28 Prague 6, Czech Republic, jana.kohoutkova@vscht.cz

Introduction

Currently, over 500 biologically active compounds are registered worldwide as pesticides. Representatives of these agrochemicals can be classified according to their molecular structure to various classes such as organohalogen compounds, organophosphates, carbamates, pyrethroids, triazoles, triazines, etc.)

With regard to their specific toxic activity against the target pests, they are known as insecticides, fungicides, herbicides, etc. Occurrence of pesticide residues (typically insecticides and fungicides) in food crops is unfortunately unavoidable and is obviously a matter of health concern. Analytical control foodstuffs and raw materials used for their production to prevent consumer exposure, is obviously needed. While within the recent years, several multiresidue methods allowing reliable screening of the major classes of pesticides in a wide range of plant matrices – mainly common fruits and vegetables – have been developed, only very few studies were concerned with analysis of such as complex matrix as is hop.

Hop is not only an important raw material used in beer productions, extracts obtained from this plant find nowadays several other uses for instance as food additives containing various health-promoting components (prenylflavonoids possessing antioxidative properties). Alike in other crops, various pesticide preparations are used during growing season for hop protection what may result in the presence of some residues in this commodity at the time of harvest. Unfortunately, a determination of residues in hops samples is an extremely complicated task. Compared to other food crops hop contains high amount of relatively non-polar natural components (resins) that can be co-extracted into organic solvents during isolation step.

With regard to the complexity of hop extract its thorough purification is essential for reliable data. Gel permeation chromatography (GPC) and/or solid phase extraction (SPE) are commonly used for removing major interferences from crude extracts. The aim of presented study was to critically assess efficiency GPC clean-up procedure in terms of the applicability of various GC detectors for examination of purified extracts.

Experimental

S t a n d a r d s

Pesticide standards (beta-cyfluthrin, bifenthrin, cypermethrin, deltamethrin, fenvalerate, lambda-cyhalothrin, permethrin, resmethrin, tau-fluvalinate, chlorpyrifos, chlor-

pyrifos-methyl and propiconazole), all 95% or higher purity, were obtained from Dr. Ehrenstorfer (Augsburk, Germany). Stock and working solutions of standards were prepared in toluene.

A n a l y t i c a l   m e t h o d

Extraction: 2 g homogenized hop sample were extracted with ethyl acetate (3×50 ml), combined extracts were evaporated to dryness and the residue was transferred by ethyl acetate – cyclohexane mixture (1:1, v/v) to volumetric flask, the final volume was made up to 25 ml.

Clean-up: 1 ml of extract was loaded onto GPC column (Bio -Beads SX3, mobile phase was ethyl acetate – cyclohexane (1:1 v/v), its flow rate 1 ml min^{-1}, volume of collected fractions were 13 ml. This "pesticide" fraction was evaporated and resolved in 1 ml of toluene.

Identification/quantification: Gas chromatographs HP 6890 combined with nitrogen-phosphorus and electron capture detectors or with mass selective detector were used. Separation of sample components was carried out either by conventional set-up (i) or employing low-pressure chromatography (ii). The conditions used were as follows:

(i)　column: Rtx -5MS (30 m $\times$ 0.25mm $\times$ 0.25 μm)
　　initial temperature: 90 °C
　　initial time: 2 min
　　temperature rate: 10 °C min^{-1},
　　final temperature: 280 °C, final time: 15 min
　　run time: 36 min

(ii)　columns: non-coated restriction column
　　(10 m $\times$ 0.18 mm) coupled with column DB -5MS
　　(15 m $\times$ 0.53 mm $\times$ 0.5 μm)
　　initial temperature: 90 °C
　　initial time: 0.5 min
　　temperature rate 1: 50 °C min^{-1},
　　final temperature: 200 °C, final time: 0 min
　　temperature rate 2 : 20 °C min^{-1},
　　final temperature: 290 °C, final time: 5 min
　　run time: 12 min

Results and discussion

In the first part of our study GPC elution profile of hop co-extracts was determined using "classic" Bio-Beads SX-3 (soft gel) column. Elution curves of pesticides typically used for crop protection were determined as well. As shown in Fig. 1. large overlap of matrix components and some early eluting pyrethroids such as lambda-cyhalothrin, tau-fluvalinate and fenvalerate occurred. Low elution volumes of these insecticides are not surprising since their molecular weight is quite high (338–505) as compared to other pesticides, for example chlorpyrifos-methyl and propiconazole (322 and 342, respectively). The dotted line in Fig. 1. shows elution curve of co-extracts corresponding to "simple" matrix in particular case apple. The comparison with zone corresponding to the hop extract illustrates the problem encountered in analysis of residues in hop, specifically pyrethroids.
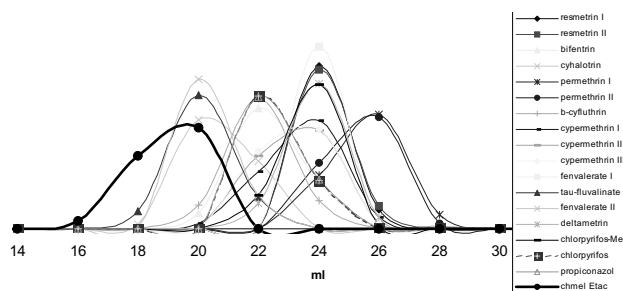
Fig. 1. **Elution profiles of matrix co-extracts contained in hop extract and selected pesticides**
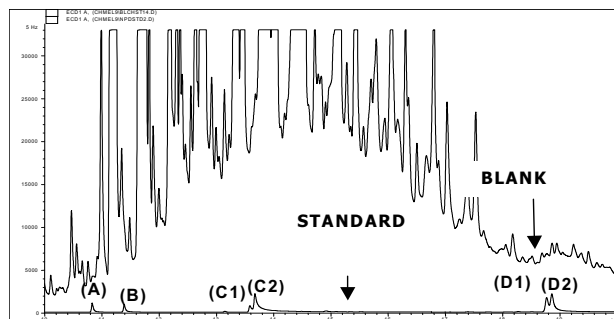

Fig. 2. **ECD chromatogram of hop blank and mixture of pesticide standards (chlorpyrifos (A; 0.35 μg ml^{-1} toluene), chlorpyrifos-Me (B; 0.32 μg ml^{-1} toluene), propiconazole (C1, C2; 5 μg ml^{-1} toluene) and tau-fluvalinate (D1, D2; 4.6 μg ml^{-1} toluene))**

As far as sufficiently high recoveries are required (according to EC directive SANCO/10476/2003 recovery should not be bellow 70 %), the collection of pesticide fraction has to be started before the removal of matrix is completed. Under these circumstances due to the high chemical noise (large amount of matrix components that are detected) electron capture detector (ECD), normally used in residue analysis of these compounds, cannot be employed. The only remaining solution is the use of mass selective detector (MSD); nevertheless detection limits are again, because of relatively high background noise fairly higher than in apple extract. Regarding organophosphates and carbamates nitrogen-phosphorous detector (NPD) can be used thanks to its good selectivity, although, LODs are again relative higher, compared to other matrices. To decrease the detection limits low-pressure chromatography (LP-GC/MS) was used for hop extracts analysis. As shown in Fig. 4. this system consisting from wide-bore column connected to injector by non-coated restriction capillary provided improved signal to noise ratio. In addition compared to conventional GC/MS procedure LP-GC/MS technique enabled faster separation and higher sample capacity. Considering these advantages, application of the latter approach in hop analysis is highly recommended.
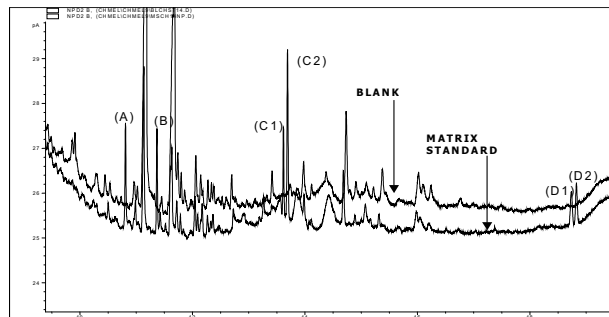

Fig. 3. **NPD chromatogram of hop blank and matrix standard (chlorpyrifos (A;0.35 μg ml^{-1} toluene), chlorpyrifos-Me (B; 0.32 μg ml^{-1} toluene), propiconazole (C1, C2; 5 μg ml^{-1} toluene) and tau-fluvalinate (D1, D2; 4.6 μg ml^{-1} toluene))**
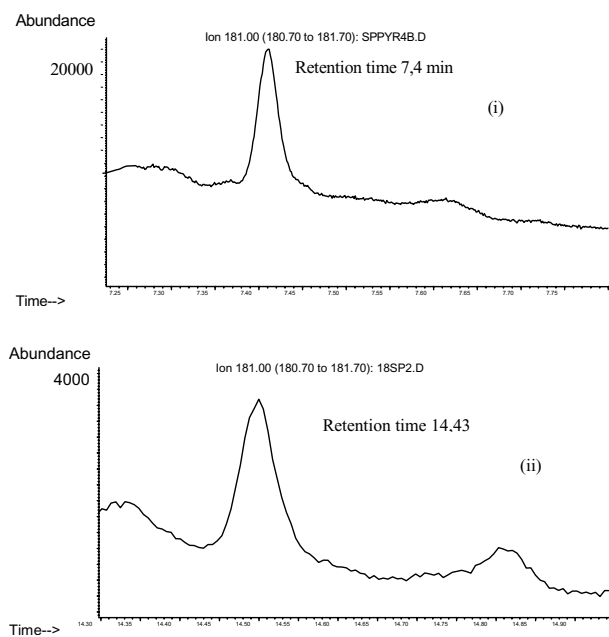
Fig. 4. **Comparsion of (i) low-pressure LP-GC/MS chromatography and (ii) conventional GC/MS chromatography for deltamethrine analysis in hop extracts (0.44 μg ml^{-1} toluene; m/z 181)**

Conclusions

Only part of co-extracts contained in hop extract can be removed by GPC, overlap of early eluting pyrethroids and matrix components elution bands occurs.

ECD cannot be used for determination of pesticide residues in hop extracts due high chemical noise.

Although NPD can be used for determination of N- and P- containing pesticides, the detection limits are relatively high for hop compared to other plant materials.

MS detection, especially in combination with low -pressure chromatography (LP-GC/MS) thanks to relatively good selectivity is the only technique enabling determination low residues of pyrethroids in hop extracts.

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P22　BIOSENSORS BASED ON SCREEN PRINTED ELECTRODES MODIFIED WITH RUTHENIUM DIOXIDE

PETRA BRÁZDILOVÁ[a], PETR KOTZIAN[a], KURT KALCHER[b] and KAREL VYTŘAS[a]

[a]*Department of Analytical Chemistry, University of Pardubice, nám. Čs. legií 565, 532 10 Pardubice, Czech Republic, petr.kotzian@upce.cz,* [b]*Institute of Chemistry – Analytical Chemistry, Karl-Franzens University, Universitaetsplatz 1, 8010 Graz, Austria*

Introduction

In present analytical research, one of the trends lies in developing simple, rapid and inexpensive sensors, which facilitate reproducible determinations of various species at low, medium as well as higher concentrations. One of the possibility is the use of screen-printed carbon electrodes (SPCEs), having widespread popularity due to their broad exploitable potential window, low background currents, chemical inertness, ease of chemical derivatization and modification and mass production[1,2].

Hydrogen peroxide, H_2O_2, represents an important intermediate in environmental and biological systems as a product of numerous enzymatic reactions (when some oxidases are applied). However, its direct oxidation at carbon electrodes requires relatively high positive potentials, which in turn causes interferences to many other oxidizable components. Therefore, the main aim is to reduce the overpotential by means of a mediator introduced as a modifier to the electrode surface or into the electrode bulk. Recently, manganese dioxide[3] was studied in the role of the mediator in details with our group. Here we report the use of ruthenium dioxide as modifier of SPCEs for determination of H_2O_2 and, in combination with enzymes, such as glucose oxidase (GOx) or xanthine oxidase (XOx), for the determination of glucose or hypoxanthine[4], respectively.

Results

To optimize the operational parameters, all experimental variables affecting the amperometric determination of hydrogen peroxide, glucose and hypoxanthine with flow injection analyses (working potential, the pH of supporting electrolyte, flow rate, etc.) were studied in detail.

For the determination of hydrogen peroxide were found these optimized parameters: working potential +0.48 V, pH of phosphate buffer 7.5, flow rate 0.2 ml min⁻¹. The relationship between the amperometric peak current and the concentration of H_2O_2 was investigated in the range 1 and 1000 mg l⁻¹. Up to concentrations of 1 mg l⁻¹ linearity was also found. The detection limit (three signal-to-noise ratio) was determined to be 3 μg l⁻¹ H_2O_2.

Hydrogen peroxide influences dominantly the electrochemical reactions on SPCEs even if glucose oxidase is entrapped in Nafion on the electrode surface. No or very low change in the shape of optimization curves was observed with GOx modified electrodes. That is why the same conditions as found for the determination of hydrogen peroxide could be applied for measurements with biosensors containing immobilized GOx. Linear relation between the concentration of glucose and the current response was found in the range 1–500 mg l⁻¹. The detection limit (3σ) was found to be 0.1 mg l⁻¹. The main interferences were from L-ascorbic acid and/or uric acid present in the sample matrix. Finally, it was observed that while both L-ascorbic and uric acids are electroactive at the applied potential, no response of those compounds occurred at –0.1 V. The calibration curve of glucose was nearly the same course when compared to experiments performed at +0.48 V, but the sensitivity was significantly lower. The glucose biosensor was applied to determine glucose content in white wine grapes and tangerines. Measurements were performed using the spiked samples; results are listed in Table I.

Table I
Determination of glucose in fruit samples and corresponding recovery studies. Working potential +0.48 V vs. Ag/AgCl; carrier phosphate buffer pH 7.5 (0.1 mol l⁻¹); flow rate 0.2 ml min⁻¹; injection volume 200 μl

Sample	Spike [mg l⁻¹]	Expected [mg l⁻¹]	Found [mg l⁻¹]	Recovery [%]
Wine grapes	0.0		23.2	
	33.0	56.2	54.2	96.4
Tangerines	0.0		11.1	
	25.0	36.1	35.7	98.9

The determination of hypoxanthine has considerable importance for the quality control of fish products in food industries. The hypoxanthine biosensor was prepared by entrapping xanthine oxidase in Nafion on the electrode surface as glucose biosensor. Employing the optimum operational parameters (working potential +0.5 V *vs*. Ag/AgCl; pH of phosphate buffer 7.5; flow rate 0.6 ml min⁻¹), linear relation between the current response and concentration was found in the range 1–15 mg l⁻¹ of hypoxanthine. At concentrations higher than 15.0 mg l⁻¹ deviation from the linearity was observed. The detection limit (3σ) was found to be 0.4 mg l⁻¹. If necessary to avoid common interferants (both ascorbic and uric acids), determination can also be realized at –0.1 V, but

with lower sensitivity. The hypoxanthine biosensor was applied to a fish sample. The homogeneous solution, prepared by mixing of homogenized salmon sample ($\approx$15 g) with phosphate buffer (150 ml), was filtered to retain proteins and solid particles, and was then directly injected to the stream of the buffer carrier. A growing amount of hypoxanthine was measured when the fish sample was kept at room temperature (25 °C). A pronounced increase of the sensor response during 24 h can be related to the continuous degradation of the meat tissue. At the end of the measuring period, the found amount of hypoxanthine was 170 % of its original content. To verify the influence of the matrix, hypoxanthine was determined also in freshly prepared homogenates of salmon meat samples spiked with known additions of the substance. The results (see Table II) can be considered quite satisfactory; the higher value indicated at the sample spiked with higher content can be attributed to the partial sample decomposition in the course of individual injections.

Table II
Determination of hypoxanthine in salmon meat and corresponding recovery studies. Working potential +0.5 V *vs*. Ag/AgCl; carrier phosphate buffer pH 7.5 (0.1 mol l^{-1}); flow rate 0.6 ml min^{-1}; injection volume 200 µl

Sample	Spike [mg l^{-1}]	Expected [mg l^{-1}]	Found [mg l^{-1}]	Recovery [%]
Salmon meat	0.0		8.1	
	2.5	10.6	10.6	100
	5.0	13.1	13.5	103

Conclusion

It has been shown that the screen-printed biosensors containing ruthenium dioxide and corresponding enzymes immobilized on the electrode surfaces in Nafion are suitable for simple and rapid FIA determination procedures of hydrogen peroxide, glucose and hypoxanthine in different matrices. For analysis of food samples, no special pre-treatment is necessary. Having in mind problems connected with analyses of practical food samples, this fact represents a big advantage.

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P23 THE USE OF C8 FATTY ACID TO BACTERIAL FLORA OF CHICKEN SKIN

DANIELA KRAMÁŘOVÁ[a], EVA LUKÁŠKOVÁ[a], PAVEL BŘEZINA[a] and MILAN MAROUNEK[b, c]

[a]*Tomas Bata University in Zlin, Faculty of Technology, Department of Food Engineering, 275 TGM, 760 01 Zlin, Czech Republic, kramarova@ft.utb.cz*, [b]*Research Institute of Animal Production Prague, Czech Republic*, [c]*Institute of Animal Physiology and Genetics, Czech Academy of Sciences Prague, Czech Republic*

Introduction

Plenty of chemical treatments reduces the bacterial population of processed carcasses, contaminated poultry products continue to be major source of human foodborne diseases. The bacterial flora of the poultry skin can include coliforms, pseudomonas, staphylococci, salmonella and other aerobic and anaerobic bacteria. Some of these bacteria are present on processed chicken carcasses, and may be foodborne pathogens capable of causing illness or even death in humans[1,2].

The effect of caprylic acid on bacterial flora of skin of processed chickens was examined. The aim of this study was to assess susceptibility of bacteria contaminating poultry skin to caprylic acid (C8:0).

Methods

Chicken carcasses were stored at 4 °C for three days. Skin was removed from carcasses every day, cut into 50 g pieces, placed in sterile bottle with glass balls and 100 ml of 2 and 5% (w/v) of caprylic acid emulsion were added. Control sample was prepared in the same way, but 0.1% peptone solution was used instead of caprylic acid. Aliquots of the rinsate were taken for microbiological analyses[3]. The standard plate count was performed using Plate Count Agar (PCA, HiMedia Laboratories) to enumerate obligately aerobic and facultatively aerobic bacteria; plates were incubated aerobically at 37 °C for 24 hours. Each experiment was repeated five times.

Results and discussion

Washing poultry skin samples in caprylic acid also reduced the number of bacteria that remained attached to the poultry skin after rinsing.

Table I
Effect of 2% concentration of caprylic acid on bacterial flora

Day	Control sample [CFU/100 g]	2% C8 [CFU/100 g]
1	$8.69 \cdot 10^2$	0
2	$7.09 \cdot 10^2$	0
3	$6.09 \cdot 10^2$	$0.51 \cdot 10^0$

Coliforms, staphylococci and micrococci were highly susceptible to the bacterial activity of caprylic acid, while the populations of pseudomonas exhibited greater resistance. Staphylococci, micrococci, pseudomonas from poultry skin were susceptible to the bactericidal activity of caprylic acid at concentration of 2–5 %. Coliform bacteria were more suspectible to caprylic acid than the others strains as 2% concentration was sufficient for their complete elimination.
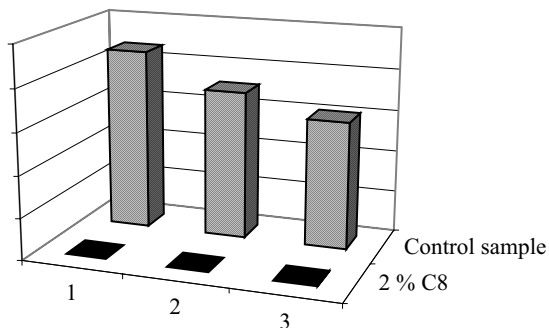

Fig. 1.   **Effect of 2% concentration of caprylic acid on bacterial flora**

Table II
Effect of 5% concentration of caprylic acid on bacterial flora

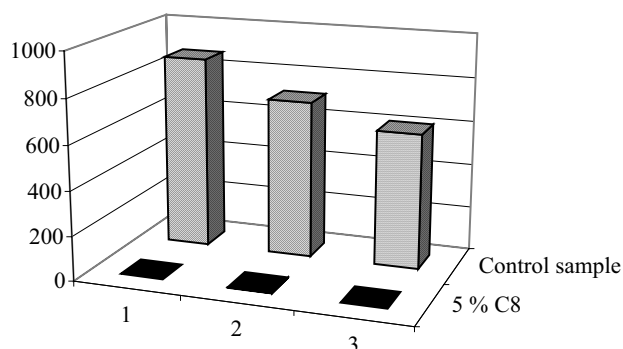| Day	Control sample [CFU/100 g]	5% C8 [CFU/100 g]
1	$8.69 \cdot 10^2$	0
2	$7.09 \cdot 10^2$	$1.98 \cdot 10^0$
3	$6.09 \cdot 10^2$	0

Fig. 2.   **Effect of 5% concentration of caprylic acid on bacterial flora**

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P24   THE DETERMINATION OF ACRYLAMIDE IN FOODSTUFFS OF FOOD BASKET OF CZECH POPULATION BY GC-MS

IRENA ŘEHŮŘKOVÁ, JOSEF SLAVÍK, SYLVIE BĚLÁKOVÁ, JOSEF ČÁSLAVSKÝ, EVA JANOUŠKOVÁ, MIROSLAVA KRBŮŠKOVÁ and JIŘÍ RUPRICH
National Institute of Public Health – Centre for Hygiene of Food Chains, Palackého 3a, 612 42 Brno, Czech Republic, rehurkova@chpr.szu.cz

Introduction

Acrylamide ($CH_2 = CHCONH_2$, CAS 79-0601) is an important industrial chemical used as a chemical intermediate in the production of polyacrylamides, which are used as flocculants for clarifying drinking water and industrial applications. The neurotoxicity of acrylamide in humans is known from occupational and accidental exposures. Experimental studies with acrylamide in animals have shown reproductive, genotoxic and carcinogenic properties.

In April 2002 the Swedish National Food Authority reported on elevated levels of acrylamide in heat-treated potato products and baked goods.

The findings concerning acrylamide formation in cooked food, however, are completely new. The chemical mechanism leading to the formation of acrylamide in foodstuffs is not completely understood. It is assumed to derive from the well-known Maillard reaction that occurs between reducing sugars and proteins/amino acids. One of the main contributing compounds seems to be asparagines, an amino acid frequently occurring in foods. The cooking process, in particular frying or roasting at high temperature, then induces the formation of acrylamide[1–3].

As acrylamide is a potential carcinogen, a worldwide surveillance of this substance in various food products has been started. In view of surveillance and subsequent exposure assessment it is essential to use analytical methods of similar performance to produce reliable and comparable data. In the Czech Republic The National Institute of Public Health in Prague, Centre for Hygiene of Food Chains in Brno is involved in "The Project of Dietary Exposure to Selected Chemical Substances" within the framework of "The Environmental Health Monitoring System in the Czech Republic". This project describes the dietary exposure of the Czech population to selected chemical substances. The findings mentioned above were the reason to include monitoring of acrylamide in this project[4,5].

Methodology

Several analytical methods for acrylamide detection and determination in food were tested[6,7]:

LC-MS/MS, LC-UV, GC-MS-EI, GC-MS bromination method. It was found that the GC-MS bromination method is robust and ensures a good recovery and accuracy[8]. Sample preparation procedure is also important.

The method is based on the extraction of acrylamid with water, multi-stage filtration and acidification followed by bromination[9,10]. The 2,3-dibromopropionanamide as a product of bromination is extracted with ethylacetate and converted into stable 2-bromopropenamide by dehydrobromination with triethylamine. The ethylacetate extract is filtered and analysed by gas chromatography coupled with mass spectrometry employing $^{13}C_3$ acrylamide as the internal standard. Standard solutions were prepared from 2,3-dibromopropionanamide and ethylacetate-triethylamine as a solvent.

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 ^{79}Br to ^{81}Br 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 is suitable for the wide range of foods.

The method was validated and quality control was implemented by participating in proficiency testing organised by Food Analysis Performance Assessment Scheme (FAPAS; UK). In addition, FAPAS samples of known acrylamide content were used as reference materials for internal testing.

The data quality is indicated by LOQ of 15 μg kg^{-1}; a very good linearity from 15 to 500 μg kg^{-1} with the correlation coefficient of 0.9998; the recovery ranges from 60 to 80 % according to the matrix, CV% of repeatability 2–3. The method was accredited according EN ISO/IEC 17025 (Czech Accreditation Institute).

Samples typical for food basket of the Czech Republic were selected for the analysis. Systematic sample collection is determined by the system of the monitoring of dietary exposure. First of all the samples were subjected to culinary treatment so that they could be analysed in the same condition as they are consumed[4,5].

Results and discussion

In total 110 matrix types were analysed in 2004. In most of them the content of acrylamide did not exceed LOQ. The abundance of the quantifiable samples amounted to 8 %. Acrylamide concentrations in those food samples in which the measured concentration of AA exceeded LOQ are given in the Fig. 1.

The highest content of acrylamide of all the 110 analysed samples was found in potato crisps (670 μg kg^{-1}), spices (220 μg kg^{-1}) and french-fries (135 μg kg^{-1}). Concentration in pastry, chocolate, cakes, ranged from 15 μg kg^{-1} to 100 μg kg^{-1}. The acrylamide concentration in 92 % of

samples (meat and meat products, raw fruit, vegetables, beverages, milk, dairy products, eggs, legumes, margarines, cereals) was lower than LOQ. These results correspond with published data. For example, in 2003 U. S. Food and Drug Administration analysed 286 samples from the USA[11] within the framework of the Total Diet Study (TDS; monitoring of dietary exposure in the Czech Republic works on the TDS principles).
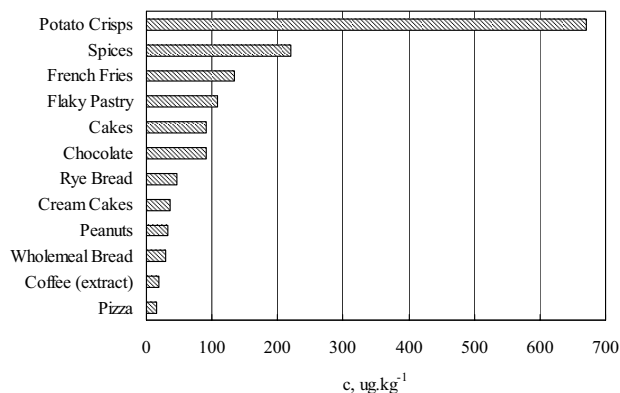
Fig. 1.　**Acrylamide content in food samples after culinary treatment (in relevant cases) exceeding LOQ**

Conslusion

110 selected composite food samples representative of the food basket were analysed in 2004. For the estimation of the dietary exposure of acrylamide more data are required. They are going to be available after completing the monitoring period of 2005.

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P25 PHYSIOLOGICAL CHARACTERISATION OF XYLOSE-UTILISING *SACCHAROMYCES CEREVISIAE* STRAINS

JITKA KUBEŠOVÁ[a],
MARIE-FRANCOISE GORWA-GRAUSLUND[b],
KAISA KARHUMAA[b] and IVANA MÁROVÁ[a]

[a]*Department of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, kubesova@fch.vutbr.cz,* [b]*Department of Applied Microbiology, Lund Institute of Technology, Lund University, P.O. Box 124, 221 00 Lund, Sweden, francoise.gorwa@tmb.lth.se*

Introduction

Bioethanol is a renewable energy source produced through fermentation of sugars. Several natural and recombinant microorganisms have been studied for ethanol production from lignocellulosic materials. *Saccharomyces cerevisiae* ferments the hexose sugars present in lignocellulose hydrolysate. It has high tolerance to ethanol and inhibitors but does not ferment the pentoses xylose and arabinose.

By overexpression of the *P. stipitis XYL1* and *XYL2* genes, encoding XR and XDH, respectively along with the native *XKS1* gene encoding xylulokinase (XK) in *S. cerevisiae*, the recombinant xylose-utilising strain TMB3001 capable of ethanol production has been generated[1]. TMB3001 is able to form ethanol from xylose anaerobically. However, the xylose consumption rate and the ethanol yield on xylose are not as high as on glucose. This has been attributed to problems with xylose transport into the cells at low substrate concentrations, low expression of the enzymes involved in the non-oxidative pentose phosphate pathway in *S. cerevisiae* and the redox imbalance that is created by the NAD(P)H-utilising XR and the NAD^{+}-utilising XDH.

In order to address these problems a new set of recombinant strains has been generated and the subject of this project was to test these strains for xylose consumption and ethanol production in anaerobic batch fermentation under well-controlled conditions.

Materials and methods

S t r a i n s

The investigated strains were TMB3001, TMB 3062, TMB3260, TMB3270, TMB3271, TMB3185 and TMB3186.

M e d i u m

A defined mineral medium[5] containing salts, sugars, vitamins and trace elements was used both for the preculture and the culture.

Table I
Strains used in this work

Strain	Relevant phenotype	Reference
TMB3001	Produces XR, XDH, XK	1
TMB3062	Produces XR, XDH, XK	Karhumaa, unpublished
TMB3260	Overproduces XR	2
TMB3270	Produces mutated XR	3
TMB3271	Overproduces mutated XR	3
TMB3185	Expresses NAD kinase. Overproduces XR, XDH, XK	4
TMB3186	Produces XR, XDH, XK	4

F e r m e n t a t i o n   s e t - u p

Cultivations were carried out in well-controlled 3L Bioflow III fermentor (New Brunswick, Edison, NJ) with a working volume of 1 l. Mineral medium containing a mixture of glucose (20 g l^{−1}) and xylose (50 g l^{−1}) was used. The pH was regulated at 5.5 by 2M NaOH addition and the temperature was kept at 30 °C. The fermentor was inoculated with a starting OD of about 0.2. A nitrogen gas containing less than 5 ppm of O_2 (ADR class2 1A, AGA, Malmö, Sweden) was used at flow rate of 0.2 l min^{−1} controlled by mass flowmeter (Bronkorst HI-TECH, Ruurlo, The Netherlands) and 200 rpm stirring. Samples were taken from the cultivation broth, filtered through 0.2 μm pore size filters (Advantec MFS, Peasanton, USA) and stored at 4 °C and −20 °C for HPLC analysis.

H P L C

Concentrations of glucose, xylose, xylitol, glycerol and acetate in the culture were measured by HPLC (Waters, Milford, Massachusetts, USA). Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA) was used at 45 °C with a mobile phase 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{−1}. A refractive index detector (RID-6A, Shimadzu, Kyoto, Japan) was used for the detection. A standard solution was prepared with 10 g l^{−1} of glucose, xylose, xylitol, glycerol, acetic acid and ethanol and then diluted at the following dilutions: 10, 7.5, 5, 2.5, 1, 0.5, 0. Ethanol concentration was calculating from redox balance because of high volatility. Yields were calculating from formula:

$$Y_{A,B} = \Delta A / \Delta B$$

where ΔA and ΔB are concentration differences of product A and substrate B between the beginning and the end of the measured period.

Results and discussion

Saccharomyces cerevisiae produces ethanol from the hexose sugars in lignocellulose hydrolysates but it cannot utilize pentose sugars such as xylose and arabinose. Recombinant *S. cerevisiae* strains that express heterologous genes

for xylose utilization (xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* or xylose isomerase (XI) from *Thermus thermophilus*) have been constructed. The aim of this work was to compare the fermentation performance of different recombinant xylose-utilising strains under anaerobic batch cultivation with 20 g l^{-1} glucose and 50 g l^{-1} xylose. Seven different strains were investigated: TMB3001 (reference strain), TMB3062, TMB3185, TMB3186, TMB3260, TMB3270, TMB3271.

TMB3062 (XK integrated; XR, XDH on multicopy plasmids) was compared with TMB3001 which had XR, XDH and XK (xylulokinase) integrated. The strain with more copies of XR and XDH on plasmid had higher ethanol yield and lower xylitol yield but the consumption of glucose and xylose was slower.

TMB3185 (expresses NAD$^+$ kinase) was compared with TMB3001. The strain TMB3185 had higher ethanol yield and similar xylitol yield. TMB3186 (unknown mutation promoting growth on L-arabinose) had enhanced ethanol yield and lower xylitol yield than TMB3001.

Strains TMB3001 and TMB3260 (more copies of XR integrated) were compared with strains with one or several copies of a mutated XR with a reduced affinity for NADPH. Enhanced ethanol yields accompanied by decreased xylitol yields were obtained in the strain carrying one copy of the mutated XR (TMB3270). The overproduction of the mutated XR (TMB3271) resulted in the highest ethanol yield combined with the lowest xylitol yield, and with a moderate xylose consumption rate.

To conclude, strain TMB3271 overexpressing the mutated XR appears as the best strain from the investigated strains for ethanol production from xylose.

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P26   IDENTIFICATION OF BARLEY PROTEINS AND GLYCOPROTEINS BY VARIOUS SEPARATION TECHNIQUES AND MALDI MS

MARKÉTA LAŠTOVIČKOVÁ[a, b], KAREL BEZOUŠKA[c], MARTINA MARCHETTI[d], GUNTER ALLMAIER[d] and JOSEF CHMELÍK[b]

[a]*Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic; stikarovska@fch.vutbr.cz,* [b]*Institute of Analytical Chemistry, Academy of Science of the Czech Republic, 611 42 Brno, Veveří 97, Czech Republic,* [c]*Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic,* [d]*Institute of Chemical Technologies and Analysis, Vienna University of Technology, Getreidemarkt 9/164, A-1060, Vienna, Austria*

Introduction

Barley (*Hordeum Vulgare*) has many advantages that made it to be a major world crop[1]. The cereal proteins have been one of the main topics of research with aim of understanding their structures and role in grain utilization. Barley grain analysis is important especially for brewing industry.

Electrophoretic analysis of proteins and glycoproteins has been used for barley cultivar discrimination[2]. It is important because malting and brewing properties, the resistance of barley plant to fungal or viral diseases and cold weather are cultivar-dependent.

There are many different strategies for separation and structural analysis of proteins based on gel electrophoresis and other techniques. Due to their microheterogenity, the isolation and separation of glycoproteins is not a simple task. Generally, all techniques used for the purification of proteins can also be applied to glycoproteins. Moreover, affinity (e. g. lectin) chromatography is available[3].

The recent the literature has reported hundreds of applications of mass spectrometry (MS) techniques in proteomics. MS has become widely used mainly after the invention of the soft ionization methods such as matrix-assisted laser desorption/ionization (MALDI)[4]. The connection of separation techniques with MS and bioinformatics has become the most important tools for protein identification[5].

This study deals with using various separation methods (affinity lectin chromatography, gel electrophoresis with different staining) and MALDI MS for the characterization of proteins and glycoproteins isolated from barley grains.

Experimental

Extraction: Barley flour (50 mg) was extracted with 500 μl deionized water. The extraction was repeated two times and the supernatants were combined.

Affinity chromatography: A glycoprotein enriched fraction was obtained by Concanavalin *A* (Con *A*) lectin chromatography (Con *A* Agarose set; Calbiochem, San Diego, CA, USA).

SDS-PAGE electrophoresis: The proteins and glycoproteins were separated by 10% or 12% SDS–PAGE gels. The visualization was carried out by Coomassie Brilliant Blue R-250 and dansylhydrazine methods[6].

Mass spectrometry: MALDI-TOF and TOF/TOF MS (MS/MS) experiments were performed with *AXIMA LNR* mass spectrometer (Shimadzu-Kratos Biotech, Manchester, UK) and Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA).

Result and discussion

Two approaches were used for characterization of proteins and glycoproteins from barley grains. The first approach was based on direct separation of proteins and glycoproteins from crude extract by SDS-PAGE with dansylhydrazine staining allowing the identification of glycoproteins (see Fig. 1.). In the second approach, the crude extract was subjected the separation on an affinity column with immobilized Con *A*. Thus the fraction of high-mannose and hybrid-types *N*-glycoproteins were enriched. The particular proteins (Con *A* non-bound fraction) as well as glycoproteins (Con *A* bound fraction) were separated by SDS-PAGE and stained by Coomassie Blue. SDS-PAGE electropherogram of both fractions is shown in Fig. 2.
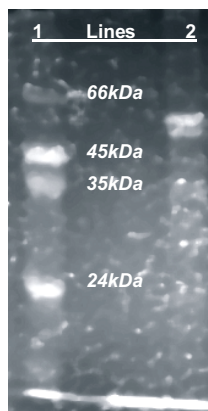

Fig. 1. **SDS gel electropherogram obtained by dansylhydrazine staining; Lines: 1 – molecular weight markers, 2 – crude water extract from barley flour**

Several intensive glycoprotein bands were detected by both staining techniques. In addition, after Coomassie staining many minor bands with molecular masses in the range of 10–120 kDa were seen.

The bands of interest (*A, B, C, D bands* in Fig. 2.) were excised and in-gel digested by trypsin. The extracted peptides were analyzed by MALDI-TOF MS. A considerable number of $[M+H]^+$ ions were found in the mass spectra and used for database search with the search programs ProFound and MASCOT.

The first investigated protein (non-bound on Con *A* column) was determined as β-*amylase* (EC 3.2.1.2). The second non-bound protein was identified as *Protein z-type*
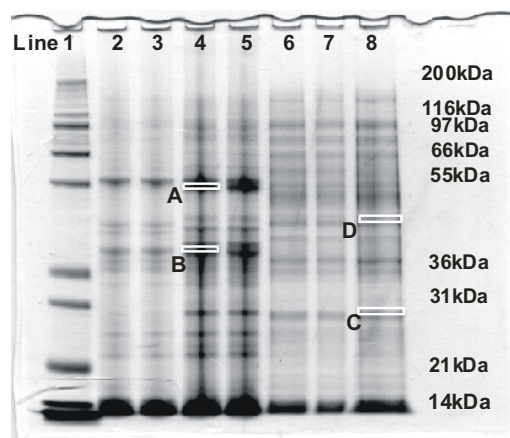
Fig. 2. **SDS-PAGE gel electropherogram stained by Coomassie Blue; Lines: 1 – molecular weight markers, 2–5 – non-bound fractions, 6–8 – bound fractions. The identified proteins were labeled: A) β-amylase; B) Protein z-type serpin; C) Con A (contaminant from the column material); D) Elongation factor**

serpin (P40076; for its peptide spectrum see Fig. 3.). Another protein, which formed an intense band in "Con *A* bounded fraction" gel line, was identified as Con *A*. It was eluted from the affinity column during chromatography. The line *D* was determined as barley *Elongation factor*.

The examples demonstrate the feasibility of the strategy and the future work will concentrate on the identification of glycoproteins from barley grains with emphasis on the characterization of both peptide and their glycan part. For the protein identification, both peptide mass fingerprinting as well as CID-generated fragmentation patterns, obtained by tandem mass spectrometry, will be used.

Fig. 3. **Positive-ion MALDI mass spectrum of Protein z-type serpin. 2,4,6–trihydroxyacetophenone (20 mg ml⁻¹ methanol) were used as the matrix and 0.2 M diammonium hydrogen citrate as co-matrix. The asterisks label the peaks of identify protein**

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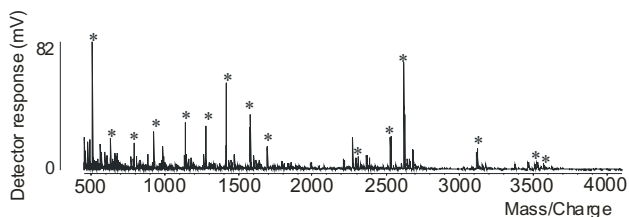2. Weiss W., Postel W., Gorg A.: Electrophoresis, *12*, 323 (1991).
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P27 AUTHENTIFICATION OF SELECTED PRESERVATIVE PRODUCTS OF THE SLOVAK MARKET

JARMILA LEHKOŽIVOVÁ[a], JOLANA KAROVIČOVÁ[a], PETER ŠIMKO[b], MILAN SUHAJ[b], ZLATICA KOHAJDOVÁ[a] and LENKA MACHAROVÁ[a]

[a]*Department of Food Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic, jarmila.lehkozivova@stuba.sk,* [b]*Food Research Institute, Priemyselná 4, 824 75 Bratislava, Slovak Republic*

Introduction

Tomatoes are an important agricultural commodity worldwide. More than 80 % of processed tomatoes are consumed in the form of tomato juice, paste, puree, ketchup, sauce, and salsa[1]. Important quality aspects of processed tomatoes are colour, taste and consistency[2]. Analytical markers of processed tomatoes are lycopene, β-carotene, pyroglutamic acid, citric acid, malic acid, K^+, formol number, content of soluble solids, etc. Most frequently way of ketchup adulteration is lowering of tomato content by adding hydrocolloids and sucrose[3].

Adulteration of fruit-based products such as fruit preparations, jams and spreads may encompass both the admixture of cheaper fruits and non-compliance with the specified fruit content. Methods reported so far for the determination of the fruit content are mostly based on the quantification of low-molecular compounds, e. g. amino acids, sugars, and minerals[4]. The determination of characteristic phenolic compounds is a common tool for the differentiation of fruit species[5].

Materials and methods

14 samples of ketchup were analysed. Eight of them were purchased from a local supermarket and six were from the firm Merx Slovakia a. s. In this samples of ketchup were determined selected physical-chemical parameters: lycopene (spectrophotometric method), citric acid (capillary isotachophoresis), K^+ (AAS), formol number (potentiometric titration), content of soluble solids (refractometric method).

Results and discussion

Results of selected physical-chemical parameters of ketchup samples were compared with requirements of AIJN[6],

Decree of Slovak Republic[7] and with the study of Shi & Le Maguer[1]. The content of soluble solids in samples ranged from 13.00 °Brix to 29.00 °Brix and therefore all samples were up to standard of Decree of Slovak Republic. However, Decree of Slovak Republic specifies the minimum tomato content in ketchup being 7 or 10 % tomato solids in total refractive solid content, which is less than or more over 30 °Brix for tomato ketchup and tomato ketchup labelled Prima, Extra, Special, respectively. So from our results, we do not know how much tomato content is in samples of ketchup. Therefore we determined other physical-chemical parameters. Shi & Le Maguer reported the values of lycopene content in tomato ketchups in range from 10.29 mg/100 g to 41.4 mg/100 g wet basis. The content of lycopene in our samples ranged from 0.23 mg/100 g to 8.65 mg/100 g wet basis. The lower content of lycopene could be caused by losses during tomato processing (isomerization and oxidation), by variety, maturity and the environmental conditions under which the fruit matured. But samples 8 and 9 are very suspicious of adulteration because amount of lycopene were 0.23 mg/100 g and 0.53 mg/100 g wet basis. The other parameters were compared with the criteria of AIJN after the results of tomato ketchup samples were calculated on 5 °Brix of soluble solids content. The criteria of AIJN for tomato juice (with 5 °Brix of soluble solids content) are: citric acid from 2 to 5 g/kg, K^+ from 1500 to 3500 mg/kg, formol number from 25 to 60 ml 0.1M NaOH/100g. In samples of ketchup citric acid ranged from 0.59 to 5.06 g/kg, formol number from 4.76 to 32.65 ml 0.1M NaOH/100g and K^+ from 581 to 2768 mg/kg. Here is a high probability that samples 8 and 9 were adulterated, because their amounts of citric acid, K^+ (only sample 9) and formol number were much lower than criteria of AIJN.

Conclusion

In the 14 samples of tomato ketchup selected physical–chemical parameters were determined. All samples fulfilled requirement for content of soluble solids. High probability of adulteration is in samples 8 and 9, because amounts of citric acid, lycopene, K^+ (only sample 9) and formol number were much lower than criteria of AIJN and values presented by Shi & Le Maguer.

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P28 DETERMINATION OF ZEARALENONE IN COW MILK, DEVELOPMENT OF THE METHOD AND VALIDATION

JAROMÍR LOJZA, VĚRA SCHULZOVÁ and JANA HAJŠLOVÁ

Department of Food Chemistry and Analysis, Institute of Chemical Technology, Prague, Czech Republic,
jaromir.lojza@vscht.cz

Introduction

Zearalenone (ZON) (Fig. 1.) is a potent estrogenic metabolite produced by several *Fusarium sp.* and recognized to be a common contaminant in cereal grains and animal feedstuffs. Various health disorders (including loss of body weight, infertility, vaginal prolapse and enlargement of the uterus and mammary glands) associated with the intake of this mycotoxin have been well documented in domestic animals. Furthermore, on the basis of tests with experimental animals ZON has been reported to be potentially carcinogenic compound[1].
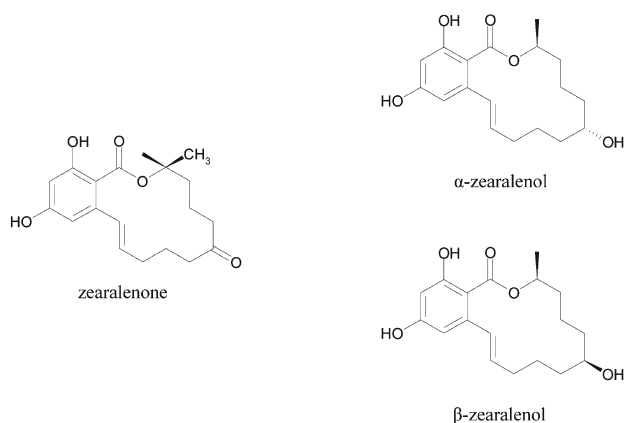

Fig. 1. **Structures of zearalenone and its major metabolites**

To prove the exposure of farm animals to ZON and monitor toxin fate during toxicological or other experiments, it is necessary to have available a simple, selective and sensitive analytical procedure enabling simultaneous identification/quantification of both parent ZON and its major metabolites, α-zearalenol (α-ZOL) and β-zearalenol

(β-ZOL) that may occur in various biological fluids[2]. Although some methods for the determination of ZON in urine or blood plasma employing dedicated immunoafinity SPE cartridges for isolation of target analytes have been published[3, 4], these straightforward procedures are not easily applicable for analysis of more complex samples, such as milk.

In presented study the newly developed method enabling simultaneous determination of trace levels of ZON, α-ZOL and β-ZOL in milk is described. The purpose of our study was to make available analytical tool for realization of follow-up experiments concerned with the fate of ZON in the food chain, particularly in dairy cows.

Experimental

M a t e r i a l s

Zearalenone (purity min. 98 %), α-zearalenol (purity min. 98 %) and β-zearalenol (purity min. 98 %) were purchased from Sigma-Aldrich (Germany). Milk samples for the analysis of zearalenone and its metabolites were supplied by project partner – Research Institute for Cattle Breeding, Rapotín, Czech Republic.

M e t h o d s

Extraction step: An aliquot of 10 ml of milk sample was mixed with 20 ml of water. Target analytes were extracted using HLB (Oasis, Waters) SPE cartridges. Extraction procedure consisted of following steps: (i) conditioning (10 ml methanol and 10 ml water), (ii) loading of diluted sample (15 ml), (iii) washing out impurities (20 ml water and 5 ml 30% methanol, *v/v*) and (iv) elution of the analytes (7 ml methanol).

Clean-up step: Crude extract (SPE fraction iv) was purified in two steps using: (i) liquid-liquid extraction with cyclohexane:ethyl acetate (1:1, *v/v*) and (ii) GPC (Bio-Beads S-X3, mobile phase cyclohexane:ethyl acetate,1:1, *v/v*). After addition 23 ml of water to 7 ml methanolic SPE eluate (fraction iv) extraction was accomplished with 50 ml of cyclohexane – ethyl acetate mixture. Upper layer was removed and evaporated. The residue was diluted in 3 ml of GPC mobile phase for. 1.5 ml of the sample (corresponds to equivalent 3.5 ml of milk) was injected onto GPC system. Analytes were eluted in 7 ml fraction collected within 13–20 ml. Eluate was evaporated and the residue was dissolved in 0.5 ml of mobile phase prior to HPLC analysis.

I d e n t i f i c a t i o n   a n d   q u a n t i f i c a t i o n

HPLC analyses were performed by HP 1100 Series (Hewlett Packard, USA) liquid chromatograph equipped with DAD detector (HP 1100 Series, Hewlett Packard, USA). C18 Discovery (150 mm × 3 mm × 5 μm) column was used for sample separation. Mobile phase (30% methanol v/v, gradient elution – 20 min 86% methanol) flow rate was 0.7 ml min^{-1}; sample injection volume was 20 μl; separation temperature was 45 °C. Analytes were monitored at 260 nm.

Results and discussion

Selective isolation of ZON and its major metabolites is a difficult analytical task because of complexity of milk matrix. Attempts to realize efficient extraction (various solvents used) failed mainly because of stable emulsions formation. As an alternative solution, solid phase extraction employing HLB cartridges was tested. However, together with target analytes, many other matrix components mainly lipids were co-isolated what caused an intensive chemical noise in HPLC/DAD chromatograms. In the next set of experiments optimum purification strategy had to be searched. Liquid – liquid extraction emplyoying cyclohexane – ethylacetate mixture enabled isolation of both parent mycotoxin and its metabolites. GPC used in the next step removed lipids occurring in this extract. The efficiency of clean-up procedure is well documented in Fig. 2.

The recoveries of the above described procedure were 83, 79, 81 % for ZON, α-ZOL and β-ZOL, respectively. The limit of quantification (LOQ) for all target analytes in cow milk (3 % fat) was 4 ng ml^{-1}, the repeatability of measurements expressed as a relative standard deviation (RSD) was 8.0 %.

The newly developed and validated analytical method was applied for the examination of milk samples collected within monitoring study initiated in the collaboration with the Research Institute for Cattle Breeding, Rapotín, Czech Republic (samples from breeding farm in the Czech Republic).
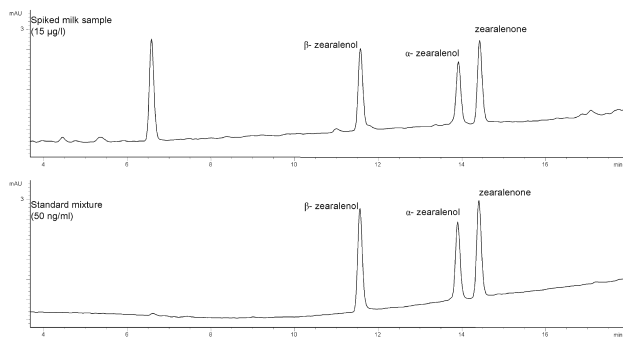

Fig. 2. **Chromatogram of the standard mixture and spiked sample**

Conclusions and follow-up studies

Although, due to the intensive multistep clean-up the new HPLC/DAD method presented in this paper is rather laborious, its wide use in many routine laboratories is enabled thanks to employing a common laboratory equipment. In the follow-up study tandem mass spectrometric detection (LC/MS/MS) will be used to improve performance characteristics (the dependence of analytes recovery on the fat content in original sample will be tested) and simplify clean-up step. As shown in preliminary experiments significant increase of detection selectivity hence very low detection limits can be obtained what makes possible a detailed investigation of zearalenone fate in human food chain.

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P29   THE SIMPLE METHOD FOR ANALYSIS OF FATTY ACIDS IN FOOD

BLANKA LOUPANCOVÁ[a], EVA VÍTOVÁ[a], JANA ZEMANOVÁ[a], FRANTIŠEK BUŇKA and MIROSLAV FIŠERA[a]

[a]*Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, loupancova@fch.vutbr.cz,* [b]*Faculty of Technology, University of Tomas Bata, T. G. Masaryka 588, 760 00 Zlín, Czech Republic*

Introduction

The purpose of this work was to develop simple, fast and cheap method for analysis of fatty acids in food.

Fatty acids, mainly unsaturated fatty acids, are important food components. Lipids serve as reserve and prompt source of energy. Palmitic and stearic acids have the greatest proportion in blood and are prime source of energy for cardiac muscle.

Humankind is, in contrast to plants, disable to synthesize unsaturated fatty acids, so they are essential for its organism[1].

Linoleic, α-linolenic, arachidic and arachidonic acids are mainly significant from nutrition perspective, because they are constituents of phospholipids and biological membranes. Relevance of phospholipids is in prevention cardiovascular and hepatic diseases and stimulation of nerve system[2].

Method and materials

We first had to choose the method for preparing of volatile esters of fatty acids usable for gas chromatography analysis. Several methods found in literature were examined and compared; they were applied to milk fat extracted from processed cheeses.

The second aim was to validate the esterification method chosen.

Extraction method

The cheese sample (5.0 g) was heated with 15 ml of hydrochloric acid in boiling water bath to dissolution and than 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 than allowed to separate the water and organic phases. The upper (organic) phase was displaced to dried weighed flask by the pipette. The water phase was next reextracted two times with 15 ml of and diethyl ether and 15 ml of petrol ether.

The rest of organic solvent was evaporated in 60 °C water bath (to dispose of smell). Flask with sample was dried for one hour at 105 °C[4].

Methanol esterification methods

I. The fat sample (5.0 g) was saponified with 50 ml of methanolic potassium hydroxide solution (c = 0.5 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 twice rinsed with 20 ml of water. 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].

II. Methanole (40 ml), methanolic potassium hydroxide solution (0.5 ml, c = 1 mol dm^{-3}) and a boiling aid were added to 4 g of the fat sample. After achieving of boiling point the sample became clear (it usually taken 5 to 10 minutes).

The flask was cooled and after transferring of the content into the separation funnel was rinsed with 20 ml of heptane. The funnel content was shaken with 40 ml of water and than allowed the two phases to separate. Esters were moved to heptane phase and water phase was shaken with 20 ml of heptane again. Both heptane portions were (twice) rinsed TWICE with 20 ml of water. 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].

General method using boron trifluoride

Approximately 350 mg of the fat sample, 6 ml of the methanolic sodium hydroxide solution (c = 0.5 mol dm^{-3}) and a boiling aid were introduced into the 50 ml distilling flask.

The mixture was boiled under reflux until the droplets of fat disappeared (this usually taken 5 to 10 minutes). Than 7 ml of methanolic boron trifluorid solution was added through the top of the condenser. Boiling continued for 3 minutes[6].

Approximately 3 ml of isooctane was added to the boiling mixture and the flask was removed from the heat source. Immediately, without allowing the flask to cool, 20 ml of sodium chloride solution was added and stopped flask was vigorously shaken at least 15 seconds.

Than more of saturated sodium chloride solution was added to bring the liquid level of the mixture into the neck of the flask and allowed the two phases to separate.

The upper isooctane layer (1–2 ml) was transferred into 4ml vial and small amount of anhydrous sodium sulphate was added to remove any traces of water. Sample was filled up to a 50 ml volumetric flask.

GC analysis

So prepared heptane or isooctane methyl esters solutions were injected to gas chromatograph using auto sampler.

GC conditions: gas chromatograph TRACE GC (ThermoQuest Italia S. p. A., I) equipped with flame ionization detector, split/splitless injector and capillary column SP^{TM} 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 and discussion

Results of each method are presented as chromatograms in Fig. 1. and 2. Tables I and II show determined fatty acids.

The second methanol esterification using potassium hydroxide catalysis was chosen for preparing of the fatty acids methyl esters. The method was verified and compared with normalized method. It is simple in respect to instrumentation and chemicals and can be used for analysis of fatty acids in various foods and could be applied to observe its contents in milk products.

Fig. 1. **Chromatogram of methyl esters prepared using the first methanol esterification method: 1. Caproic acid (13.9), 2. Caprylic acid (15.9), 3. Capric acid (17.9), 4. Undecanoic acid (18.7), 5. Lauric acid (19.7), 6. Tridecanoic acid (20.5), 7. Myristic acid (21.5), 8. Myristoleic acid, 9. Pentadecanoic acid (22.3), 10. Palmitic acid (23.4), 11. Palmitoleic acid (23.9), 12. Margaric acid (24.1), 13. Heptadecenoic acid (24.8), 14. Stearic acid (25.3), 15. Oleic acid (26.0), 16. Linoleic acid (27.0), 17. Linolenic acid (28.5)**
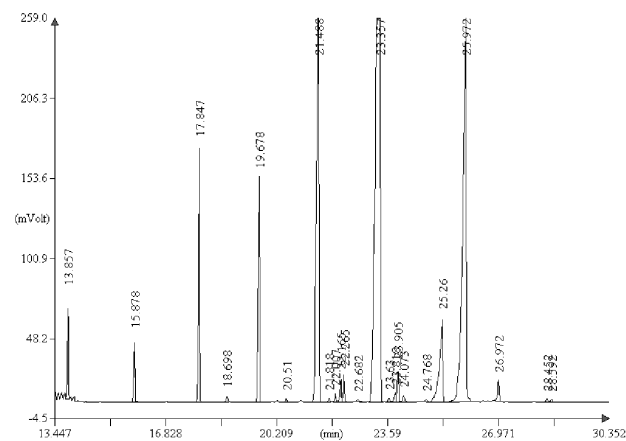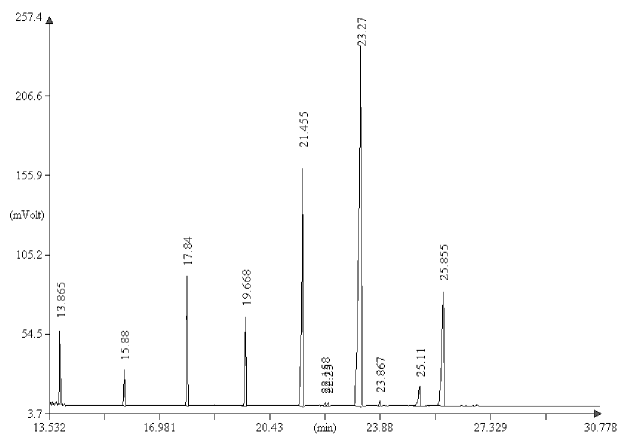
Fig. 2. **Chromatogram of methyl esters prepared using the second methanol esterification method: 1. Caproic acid (13.9), 2. Caprylic acid (15.9), 3. Capric acid (17.8), 4. Lauric acid (19.7), 5. Myristic acid (21.5), 6. Myristoleic acid (22.2), 7. Palmitic acid (23.3), 8. Palmitoleic acid (23.9), 9. Stearic acid (25.1), 10. Oleic acid (25.9)**

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P30 FERMENTATION OF CABBAGE JUICES BY TWO STRAINS OF LACTIC ACID BACTERIA

DRAHOMÍRA LUKÁČOVÁ, JOLANA KAROVIČOVÁ, ZLATICA KOHAJDOVÁ and MÁRIA GREIFOVÁ
Department of Food Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic,
drahomira.lukacova@stuba.sk

Introduction

The fundamental reason for the development and acceptance of fermented foods can be variably ascribed to preservation, improved nutrituonal properties, better flavour, aroma, upgrading of substrates to higher value products, and improved health aspects[3]. Inulin and oligofructose are functional food ingredients. Their incorporation in food improves organoleptic properties of food, improving both taste and mouthfeel in a wide range of food applications. They belong to non-digestible oligosacharides that are classified as prebiotics[1,4].

The objective of presented work was compared the lactic acid fermentation of cabbage juices inoculated by two various strains with addition of various powdered prebiotic preparations containig inulin and oligofructose.

Experimental part

Samples (prepared juices): cabbage juices fermented by *Lactobacillus plantarum* CCM 7039, cabbage juices fermented by *Lactobacillus plantarum* CCM 7039 with 2% addition of Raftilose Synergy 1, cabbage juices fermented by *Lactobacillus plantarum* CCM 7039 with 2% addition of Raftiline HP, cabbage juices fermented by *Lactobacillus plantarum* CCM 7039 with 2% addition of Raftiline GR, cabbage juices fermented by *Bifidobacterium longum* CCM 4990, cabbage juices fermented by *Bifidobacterium longum* CCM 4990 with 2% addition of Raftilose Synergy 1, cabbage juices fermented by *Bifidobacterium longum* CCM 4990 with 2% addition of Raftiline HP, cabbage juices fermented by *Bifidobacterium longum* CCM 4990 with 2% addition of Raftiline GR.

Preparation of vegetable juices. The juices were inoculated by *Lactobacillus plantarum* CCM 7039 (in concentration 10^6 CFU ml^{-1}) or *Bifidobacterium longum* CCM 4990 (in concentration 10^6 CFU ml^{-1}) and fermented during 16 h at temperature 21 °C and study changes in analytical and sensory properties in sample of juices.

Sensory evaluation. The samples were evaluated according to the analytical (pH, reducing sugars, total acidity, organic acids – lactic, acetic, citric, D-ascorbic) and sensory parameters (appearance, colour, sediment, odour, taste, acceptance of odour, taste and flavour) every 24 hours. For odour evaluation of cabbage juices, the following descriptors were chosen: sweet, acid, cabbage, sharp, spice, smell, sweet-acid. For taste evaluation, the following descriptors were chosen: sweet, acid, cabbage, salt, spice, bitter, sharp, harmonic, sweet-acid.

Analytical methods. The purpose of this work was use of capillary isotachophoresis for determination of organic acids (lactic, acetic, citric acid and L-ascorbic) in the lactic acid fermented cabbage juices by isotachophoretic analyzer (ZKI 01 Villa Labeco Spišská N. Ves, with conductivity detector and double line recorder TZ 4200) for identification and determination of organic acids using following electrolytic system: leading electrolyte – HCl in concentration $1\cdot10^{-2}$ mol l^{-1}, counter-ion 6-aminocapronic acid, pH 4.25, additive 0.1 % methylhydroxyethylcellulose, terminating electrolyte – capronic acid $5\cdot10^{-3}$ mol l^{-1}.

The samples were analysed at a driving current of 250 µA in the pre-separation column. Quantitative analysis was performed by calibration[2].

Conclusion

On the basis of analytical and sensory results we suggested addition of polysaccharide preparates containing more than 99.5 % of inulin into the cabbage juices inoculated with *Lactobacillus plantarum* CCM 7039. We recommended the stop of fermentation process of this juice in the 72th h of fermentation because in this hour the juice had the highest intensity of selected sensory parameters and juice proved adequately low pH value (3.65) for guarantee of preservative effects. We suggested that for lactic acid fermentation by *Bifidobacterium longum* CCM 4990 of to use of RAFTILOSE Synergy 1 preparation and to stop of fermentation process in the 72th h of fermentation because in this hour was juice the most acceptable for assessors (92 % from scale) and contained sufficient concentration of lactic acid (5.89 g l^{-1}) and had adequately low pH value.

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P31 THE APPLICATION OF FATTY ACIDS ON THE CHICKEN SKIN

EVA LUKÁŠKOVÁ, DANIELA KRAMÁŘOVÁ and LEONA ČECHOVÁ
Tomas Bata University in Zlin, Faculty of Technology, Department of Food Engineering, 275 TGM, 760 01 Zlin, Czech Republic, lukaskova@ft.utb.cz

Introduction

Several chemicals have been proposed as sanitizers for the creasing the level of microbial contamination of processed poultry and poultry processing water. In the presence of lactic acid, monolaurin was able to suppressed growth of *Staphylococcus* aureus on meat products, to increased sensitivity of *Bacillus cereus* spores in thermal inactivation and, in mixture with monocaprin, to inhibit growth of *Listeria monocytogenes*. Monocaprin also displayed excellent results against Gram-positive cocci by action consisted of disintegration of the cytoplasmic membrane. It was found, the growth of all tested Gram-positive bacteria, all yeasts and also all filamentous fungi excepting the species *Mucor racemosus* was inhibited[1,2].

The aim of this study was to assess susceptibility of bacteria contaminating poultry skin to caprylic acid (C8:0).

Methods

Chicken carcasses were stored at 4 °C for three days. Skin was inuncted by emulsion of caprylic acid (concentration of 2 % w/v) and then removed from carcasses every day, cut into 50 g pieces, placed in sterile bottle with glass balls and 100 ml of 0.1% peptone solution were added. Control sample was prepared in the same way, but skin was not inuncted by emulsion of caprylic acid. Aliquots of the rinsate were taken for microbiological analyses[3,4].

The standard plate count was performed using Plate Count Agar (PCA, HiMedia Laboratories) to enumerate obligately aerobic and facultatively aerobic bacteria; plates were incubated aerobically at 37 °C for 24 hours. Each experiment was repeated five times.

Results and discussion

The caprylic acid (concentration of 2 % w/v) produced significant reduction in the number of aerobic bacteria.

Table I

Effect of 2% concentration of caprylic acid on bacterial flora – sample 1

Day	Control sample [CFU/100 g]	2% C8 [CFU/100 g]
1	$2.7\cdot10^{0}$	$6.9\cdot10^{0}$
2	$5.2\cdot10^{2}$	$4.5\cdot10^{1}$
3	$3.6\cdot10^{2}$	$7.4\cdot10^{1}$

Coliforms, staphylococci and micrococci were highly susceptible to the bacterial activity of caprylic acid, while the populations of pseudomonas exhibited greater resistance. Coliform bacteria were more suspectible to caprylic acid than the others strains as 2% concentration was sufficient for their complete elimination.
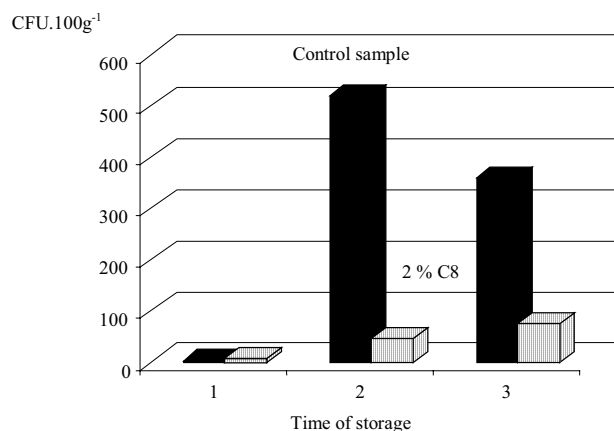
CFU.100g⁻¹

Fig. 1. **Effect of 2% concentration of caprylic acid on bacterial flora – sample 1**

Table II
Effect of 5% concentration of caprylic acid on bacterial flora – sample 2

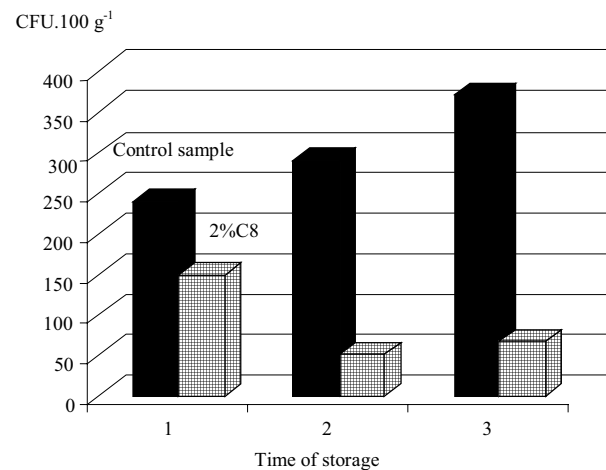| Day	Control sample [CFU/100 g]	2% C8 [CFU/100 g]
1	$2.4 \cdot 10^2$	$1.5 \cdot 10^2$
2	$2.9 \cdot 10^2$	$5.1 \cdot 10^1$
3	$3.7 \cdot 10^2$	$6.7 \cdot 10^1$

CFU.100 g⁻¹

Fig. 2. **Effect of 2% concentration of caprylic acid on bacterial flora – sample 2**

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P32 INFLUENCE OF POLYUNSATURATED FATTY ACIDS INTAKE ON LIPID METABOLISM IN PATIENTS WITH HYPERLIPIDAEMIA

SIMONA MACUCHOVÁ[a], IVANA MÁROVÁ[a] and ROSTISLAV KOTRLA[b]

[a]*Department of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00, Czech Republic, macuchova@fch.vutbr.cz*, [b]*Department of Klinical Biochemistry, Regional Hospital, Kyjov, Czech Republic*

Introduction

Cardiovascular disease has by far the highest prevalence of all diseases in affluent industrialized countries. Cardiovascular has also the highest mortality across Europe and in the USA.

Atherosclerosis is regarded to be a multifactorial disease. The main risk factor is an increasing age, other well established risk factors are high blood pressure, consumption of food rich in cholesterol or fat, inflammatory diseases, attack of microorganisms, mechanical injury, and smoking.

Atherosclerosis is characterized by deposition of cholesterol rich plaques in the endothelium. This observation stimulated research on the metabolism of cholesterol and revealed that cholesterol is transported in esterified form to cells by the low density lipoprotein (LDL). LDL is recognized by an endothelial cell receptor and introduced into the cell by endocytosis. There the esters are cleaved. The resulting free cholesterol is transferred to the cell walls. The process is strictly regulated.

In LDL of atherosclerotic patients LDL is altered by oxidation. This altered LDL is taken up in unlimited amounts by macrophages. Dead macrophages filled with cholesterol esters are finally deposited in arteries[1].

Polyunsaturated fatty acids (PUFAs) esterified to cholesterol or present as phospholipids represent the most oxygen sensitive compounds of all these LDL constituents.

Dietary polyunsaturated fatty acids (PUFA) have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, and neuronal development and visual function.

Ingestion of PUFA will lead to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoid synthesis, and signaling as well as the regulation of gene expression.

Cell specific lipid metabolism, as well as the expression of fatty acid-regulated transcription factors likely play an important role in determining how cells respond to changes in PUFA composition.

Chemically, PUFA belong to the class of simple lipids, as are fatty acids with two or more double bonds in cis position. There are two main families of PUFA: n-3 and n-6. These fatty acids family are not convertible and have very different biochemical roles. Dietary n-3 PUFA have several beneficial properties:
– act favorably on blood characteristics by reducing platelet aggregation and blood viscosity;
– are hypotriglyceridemic;
– exhibit antithrombotic and fibrinolytic activities;
– exhibit antiinflammatory action;
– reduce ischemia/reperfusion-induced cellular damage. This effect is apparently due to the incorporation of eicosapentaenoic acid in membrane phospholipids.

Linoleic acid (n-6) (LA) and alfa-linolenic acid (n-3) (LNA) are two of the main representative compounds, known as dietary essential fatty acids (EFA) because they prevent deficiency symptoms and cannot be synthesized by humans[2].

Clinical experiment

Influence of complex food supplement containing tocopherol as antioxidant component and polyunsaturatd fatty acids as hypolipidaemic component on antioxidant status and parameters of lipid metabolism in 30 patients with hyperlipidaemia was studied. Food supplement (180 mg of eicosapentaneic acid EPA, 120 mg of docosahexaneic acid DHA, 1.2 mg of vitamin E in 1 tbl.) was taken for 3 months, two tbl. daily; blood samples of each subject were taken in regular intervals.

Methods
Biochemical parameters

A set of biochemical parameters characterizing lipid metabolism was determined using automatically system HITACHI 717.

Determination of antioxidant activity

Total antioxidant status was determined using ABTS method (Randox Laboratories, USA). Serum AGE (Advanced glycation end products) were analysed fluorimetrically at 350 nm/440 nm (ref.[3]) Total amount of serum oxidation products „AOPP" was analysed spectrophotometrically according Witko-Sarsat *et al*. 1996, in Kalousová *et al*. 2001 modification[3].

HPLC analysis

Parameters of antioxidant status levels of serum carotenoids, tocopherol and retinol were measured using HPLC

method. For the chromatographic analysis The Biospher C18 4.6 mm × 150 mm, 7 μm column was used. Separation of carotenoids, retinol and tocopherol was carried out using methanol as the mobile phase and flow rate 1.1 ml min^{-1}. Content of *trans-all*-retinol was detected at 325 nm, α-tocopherol at 289 nm, carotenoids at 450 nm.

Results

Biochemical analysis have shown, that levels of lipid parameters decreased after 3 month intake of food supplement (see Fig. 1.). The level of total antioxidant status has increased, as well as the levels of some individual antioxidants above all α-tocopherol and *trans-all*-retinol. Simultaneously, the levels of serum AGE and AOPP decreased.

Our results indicated, that intake of food supplement containing PUFA and tocopherol can positively influence lipid metabolism and antioxidant status in patients with hyperlipidaemia.
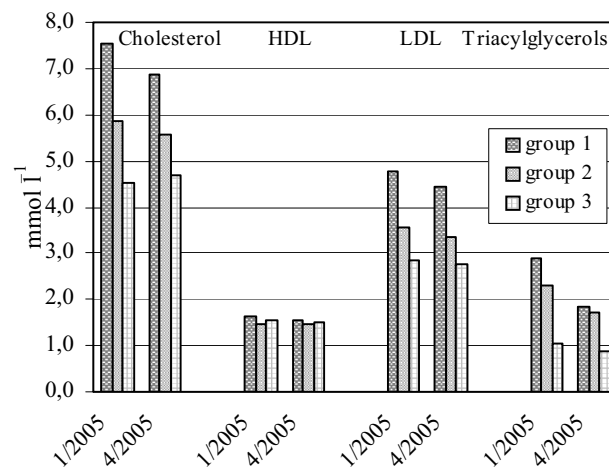

Fig. 1. **Levels of lipid parameters before and after intake of food supplement**
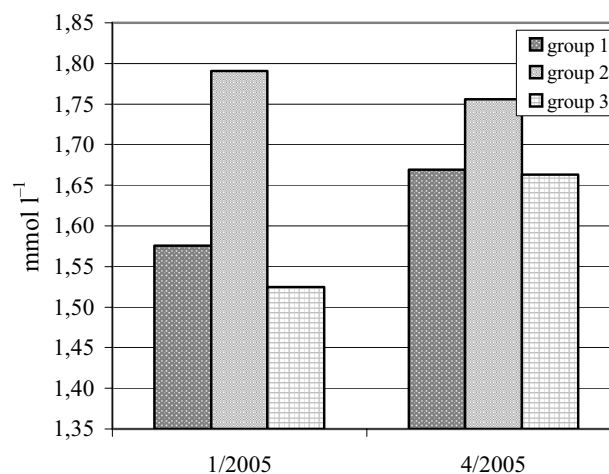

Fig. 2. **Levels of total antioxidant status before and after intake of food supplement**

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P33 ANTIOXIDANT AND ANTIMUTAGENIC PROPERTIES OF SEVERAL RED AND WHITE WINES: A COMPARATIVE STUDY

SIMONA MACUCHOVÁ, ANDREA MIKULCOVÁ, EDITA TIHLÁŘOVÁ, RADKA CHVÁTALOVÁ and IVANA MÁROVÁ

Department of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00, Czech Republic, macuchova@fch.vutbr.cz

Introduction

Free radicals are extremely harmful to living organism in that they attact different constituents of the cell, which leads to acceleration of the aging process and sometimes, even, cell destruction or, if the DNA is affected, irreversible malfuctions. Growing evidence of the role of free radicals and antioxidants in health and ageing has focused great interest on these compounds[1].

Flavonoids and phenolic compounds are widely present in higher plants and form part of the human diet. As well as plant phenolics, they have been reported to have multiple biological effects such as antioxidant or antimicrobial activities. Grapes and wine contain large amounts compounds, mostly flavonoids[2]. Phenolic compounds are responsible for some of the major organoleptic properties of wines, in particular colour and astringency. Wine phenolic composition depends on the grapes used to make wines the wine on the vinification conditions. The flavonoid content of red wine has been suggested as an explanation of the "French paradox" i. e. the fact that French people have low incidence of the coronary heart deseases, despite having a diet high in fat and being heavy smokers[1].

The aim of this work was comparison of content of selected antioxidants in 4 kinds of white and 6 kinds of red wines from region of South Moravia. Using HPLC-UV/VIS contents of *trans*-resveratrol, catechins, rutin, quercetin, morin, fisetin, ascorbic acid, luteine, α-tocopherol were analysed. The total antioxidant status of wines was determined using ABTS method. In the work contents of total polyphenols and total flavonoids were observed too.

Antimutagenic effect of some wines was observed using Saccharomyces cerevisiae D7 test.

Methods

H P L C a n a l y s i s

For the chromatographic analysis of catechin and catechingallate HPLC column Supelcosil™ LC 18 4.6 mm×250 mm, 5 µm was used. As the mobile phase MetOH: water (45:55) and flow rate 0.6 ml min⁻¹ was used. Detection of content of components was done at 280 nm. The content of ascorbic acid was analysed by Hypersil APS-2 4.6 mm×150 mm, 5 µm using mobile phase acetonitrile and 0,05 mol l⁻¹ CH3COONa (5:95), flow rate 0.6 ml min⁻¹ at 254 nm. The Biospher C18 4.6 mm×150 mm, 7 µm column was used for determination of content of rutin, quercetin, morin, fisetin and resveratrol. Separation of components was carried out using methanol, acetonitrile, water and ortho-phosphoric acid (20:30:49.5:0.5) as the mobile phase at flow rate 0.6 ml min⁻¹. Trans-resveratrol was detected at 306 nm, rutin, quercetin and morin at 370 nm, fisetin at 240 nm.

D e t e r m i n a t i o n o f a n t i o x i d a n t
a c t i v i t y

Total antioxidant status of analyzed wines was determined using ABTS method (Randox Laboratories, USA). Incubation of ABTS® with a peroxidase (metmyoglobin) results in production of the radical cation ABTS⁺®. This species is blue-green in colour, and can be detected at 600 nm. Antioxidants in the added sample cause inhibition of this colour production to a degree that is proportional to their concentration.

A n a l y s i s o f t o t a l p o l y p h e n o l s a n d
f l a v o n o i d s

For the determination of total polyphenols content was used photometric method using Folin-Ciocalteau reagent[3]. Gallic acid was used as a standard. Samples were measured at 750 nm. For the determination of total flavonoids photometric method using $NaNO_2$ and $AlCl_3$ was used[4]. Catechin was used as a standard. Samples were measured at 510 nm.

A n t i m u t a g e n i c i t y a s s a y

In the *Saccharomyces cerevisiae D7* test biological effects of red (Modrý portugal pozdní sběr, Svatovavřinecké) and white (Vetlínské zelené, Rulandské šedé) wines were observed as ability to inhibit the formation of mutant colonies (tryptophane-conversions, isoleucine-reversions) caused by affect of standard mutagen 4-N-nitroquinoline-N--oxide (4-NQNO). The suspension of *S. cerevisiae D7* in the logarithmic phase of growth in the liquid YPD medium were centrifugated (4500 rpm, 5 minutes, 20 °C) and cell sediment was washed twice by the Sörensen (phosphate) buffer pH 6.98. In the assay the influence of 0.3 ml red or white wines and 0.1 ml 0.06 mg ml⁻¹ 4-NQNO in the 10 ml of the yeast suspension in the Sörensen (phosphate) buffer pH 6.28 was evaluated.

The yeast suspension (0.1 ml of 10⁶ cells ml⁻¹) was inoculated in the selective medium without tryptophane. In the case of selective medium without isoleucine 0.1 ml of 10⁷ cells ml⁻¹ of yeast suspension was inoculated. The number of the tryptophane conversions was evaluated after 5 days while the colonies of isoleucine reversions were counted after 10 days of incubating of Petri dishes at 28 °C.

Results

HPLC analysis of active components

The amount of all tested antioxidants (carotenoids, flavonoids, catechins) was substantially highes in red wines when compared with white wines (see Fig. 1. and 2.).
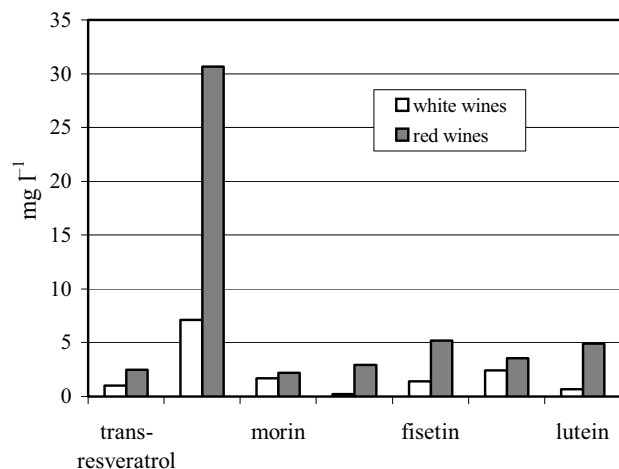

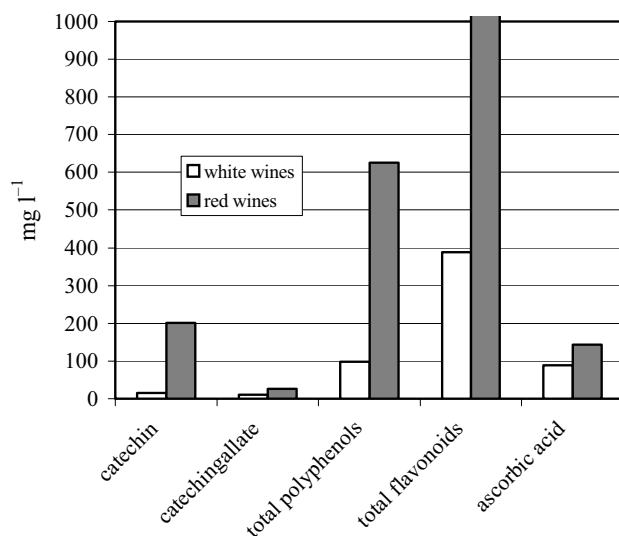Fig. 1. **Average levels of tested antioxidants in wines**

Fig. 2. **Average levels of tested antioxidants in wines**

Total antioxidant status

The antioxidant capacity of the wine samples was analysed in the automatic system HITACHI using Randox TAS kit. Red wine samples were diluted 1 : 15. In samples of red wines the antioxidant capacity was about ten times higher than in white wines.

The sample of pure 12% ethanol was measured in order to find out if the ethanol in wine influences the levels of antioxidant capacity. The measurement has shown, that ethanol doesn't cause inhibition of production of the radical cation, thus, no antioxidant capacity was observed.

Analysis of total polyphenols and flavonoids

The analysis exhibited, that red wines contain higher levels of total polyphenols and total flavonoids than white wines. Measured values were from 100 to 1000 mg per litre.

Antimutagenicity assay

In the *S. cerevisiae D7* test antimutagenic effect of red (Modrý portugal pozdní sběr, Svatovavřinecké) and white (Vetlínské zelené, Rulandské šedé) wines was evaluated. The results were obtained by comparision of the number of the yeast colonies in the Petri dish grown in the presence of the wine and the standard mutagen (4-NQNO) and the number of the yeast colonies in the Petri dish which were influenced by the standard mutagen only. The *S. cerevisiae D7* test showed that tested red wines and white wines were antimutagens.

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**P34   DETERMINATION OF TRACE ELEMENTS
          IN RUMEN FLUID**

MIROSLAVA MAHROVÁ[a], JIŘÍ PÁČ[a],
EMANUEL ŠUCMAN[b], RUDOLF DVOŘÁK[a],
MILADA VÁVROVÁ[b] and PETER KETTISCH[c]
[a]*Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Science Brno, Palackého 1–3, 612 42 Brno, m.mahrova@centrum.cz,* [b]*Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno,* [c]*Anton Paar, GmbH, Graz, Austria*

Introduction

The microflora of rumen ecosystem needs to be supplemented with various mineral matters including microelements[1]. The most important are essential microelements e.g. Mn, Cu, Zn, Mo, Co. On the other hand animals can also be exposed to effects of toxic trace elements like Cd, Pb, Hg etc. Therefore there is a continuous need for the development of new accurate, fast and reliable methods for determination of such trace elements. The aim of this work was to apply the method of differential pulse anodic stripping voltammetry (DPASV) for the determination of Cu, Zn, Cd, Pb. Principles

of this technique are described in[2,3]. For sample preparation the microwave assisted high pressure wet digestion technique was chosen[4].

Experimental part

Instrumentation

The microwave assisted digestion was performed using the Multiwave 3000 with both quartz (digestion step) and Teflon (evaporation step) vessels (Anton Paar, Austria).

The voltammetric measurements were performed at the hanging mercury drop electrode (HMDE) using 757 VA Computrace connected to Multi-Mode Electrode 6.1246.020 (Metrohm, Switzerland).

Reagents and solutions

Concentrated nitric acid (65%), hydrogen peroxide (30%), acetic acid (100%) and anhydrous sodium acetate were used. All reagents were of Suprapur grade (Merck, Germany).

Ultrapure water (specific conductance $<1\ \mu S\ m^{-1}$), NANOpure (Barnstead, USA) was used for all sample and/or standard solution preparations.

Standard solutions of Cu, Zn, Cd, Pb were prepared by diluting of the stock solutions Astasol ($1.000\pm0.005\ g\ l^{-1}$) (Analytika, Czech Republic).

Trace elements were analysed in 12 samples of rumen fluids. The Group 1–6 samples were obtained at the university clinic (University of Veterinary and Pharmaceutical Sciences Brno). The Group 7–12 samples were from the free naturally breeding at the university farm in Nový Jičín.

Microwave digestion

20 ml of rumen fluid were digested in the quartz vessels with 6 ml HNO_3 and 6 ml H_2O_2. The maximum temperature was 280 °C. The digestion program parameters are shown in the following Table I.

Table I
The digestion parameters

Step	Power [W]	Ramp [min]	Hold [min]	Fan
1	900	5	60	1
2	0		30	3

After the digestion step the solutions were transferred into Teflon vessels and evaporated to dryness (60 min, 900 W). The dry residues were dissolved in 40 μl of HNO_3 (6 mol l^{-1}) and 12 ml of water. Thus prepared solutions were ready for voltammetric determination. All operations with sample and/or standard solutions were performed under the laminar flow of cleaned air.

Voltammetric determination

5 ml of a digested sample and 12 ml of the acetate buffer (pH = 5.6) were taken for measurements.

Instrumental parameters for the DPASV: Potentials: Deposition: −1.2 V, Initial: −1.2 V, Final: 0.15 V; Scan rate: 20 mV s⁻¹; Deposition time: 180 s.

Results and discussion

The rumen fluid is not a common biological material for analysis. First of all it was necessary to optimize conditions of the digestion method in terms of HNO_3/H_2O_2 ratio, power and digestion temperature/time programme. Parameters of the digestion programme finely used are shown in Table I. For the determination of Cu, Zn, Cd, Pb the technique of DPASV was applied. The measurements were evaluated by the method of standard addition. En example of the voltammogram is shown in Fig. 1. and the evaluation by the technique of standard addition in Fig. 2.
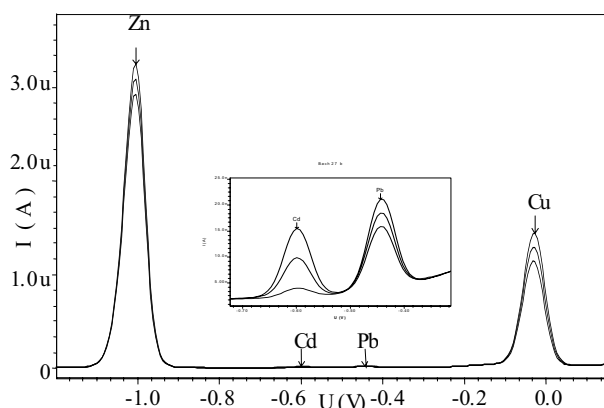
Fig. 1. **Determination of Zn, Cd, Pb and Cu in rumen fluid by DPASV. Curves represent sample and standard additions of Zn (30, 60 μg l⁻¹); Cd and Pb (1, 2 μg l⁻¹); Cu (10, 20 μg l⁻¹).**
(acetate buffer pH = 5.6; deposition time: 180 s; deposition potential: −1.2 V, scan rate 20 mV s⁻¹)

Regarding the fact that there is no standard reference material available for our kind of the biological material, the accuracy of the method was checked by two procedures.

Firstly known standard additions of each analyte were added to the digested samples before voltammetric step: 100 μg l⁻¹ Cu, Zn and 50 μg l⁻¹ Cd, Pb. The average recoveries with RSD (%) in this step were Cu 95.8 % (1.27 %); Zn 98.1 % (0.7 %); Cd 99.4 % (0.12 %) and Pb 104 % (1.64 %). Secondly known standard additions of each analyte were added to the samples before digestion step: 100 μg l⁻¹ Cu, Zn and 50 μg l⁻¹ Cd, Pb. The average recoveries with RSD (%) in this step were Cu 90.4 % (1.42 %); Zn 94.8 % (1.33 %); Cd 95.1 % (0.71 %) and Pb 110 % (0.71 %). In terms of these values one can conclude that the accuracy of the method is satisfactory and that the method is sensitive and reliable enough for the analysis of rumen fluid samples.

The method was then applied for the determination of trace elements in rumen fluids. Experimental results are compiled in Fig. 3.

(A)　　　　　　　　　　　　　　　　　　　　**(B)**
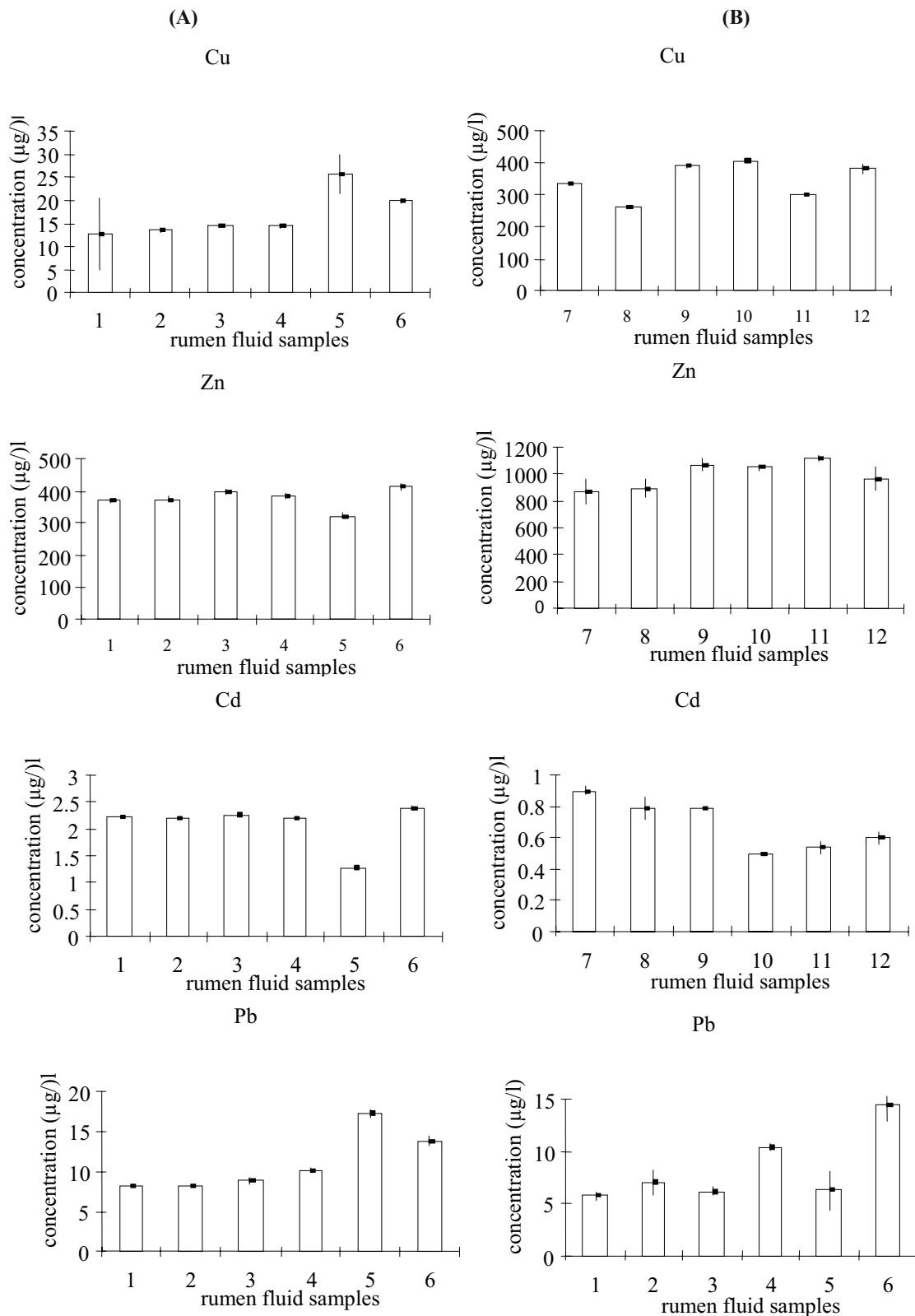
Fig. 3.　**Concentrations of Cu, Zn, Cd and Pb in rumen fluids of animals breed at the University of Veterinary and Pharmaceutical Sciences Brno** (A) **and at Nový Jičín farm in free naturally breeding** (B) **(with confidence interval 95%)**

Cu
c = 618.595 μg/L
+/- 23.999 μg/L (3.88%)

Zn
c = 1721.591 μg/L
+/- 164.855 μg/L (9.58%)

Cd
c = 0.998 μg/L
+/- 0.029 μg/L (2.86%)

Pb
c = 16.321 μg/L
+/- 0.188 μg/L (1.15%)
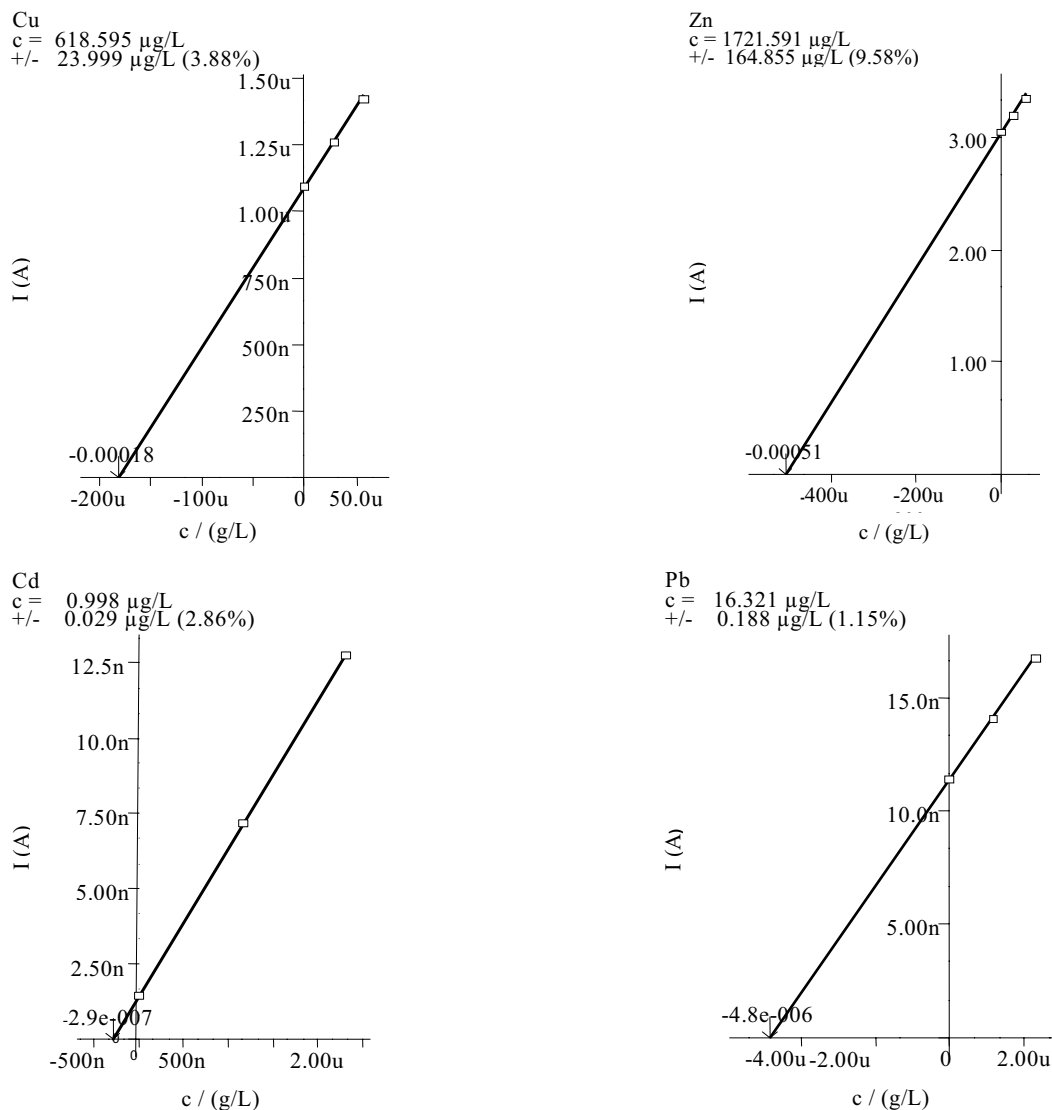
Fig. 2.   **Standard addition curves for evaluation of Cu, Zn, Cd, Pb concentration in rumen fluid concentrations (respectively)**

The average concentrations for particular trace elements and corresponding minimum and maximum values are as follows [μg l^{-1}]: Cu_{1-6} 16.8 (13.0 to 25.7) and Cu_{7-12} 346 (264 to 407); Zn_{1-6} 377 (322 to 412) and Zn_{7-12} 997 (874 to 1120); Cd_{1-6} 2.10 (2.20 to 2.40) and Cd_{7-12} 0.69 (0.50 to 0.79); Pb_{1-6} 11.2 (8.28 to 17.3) and Pb_{7-12} 8.38 (5.80 to 14.5). Generally are the concentrations of all elements found in samples 1–6 lower than in samples 7–12. Nevertheless within this pilot study in which the attention was payed to the development of the voltammetric measurement method it is not possible to make any conclusion regarding to explanation of the differences observed. For the interpretation of values analysis of more samples from different environments must be done taking into account complex data about the animals.

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P35 BIOTECHNOLOGICAL PRODUCTION OF CAROTENOIDS BY TRANSGENIC BACTERIA AND RED YEASTS

IVANA MÁROVÁ, JANA HRDLIČKOVÁ, RADKA KOČÍ, MICHAELA DRÁBKOVÁ, JITKA KUBEŠOVÁ, TEREZA VIDLÁKOVÁ and LIBOR BABÁK

Department of Food Chemistry and Biotechnology, Faculty of Chemistry, Technical University Brno, Purkyňova 118, 612 00 Brno, Czech Republic, marova@fch.vutbr.cz

Introduction

Carotenoids are isporenoid membrane-protective antioxidant pigments produced by plants, algae, bacteria and fungi. They belong to the most widespread natural pigments with many important biological activities and applications mainly in food and feed industry. It has been estimated that more than 100 million tons of a great variety of natural carotenoids are produced per year. Carotenoids are produced by specific branch of common isoprenoid biosynthetic pathway occuring in all types of organisms. To date, the commercial demand of carotenoids is mainly met by chemical synthesis and to minor extent by extraction from natural sources. Attention is now being focused on the natural production of carotenoids by microbial technology using yeast and/or bacteria. Factors that influence the efficiency of natural carotenoid biosynthesis, or that determine which carotenoids are accumulated, are therefore important for these commercial applications.

There are two major ways to influence the microbial production of carotenoids: i) by modification of cultivation conditions and ii) by construction of genetically modified overproducers. In this work these two possibilities are compared using carotenogenic yeasts *Rhodotorula and Sporidiobolus* and transgenic bacteria obtained by transformation of *E.coli* by crt genes from *Erwinia carotovora*.

Materials

Yeast strains *Rhodotorula glutinis* CCY 20-2-26, *Sporidiobolus salmonicolor* CCY 19-4-8 were studied. For isolation of crt genes bacteria *Erwinia carotovora* CCM1008 was used. As recipient cells *E.coli* DH5a were tested.

Methods

Yeast strains were cultivated on glucose medium[1] aerobically at 28 °C. Exogenous stress was induced by 2–5 % NaCl and 2–5 mM H_2O_2. As metabolic parameters levels of carotenoids – lycopene, alpha-carotene, beta-carotene, torulen, (RP-HPLC, 450 nm); phytoene (RP-HPLC, 280 nm), ergosterol (RP-HPLC, 280 nm) and glycerol (Boehringer kit) were analyzed.

Erwinia carotovora cells grown in NB medium under permanent lighting at 28 °C. Restriction cleavage of cDNA was performed for isolation of crt genes. Fragments were separated by PFGE and amplified by PCR. For transformation of chemically competent recipient *E.coli* DH5α cells vector pHSG298 was used. Selection of transformants was performed according to genotype as well as phenotype (yellow colonies). Cultivation of transformants for large-scale carotenoid production was carried out in laboratory fermentor Biostat B for 24–27 hours.

Results

All types of used exogenous stress factors led to increased production of beta-carotene and ergosterol according to growth phase or concentration of stress factor. Carotenoids are overproduced mainly after application of combined stress factors. This phenomenon acts as an adaptive mechanism and could be used also for potential biotechnological application. The yield of biomass and carotenoids in *R. glutinis* cultivated in laboratory fermentor (minimal medium; stress) was comparable to other industrial strains (about 6 mg l^{-1} of carotenoids, 36 g l^{-1} of biomass, see Table II).

Transformation of *E.coli DH5a* by crt genes from *E. carotovora* was succesful; yellow colonies were selected and transferred into laboratory fermentor. The yield of individual carotenoids (lutein, beta-carotene, lycopene) obtained from various transformants was substantially higher than in natural producer and/or stressed cells (see Table I).

Table I

Comparison of carotenoid production in *E.carotovora* (natural strain and stressed cells) and in *E.coli* transformed by crt genes.

	Lutein/zeaxanthin [mg g^{-1} of d.w.]	Lycopene [mg g^{-1} of d.w.]	Beta-carotene [mg g^{-1} of d.w.]
Erwinia carotovora: natural production	1.2	0.2	1.4
Erwinia carotovora stressed by 5 mmol l^{-1} of hydrogen peroxide	5.3	0.4	2.9
E. coli/crt genes from *E. carotovora*	127.7	30.6	88.2

Table II

The yield of industrially significant metabolites (in mg l^{-1} of medium) produced by *Rhodotorula glutinis* grown under exogenous stress in mg l^{-1} of medium

Stress factor	Beta-carotene	Ergosterol [mg l^{-1}]	Total carotenoids
control	1.27	8.9	1.76
2% NaCl/inoculum II	4.60	22.3	5.60
2 mM H_2O_2/inoculum II	4.69	22.2	5.93
H_2O_2 + 5 μM H_2O_2	0.27	1.56	0.32
NaCl + 5 mM H_2O_2	2.12	34.78	2.69

Discussion

While transgenic bacteria may be engineered above all for overproduction of individual carotenoids, cultivation of yeasts under stress could be suitable for large scale production of the group of carotenoids and/or enriched biomass.

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P36 DETERMINATION OF PHYTOESTROGENS IN BEER AND BREWING MATERIALS

RENATA MIKULÍKOVÁ[a], ALEXANDR MYKIŠKA[a] and ONDŘEJ KOSÍK[b]

[a]*Research Institute of Brewing and Malting PLC, Lípová 15, 120 44 Praha, Czech Republic: mikulikova@brno.beerresearch.cz,* [b]*Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, University of Technology Brno, Purkyňova 118, 612 00 Brno, Czech Republic*

Introduction

Phytoestrogenic compounds are secondary plant metabolites which have estrogen-like properties and have been associated with lesser incidence of steroid-hormone dependent cancers, eg.those of the breast, prostate and colon. They can affect more or less favourably the human organism. For these reasons it is necessary to know their content in foodstuffs and control their consumption[1].

Phytoestrogens are phenolic substances imitating the structure of natural mammalian estrogens and exhibiting weak estrogen activity. They are found not only in plants but also in products made from these plants. Up to date are 30 known estrogenic compounds, spread in almost 300 plant species. Phytoestrogens and their metabolites have been found also in human and animal biological fluids. They were also detected in beer[2].

The detected phytoestrogens in beer belong to the subgroup called isoflavones and namely are this Daidzein, Genistein, Formononethin and Biochanin A. Isoflavones are present as glucoside conjugates (7-β-glucosides). What more, glucoside units are often esterified with acetylic or malonyl group and they build acetyl- and malonylglucosides. Except this glucosides, also free isoflavones in much lower concentrations are present[3].

For determination of total phytoestrogens content the enzymatic hydrolysis, using β-glucuronidase (EC.3.2.1.31) from *H. pomatia* in mixture with sulfatase is needed. The GC-MSD method was used for determination of phytoestrogens (Daidzein, Genistein, Formononetin, and Biochanin A)[4, 5].

Experimental

The phytoestrogens from the solid samples were extracted from the biological matrices using extraction mixtures of organic solvents and sonification (30 min). The extracted samples or the liquid samples were then centrifugated. To the upper, aqueous part, β-glucuronidase was added and samples were incubated at 37 °C over night.

After the incubation the hydrolysed samples were purified using the SPE (solid phase extraction) method. C18 columns were used and the samples were rinsed once, due to eliminate the impurifications. Purified samples were evaporated to dryness under nitrogenous atmosphere.

All phytoestrogens precursors as their exit in the plant and their metabolites in biological matrices contain polar hydroxyl and/or carboxyl groups. Derivatization is therefore needed to increase the volatility of each analyte prior to their GC-MSD analyse. The common derivatization reagents include N,O-bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA-TMCS) and produce the derivatives of trimethylsilyl ether (TMS). The derivatization was done at 60 °C for 60 minutes.

The hydrolysed, purified and derivatized samples were injected into the GC-MSD apparatus. The gas chromatograph (Trace GC Ultra Thermo Finnigan) connected to the mass detector (Trace DSQ Thermo Finnigan) was used to assess the phytoestrogen content. The separations were performed using a capillary colum DB5–MS (30 m × 0.25 mm i. d., 0.25 μm film thickness) with an oven temperature program of 160 °C (1 min), 7 °C min⁻¹ to 300 °C (5 min). Transfer line temperature was 300 °C. The temperature of the injector was 280 °C – Splitless mode (1 min). Mass detector conditions were: SIM mode (EI+). Biochanin A–413, 414, 415, 416 m/z, Daidzein – 398, 399, 400, 401 m/z, Formononethin – 339, 340, 341, 342 m/z, Genistein – 471, 472, 473, 474 m/z. The carrier gas was helium with a flow 1.5 ml min⁻¹. Detection limits for analysed substances are the following ones: for Biochanin A is 0.7, for Daidzein is 0.5, for Formononethin and Genistein 0.3 μg kg⁻¹, μg l⁻¹, respectively.

Results

The concentration ranges of 4 analyze phytoestrogens for hop, malt, unhopped wort, hopped wort and beer are summarised in following Table I.

Table I
Contents of phytoestrogens in raw material, intermediate product and beer determined by GC-MS

	Biochanin A [μg kg^{-1}]	Daidzein [μg kg^{-1}]	Formononethin [μg kg^{-1}]	Genistein [μg kg^{-1}]
hop	0.7–1064.4	0.5–51.5	0.3–854.8	0.3–367.3
malt	0.7–76.5	0.5–0.5	0.3–0.4	0.3–0.7
	Biochanin A [μg kg^{-1}]	Daidzein [μg kg^{-1}]	Formononethin [μg kg^{-1}]	Genistein [μg kg^{-1}]
unhopped wort	0.7–1.0	0.5–0.7	0.3–4.0	0.3–2.6
hopped wort	0.7–1.6	0.5–2.2	0.3–5.6	0.3–5.6
beer	0.7–0.8	0.5–2.0	0.3–2.3	0.3–3.1

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P37 DETERMINATION OF MARKERS FOR DISTINGUISHING FRUIT DISTILLATES

RENATA MIKULÍKOVÁ[a], RENATA WINTEROVÁ[b] and DANA FLODROVÁ[c]

[a]*Research Institute of Brewing and Malting PLC, Lípová 15, 120 44 Praha, Czech Republic,
mikulikova@brno.beerresearch.cz,* [b]*Food Research Institute Prague, Radiová 7, 102 31 Praha,
renata.winterova@vupp.cz,* [c]*Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, University of Technology Brno, Purkyňova 118, 612 00 Brno, Czech Republic*

Introduction

Adulteration of single-species fruit distillates has been an actual problem in recent years. Purposeful adulteration based mainly on the use of a cheaper kind of fruit as a partial substitute of a more expensive one and use of some other than the declared spirit leads not only to deceiving a consumer but it also has an economic impact in the area of customs and tax charges. In this connection, protection of "honest" producers from unfair competition is important too. According to the requirements of the current CR laws fruit distillate is only that spirit whose ethanol comes purely from fruit and which meets the principal legal requirements (content of volatile substances, methanol content and hydrogen cyanide in case

of distillates from stone fruit). However, these requirements themselves are not sufficient for distinguishing the origin of the fruit used.

The aim of our study was to identify in samples of the same type of fruit distillates flavour substances that might be specific for particular kinds of fruit[1].

To concentrate trace flavor substances that could characterize individual fruit distillates in a more complex way, micro-extraction on solid phase (SPME- Solid Phase Micro Extraction) was used[2,3].

Gas chromatography with mass detection was used for identification of particular components extracted[4,5].

Altogether 8 types of fruit distillates were analysed with this method (peach brandy, pear brandy, apple brandy, apricot brandy, slivovitz, cherry brandy, wine brandy, sour-cherry brandy). Distillates were from three producers located in different areas of the Czech Republic.

We identified 30 analytes that might potentially serve to characterize individual types of fruit distillates. Substances were determined that occur in most types of distillates and substances that might be identified as specific for the particular types of distillates. Flavour substances were determined using NIST library of spectra. Benzaldehyde and selected ethyl esters of fatty acids were quantified using standards.

Methods

Samples of fruit distillates were diluted to the same concentration of 40% v/v, filled into 4ml vials (added 100 μl internal standard [istd.] – heptane acid) and hermetically closed. The prepared samples were tempered at 25 °C for 15 min. We used SPME (Solid Phase Micro Extraction) for isolation of trace flavour substances. For DI-SPME the 75 μm CAR^{TM}/PDMS fiber was chosen. 4 ml 40% v/v fruit distillate and 100 μl of istd. was extracted at 25 °C for 15 min. After that desorption in PTV injector at 280 °C for 3 min (splitless) was done.

Standards of ethyl esters of fatty acid, ethyl ester of caprylic acid, ethyl ester of palmitic acid, ethyl ester of myristic acid, and benzaldehyde were diluted with 40% v/v of ethanol to concentration 1 mg l^{-1} and used for quantification.

The gas chromatograph was used to assess the content of flavour substances in fruit distillates (Trace GC Ultra Thermo Finnigan), it was connected to the mass detector (Trace DSQ Thermo Finnigan). The separations vere performed using a capillary colum DB – WAX (30 m × 0.25 mm i. d., 0.25 μm film thickness) with an oven temperature program of 55 °C (3 min), 10 °C min^{-1} to 150 °C (5 min), 10 °C min^{-1} to 200 °C (1 min). Transfer line temperature was 280 °C. The temperature of the injector (PTV) was 280 °C – Splitless mode (3 min). Mass detector conditions were: full-scan mode 30 00–40 000 m/z (EI+). The carrier gas was helium at 2.0 ml min^{-1}.

Results

Occurrence of the followed flavour substances ethyl ester of caprylic acid, ethyl ester of palmitic acid, ethyl ester of

myristic acid, and benzaldehyde analyzed in individual types of fruit brandies shows Fig. 1.
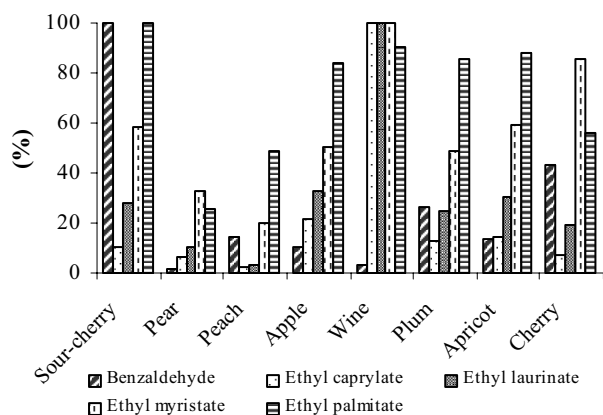

Fig. 1. **Comparison of analytes in individual types of fruit distillates**

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P38   PRODUCTION OF GLUCONIC ACID USING LIVING CELLS AND ENZYMES ENTRAPPED IN LENTIKATS®

ZUZANA MLICHOVÁ, MICHAL ROSENBERG and ĽUDMILA KRIŠTOFÍKOVÁ

Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic, zuzana.mlichova@stuba.sk

Introduction

Gluconic acid and its salts are important materials widely used in pharmaceutical, food, feed, detergent, textile, leather, photographic, and other biological industries[1]. The future of a majority of these applications depends mainly on the commercial availability of gluconates. There are different approaches available for the production of gluconic acid: chemical, electrochemical, biochemical, and bio-electrochemical processes. Fermentation is one of the dominant routes for manufacturing gluconic acid at present[2]. Glucose oxidase (GOD, β-D glucose: oxygen 1-oxidoreductase, EC 1.1.3.4.) with molecular oxygen catalyzes conversion of β-D-glucose into δ-D-gluconolactone, which is subsequently hydrolyzed to gluconic acid (Fig. 1.).

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOD}} H_2O_2 + \delta\text{-D-gluconolactone}$$

$$\underset{\text{CATALASE}}{\downarrow} \qquad \underset{H_2O}{\downarrow}$$
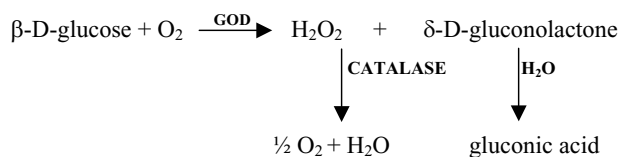
$$\tfrac{1}{2}\,O_2 + H_2O \qquad \text{gluconic acid}$$

Fig. 1.   **Conversion of β-D glucose by glucose oxidase**

This enzyme was described in 1928 by Muller in cultures *A. niger* and later detected in diverse sources (insects, honey, different fungi), and now is obtained on an industrial basic from *Penicillum amagasakiense* and especially *Aspergillus niger*, although a high level of production has recently been obtained from *Penicillum variabile*[3].

Gluconic acid fermentation by cells *A. niger* belongs to aerobic fermentations with high oxygen demand. The properties of *A. niger* allow gluconic acid production under both growth and non-growth conditions[2]. It is known, that glucose oxidase of *A. niger* is required for gluconic acid production either as whole cells or an enzyme. Nowadays, there is a great interest in using immobilized systems because of multiple, repetitive use and the operational stability of imobilizates. As reported recently, entrapment of enzymes in polyvinylalcohol (PVA) hydrogel is widely useful and effective. This matrix for immobilization combine several advantages like a gentle encapsulation method in an elastic, non-toxic and stable polyvinyl alcohol matrix and superior mass transfer properties due to their reduced thickness, easily separation by sieves[4,5,6].

The aim of this work was to consider the possibility to immobilized A. niger and GOD into PVA lens-shaped particulars, called LentiKats® in order to production of gluconic acid and to check the efficiency and long-term stability of these system.

Materials and methods

The microorganism *Aspergillus niger* CCM 8004 (Czech Collection of Microorganism) and commercial enzyme glucose oxidase – catalase preparation (isolated from *A. niger*) from Novozymes a. s. were used in this work.

The conversion of glucose to gluconic acid by immobilized biocatalyst was studied in gently stirred batch bioreactor, at 28–30 °C and pH 5.5, on glucose medium. Oxygen was supplied by hydrogen peroxide addition (immobilized cells) or air supply (immobilized enzyme). The concentration of gluconate was determined by an isotachophoretic method. Catalase activity was monitored by using a method based on disappearence of hydrogen peroxide which can be measured spectrophotometrically at 240 nm.

Preparation of LentiKats®: The biocatalyst was added to LentiKats® liquid, a polyvinylalcohol – containing aqueous solution and mixed thoroughly. Lens-shaped gels were obtained after dropping the polymeric suspension on a plate using a LentiKats® printer. Gelation and hardening of these droplets was initiated by partial controlled drying and was completed after approx. 30 minutes.

Results and discussion

In this work two different types of gluconic acid production systems during repeated batch bioconversions have been tested using LentiKats® as above mentioned attractive method for immobilization.

In the first system the immobilized spores of *Aspergillus niger* were used for fermentation. The experiment was carried out in the presence of 10 or 15% glucose at 28 °C. Due to the high catalase activity of *A. niger*, the oxygen concentration was regulated by the addition of 30 % hydrogen peroxide (the O_2 saturation remained constant 40 %). The use of hydrogen peroxide as described[7] indicates the possibility to avoid the problematic oxygen saturation in fermentation broth during gluconic acid production. On the other hand, presence of hydrogen peroxide brings about inactivation of glucose oxidase. After 14 bioconversions the specific production rate of gluconic acid decreased from 0.098 to 0.037 g_{GA} h^{-1} g_{Lent}^{-1} (gram of produced gluconic acid per hour in 1 g of LentiKats®, Fig. 2.). A. niger immobilized in polyvinylalcohol gel had high stability and good production rate in these extreme conditions (high hydrogen peroxide concentration).
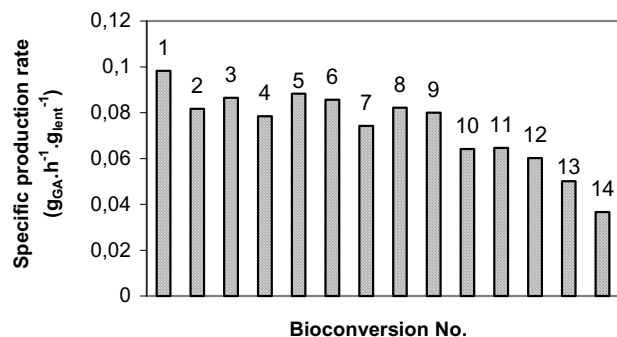

Fig. 2. **Specific production rate of gluconic acid by *A. niger* immobilized in LentiKats®, using hydrogen peroxide (30%) as an oxygen source.**
***Fermentation conditions*: pH 5.5, temperature 28°C, substrate: glucose 100 g l⁻¹ (conversions No 1–7), 150 g l⁻¹ (conversions No 8–14), 2.5 l of media, 141 g of LentiKats® with immobilized cells**

Unfortunately, because of ineligible contamination of products the use of living cells in food industry is limited. This problem can be overcome by the use of immobilized glucose oxidase. Because of increasing hydrogen peroxide concentration during the catalysis, glucose oxidase has to be coimmobilized with catalase, which is able to utilize it. Long-term experiments of coimmobilized enzymes in LentiKats® were performed in aerated batch bioreactor using a real substrate (glucose 10 g l⁻¹). The substrate conversions were maintained between 90–100%. After 10 repeated conversions, the immobilized enzyme retained about 56% of their initial activity. The decrease in relative enzyme activity can be caused by both deactivation of enzyme with hydrogen peroxide, or washing out of enzymes from immobilizates. Use of cross-linking reagent should be successive solution of

enzyme stability within LentiKats®, which will be investigated in the future studies. It is evident, that the biosynthesis is strongly dependent on the experiment conditions as well as the method of immobilization.

The results show that it is possible to produce satisfactory quantities of gluconic acid by both types of immobilizates. It allows to considerable interest in the possible application of the immobilized systems in industrial scale.
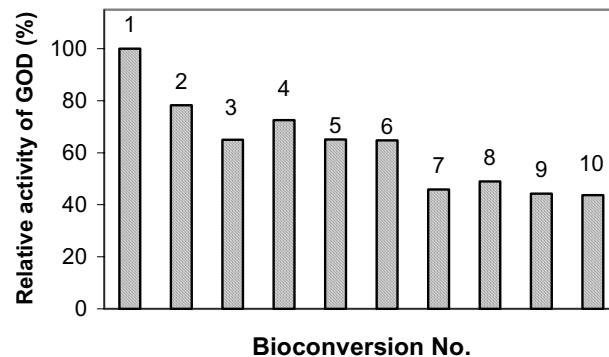
Fig. 3. **Relative activity of GOD immobilized in LentiKats® during the repeated batch conversions**
Experiment conditions: pH 5.5, temperature 30°C, aerated stirred reactor, substrate: dextran with (glucose concentration 10 g l⁻¹), 120 ml of media, 12 g of LentiKats® with immobilized GOD

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P39 STUDY OF THE DEGRADATION OF SOME BIOPOLYMERS WITH THE GLYCANASES

JIŘINA OMELKOVÁ[a], IVAN ŠIMKOVIC[b] and EVA STRATILOVÁ[b]

aFaculty of Chemistry, Brno University of Technology, Purkyňova 118, CZ-612 00 Brno, Czech Republic,b Institute of Chemistry of SAS, Dúbravská cesta 9, SK-845 38 Bratislava, Slovakia

Introduction

Cell wall of plant netting is composed of compact extra-cellular complex of biopolymers, in particular of cellulose, hemicellulose, pectin and proteins. Polygalacturonase participates on the degradation of pectic substances and is considered to be very important virulent factor of fungi. Natural substrate of pectolytic enzymes is pectin and pectic acid. Within this paper we deal with the study of the influence of pectic acid modification on its biodegradation. Consecutively, we compared the mode of polygalacturonase action on modified pectic acid and soluble form of pectic acid.

Materials and Methods

Pectic acid was applied either in soluble form on in modified insoluble version, prepared by network using 1,3-bis-(3-chloro-2-hydroxypropyl)imidazole hydrogen sulfate (BCHIHS) in the presence of NaOH and H_2O. Commercial pectolytic preparation Rohament P (Röhm) with polygalacturonase as major enzyme (produced by *Aspergillus species*) was employed.

The enzymatic degradation of water-insoluble and water-soluble polymers was investigated by analysis of the amount of reducing groups. The results obtained by this method were used to determine the kinetic constants. Degradation products were determined using TLC.

Results and discussion

The dependence of amount of reducing groups (absorbance at 530 nm) on the content of nitrogen in biopolymer (in aliquots taken in 30 min. interval) is shown in Fig. 1. From Fig. 1. follows that all biopolymers were degradated by enzyme. The highest degree degradation was found out by biopolymer with nitrogen content 7.33 %, while the lowest extent of degradation was observed by polymer with nitrogen content 1.7 %.

Based on specific activity of polygalacturonase with corresponding substrates it can be concluded that the lowest rate of degradation occured by polymer with nitrogen content 1.7 %. The most rapid degradation proceeded by polymer with nitrogen content 7.33 %.

The experimental results are summarized in Table I.

Table I
The specific activity of polygalacturonase

Nitrogen content in polymer [%]	Specific activity [μmol min^{-1} mg^{-1}]
1.70	0.004
6.18	0.024
6.45	0.028
6.73	0.036
7.33	0.042
7.35	0.035
7.52	0.031
7.59	0.025
7.79	0.024
8.44	0.024
8.48	0.020

The obtained kinetic parameters for both soluble and insoluble form do not significantly differ. By selected forms of modiffed pectic acids the mode of action of polygalacturonase was studied. It was confirmed that the main product was D-galactopyranuronic acid.

By soluble form of biopolymer the degradation prevailably leads to the products with higher degree of polymerization Fig. 2.

soluble form   insoluble form

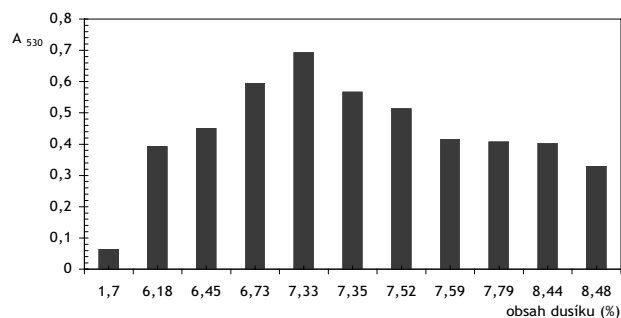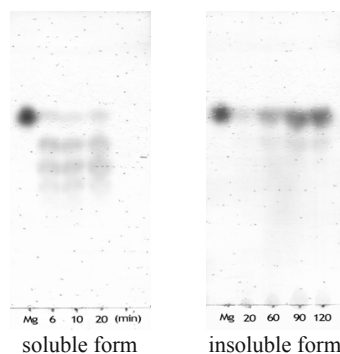Fig. 2. **Degradation products determined by TLC: soluble form (left), insoluble form (right)**

Fig. 1. **The dependence of amount of reducing groups (absorbance at 530 nm) on the content of nitrogen in biopolymer**

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P40 COMPLEX CHARACTERIZATION OF NATURAL FRUIT JUICES

PETR PTÁČEK[a], RADKA CHVÁTALOVÁ[a],
SIMONA MACUCHOVÁ[a], MILOSLAV PEKAŘ[b]
and IVANA MÁROVÁ[a]

[a]Department of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00, Czech Republic, mikulcova@fch.vutbr.cz, [b]Department of Physical and Applied Chemistry, Brno University of Technology, Purkyňova 118, 612 00, Czech Republic, kucerik@fch.vutbr.cz

Introduction

About 400 years B. C. Hippocrates wrote: „Let food be your medicine and medicine be your food". In recent years numerous studies indicate the beneficial effects of many plant foods on human health and protective effect against progression of many serious diseases, as cardiovascular injure, atherosclerosis, diabetes, cancer etc. As the most important acive components in plant foods vitamins (e. g. vitamin C, E), provitamins (e. g. carotenoids) ad other substances (e. g. polyphenols, flavonoids etc.) with antioxidant effect are described.

In Czech Republic, strong seasonal differences in plant food intake were observed depending on availability of local natural sources, especially fruits and vegetables. Thus, in winter-spring season increased intake of processed foods (e. g. fruit juices) and food supplements could be recommended.

In this work, analysis of antioxidant composition and total antioxidant status in several kinds of common natural juices was done. Further, some model mixtures of fruit juices were done and their antioxidant, antimutagenic and rheological characteristics were measured. Both types of fruit juices were analyzed using simple sensory tests.

Methods

Extraction: The 10 g of plant materials (carrot, tomato, paprika, spinach) were soaked in acetone, than were extracted with petroleum ether. Extracts were evaporated to dryness and samples were dissolved in 2 ml of DMSO. Extracts were sterilized by filtration through two Milipore filters (0.45 and 0.22 mm).

HPLC analysis of antioxidant content: Content of antioxidants in plant extracts was analyzed using RP-HPLC method. Separation of flavonoids was performed with water: phosphoric acid (99:1) as mobile phase, identification was proved at 259 nm using external standards: rutin, morin quercetin. For analysis of catechins the Nucleosil C18 column at isocratic elution by 30% MeOH/H_2O with 0.05 % CF_3COOH as mobile phase were used. HPLC analysis of carotenoids was performed with Waters RP C18 column (5 µm, 4.6×250 mm) and methanol as mobile phase. Detection and quantification were proved using external β-carotene, lutein and lycopene standards at 450 nm. Ascorbic acid was analysed using Hypersil APS-2 column (7 µm, 4.6×150 mm). All

results were evaluated and calculated using software CSW 1.7. (DataApex, CZ).

The antioxidant status analysis: Antioxidant capacity of plant extracts was analyzed using the Total radical-trapping antioxidant parameter (TRAP) and compared with ABTS test (TAS – Total Antioxidant Status, Randox kit).

Analysis of antimutagenicity: Yeast strain Saccharomyces cerevisiae D7 was used a simple eukaryotic system to test antimutagenic/genotoxic effects of fruits.

Rheological parameters: Rheometr HAAKE RS 100 was used for viscosity measurements.

Sensory analysis: A total of 30 subjects were enrolled in a simple sensory study. Basic sensory parameters (e. g. colour, acidity, flavour etc.) of mixed juices were evaluated.

Results and discussion

Analyzed fruit extracts exhibited high content of individual antioxidants according to kind of fruit. The highest amount of ascorbate and flavonoids was found in berries, grapefruit and orange, high content of carotenoids was detected in carrot, orange and pineapple. Most of fruit extracts exhibited positive (20–40 % of inhibition) or high positive (more than 40 %) antimutagenic activity in *Saccharomyces cerevisiae* D7 test. No correlation between total antioxidant status of plant extracts and their antimutagenic activity was found, although foods with high antioxidant potential obviously act as positive antimutagens.

Mixed natural juices with middle values of viscosity were the best sources of antioxidants and, simultaneouly, these juices exhibites the best sensory evaluation (e. g. mixtures F, C and G). According to antioxidant content, an optimal supplement should contain a complex mixture of naturally occuring antioxidants to ensure adequate ratio for synergistic biological effect of whole group of antioxidants.

Table I
Composition of mixtures of natural juices

Mixture	Composition	Ratio of individual components
A	Orange, grapefruit, pineapple	2:1:1
B	Orange, apple, raspberry, blackberry	1:2:0.5:0.5
C	Apple, carrot, raspberry, blackberry	2:1:0.5:0.5
D	Apple, pineapple, carrot	2:1:1
E	Orange, apple, pineapple, raspberry, blackberry	2:2:1:0.5:0.5
F	Orange, apple, carrot, raspberry, blackberry	2:2:1:0.5:0.5
G	Apple, grapefruit, pineapple, orange	2:2:1:1
H	Apple, orange, grapefruit, pineapple, carrot, raspberry, blackberry	2:2:1:1:1:0.5:0.5

Table II
Antioxidant activity of selected fruit extracts using TAS and TRAP method

Extract	TAS [mmol l^{-1}]	TRAP [mmol l^{-1}]
Orange	0.54 ± 0.121	2632.0 ± 86.27
Grapefruit	1.29 ± 0.231*	4524.7 ± 243.86
Apple	0.70 ± 0.116	864.0 ± 62.00
Berries	1.32 ± 0.145	3859.3 ± 316.29
Carrot	0.42 ± 0.997	976.3 ± 50.93
Pineapple	1.17 ± 0.298	5287.0 ± 268.57

Table III
Viscosity of mixed juices

Mixture	ç$_{ap}$ [Pa s] viscosity
C	0.0948
B	0.0753
F	0.0697
E	0.0339
H	0.0321
D	0.0295
A	0.0215
G	0.0168

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P41 IMMOBILIZATION OF GLUCOAMYLASE INTO LENTIKATS®

MARTIN REBROŠ, MICHAL ROSENBERG,
ĽUDMILA KRIŠTOFÍKOVÁ and ZUZANA MLICHOVÁ
Department of Biotechnology, FChPT-STU, Radlinského 9, 812 37 Bratislava, Slovakia; martin.rebros@stuba.sk

Introduction

Glucoamylase is an exoenzyme which hydrolyze dextrin and release single glucose molecules by hydrolyzing successive α-1,4- and α-1,6- linkages, beginning at the non-reducing end of dextrin chain. In the fermentation technologies this enzyme is used for saccharification of starch after cooking and liquefaction step[1]. Approximately 1 l of glucoamylase is used per 1 t of starch in industrial scale processes. The enzyme is not recycled, which overcharge the process of starch substrate pretreatment. The suitable immobilization of enzymes, which allows their multiplicative use, can significantly reduce the cost of the substrate preparation in the fermentation technologies. A very effective and useful matrix for enzyme entrapment is polyvinylalcohol (PVA). The gelation of the immobilizates, based on partial drying at room temperature is very gentle to biological systems and form lens-shaped immobilizates called LentiKats® (ref.[2]). As reported recently, due to the thickness (200–400 μm) of immobilizates there are low diffusion limitations, which can be crucial in enzyme biocatalyses[3]. This type of enzyme was successfully investigated in polyelectrolyte-enzyme-complexes entrapment in LentiKats® (ref.[3]). In this work we have studied the application of LentiKats® for immobilization of glucoamylase in long term experiments.

Material and methods

Immobilizated glucoamylase was prepared on pilot scale equipment in MEGA a. s. according to the manufacturer (Czech Republic, www.mega.cz). Reaction conditions: substrate – 100 ml of 10% maltose syrup Glucomalt (Amylum Slovakia) dissolved in acetate buffer; pH 4.5; temperature 30 °C; 12 g of immobilizates; gently agitated bioreactor.

Results and discussion

As reported before, the drop in activity might be attributed to limitations in diffusion of the reaction parameters[3]. In our case the activity of immobilized enzyme was reduced down to 36 % compared to free enzyme. This activity reduction can be also influenced by the inactivation of enzyme during the immobilization process. In spite of this phenomenon immobilized enzyme has better stability at the different pH (Fig. 1.). Due to variable pH conditions in the saccharification of starch substrates in industrial scale this attribute of immobilized enzyme have positive effect to the whole process. The main advantage of entrapped enzymes is the repeated use of immobilizates. This was examined in successive batch hydrolysis process. The relative enzymatic activity was stable for long time period and after 100 repetitive batch conversions, enzyme entrapped in LentiKats® retain 74% of the initial activity (Fig. 2.).
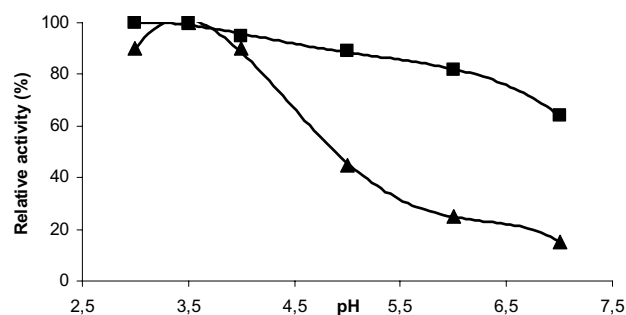

Fig. 1. **Relative enzymatic activity of free (▲) and immobilized (■) glucoamylase at different pH**
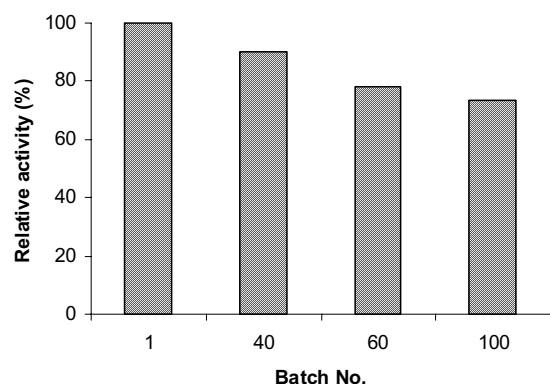
Fig. 2. **Relative enzymatic activity of glucoamylase entrapped in LentiKats® during repetitive batch conversions**

Conclusion

Glucoamylase entrapped in polyvinylalcohol immobilizates LentiKats® retain good stability in 100 successive batch conversions. Moreover, immobilized enzyme performed better activity profile within range of pH 3.6–6.

This work was supported by MEGA a. s. (Czech Republic) (www.mega.cz) and following VEGA Grants: 1/2391/05 and 1/2390/05.

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P42 EFFECT OF NITROGEN AND PHOSPHORUS ON CONTENT OF MERCURY IN LETTUCE AND KOHLRABI PLANTS

OTAKAR ROP and PAVEL VALÁŠEK

Department of Food Engineering and Chemistry, Faculty of Technology, Tomas Bata University in Zlin, nám T. G. M. 275, 762 72 Zlin, Czech Republic, rop@ft.utb.cz

Introduction

Mercury belongs to the group of heterogeneous elements known by the high toxicity for live organisms. Its cumulation in an anatomy produces hard health disorders. Due to their high ability to dissolve the lipids, mercury fumes may directly overcome blood and brain barriers and get to the central nervous system[1]. The natural level of mercury ranges in 0.02–0.2 mg kg^{-1} of the soil. In case of plants, the toxicity shows itself by the occurrence of freen-sicknesses and by the limitation of the grow of the root system. Further, mercury causes the failures of the internal arrangement of chloral plastics and conspicuous enlargement of endoplasmatic reticula and mitochondria; the lowered content of chlorophyll may occur either. Average contents of mercury in the plant ashes range around 0.1 mg kg^{-1} dry matter[2].

Methods

The aim of the experiment was to follow the content of mercury in experimental plants in dependence on the different levels of the content of mercury in the soil and on graduated doses of nitrogen and phosphorus. The experiment was performed in plastic vegetation pots. To each pot, there was weighed 10 kg of soil. Nitrogen and phosphorus application was performed before the planting; in case of nitrogen in the form of ammonia sulphate and in case of phosphorus in the form of dihydrogen potassium phosphate. Mercury was applied before the planting in the form of mercuric oxide, which was dissolved in minimum amount of nictric acid. After the application, the soil was mixed up thoroughly. Consecutively, to the pots there were planted out the plants of lettuce (sort Lednicky) and kohlrabi (sort Moravia) according to the diagram shown in the Table I. Each variant was repeated 4 times. The analyses of the vegetable mass samples were done on atomic absorption spectrophotometer (AMA-254 analyser). The results of the measurement were tested statistically.

Table I
Layout of the experiment

Variant	Content of mercury in the soil [mg kg^{-1}]	Content of nitrogen in the soil [mg kg^{-1}]	Content of phosphorus in the soil [mg kg^{-1}]
Control	Control	–	–
1	Control	20	–
2	Control	40	–
3	Control	–	100
4	Control	–	200
5	0.8	20	–
6	0.8	40	–
7	0.8	–	100
8	0.8	–	200
9	3.2	20	–
10	3.2	40	–
11	3.2	–	100
12	3.2	–	200

Results

The results of chemical analyses of lettuce leaves and kohlrabi leaves and bulbs are shown in the Table II. In our experiment, there was statistically proved the detectability of higher acceptance of mercury in dependence on increasing content of nitrogen in the soil. As well as it was successfully statistically proved that also the higher content of phosphorus

in the soil effects on the higher acceptance of mercury by plants. It was confirmed by analogy, that increasing amount of mercury in the soil effects its increasing absorption to the vegetable organism. The lettuce growing in the soil with the dose of phosphorus of 3.2 mg Hg kg^{-1} of the soil contained, in average, almost double amount of mercury, than variants with 0.8 mg Hg kg^{-1} of the soil (increase from 0.773 to 1.255 mg Hg kg^{-1} of the dry matter) and almost threefold amount of mercury, than at control variants with addition of phosphorus. In lettuce leaves, that grew in the soil containing 3.2 mg Hg kg^{-1} and 200 mg P kg^{-1} of the soil it was found 3.3 times more of mercury, than in case of the lettuce grown in the control soil, which contained only the natural amount of mercury and phosphorus. At kohlrabi bulbs there was statistically proved the significant effect of increased amount of nitrogen and phosphorus in the soil on the content of mercury. There was also confirmed the increased acceptance of mercury in dependence on its content in the soil and it was at variant with the highest content in comparison with the check variants and with 0.8 mg Hg kg^{-1} of the soil. For example, at comparison of variant, where there was the level of 3.2 mg Hg kg^{-1} and 40 mg N kg^{-1} of the soil threefold increase of the content of mercury occurred in bulbs in comparison with the variant with the same content of mercury in the soil, but with addition of 20 mg kg^{-1} of the soil. At comparison of average values measured out at check variants with phosphorus with variants, where the content was 3.2 mg Hg kg^{-1} of the soil and phosphorus was added, the twice higher difference in the content of mercury in bulbs was found out. By the statistic testing it was confirmed, that the increased amount of phosphorus in the soil has a signi-

Table II
Average contents of mercury in analysed organs of experimental plants

Variant	Content of mercury in lettuce leaves [mg kg^{-1} of dry matter]	Content of nitrogen in kohlrabi bulbs [mg kg^{-1} of dry matter]	Content of phosphorus in kohlrabi leaves [mg kg^{-1} of dry matter]
Control	0.463	0.185	0.642
1	0.461	0.124	0.546
2	0.561	0.165	0.468
3	0.457	0.266	0.409
4	0.436	0.163	0.762
5	0.632	0.159	0.603
6	1.052	0.153	0.556
7	0.824	0.177	0.487
8	0.722	0.133	1.464
9	0.704	0.166	0.577
10	0.633	0.487	0.627
11	1.100	0.438	0.512
12	1.411	0.355	0.834

ficant effect on the mercury acceptance by kohlrabi leaves. The higher amount of mercury contained in the soil had no statistically significant effect on its increased absorption to the leaves. Conclusive differences occurred at variants with the same content of mercury in the soil, but with different content of phosphorus. At check variants, in average, the content of mercury of kohlrabi bulbs was approximately 3 times smaller, than of kohlrabi and lettuce leaves. The lettuce easily cumulates the over-normative contents of heterogeneous elements[3], what was shown also in our experiment.

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P43 PRODUCTION OF LACTIC ACID WITH FREE AND IMMOBILIZED THERMOPHIL BACTERIA *BACILLUS COAGULANS*

MICHAL ROSENBERG, ĽUDMILA KRIŠTOFÍKOVÁ, MARTIN REBROŠ and LUCIA SLÁVIKOVÁ
Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak Technical University, Radlinského 9, 812 37 Bratislava, Slovak Republic,
michal.rosenberg@stuba.sk

Introduction

Lactic acid (LA) fermentation has received extensive attention for over a long period of time. Lactic acid bacteria (LAB), especially the genera *Lactobacillus, Lactoccocus, Streptococcus* have the property of producing lactic acid[1,2]. On glucose medium homofermentative LAB produce more than 85 % of lactic acid, whereas heterofermentative produce only 50 % lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide. These organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates. These mesophilic bacteria are not adapted for industrial production of lactic acid because of high contamination risks. This problem can be overcome with thermophilic microorganisms, because the sterile conditions are not necessary anymore[3]. For this reason, we focused on the production of lactic acid with thermophile gram negative bacteria *Bacillus coagulans*, which is able to produce thermostabile spores. Production optimum, which is between 52–55 °C, is the main advantage of this microorganism. The fermentation ability of thermophilic strain Bacillus coagulans has been studied in batch cultures with free cells, and cells entrapped into polyvinylalcohol lens-shaped capsules on glucose and sucrose mediums.

Materials and methods

Bacillus coagulans CCM 4318 was used in this work.

Spore suspension was prepared on MRS agar. The spore concentration of about 10^6 cell/ml was used as an inoculum. Spores of microorganism have been immobilized to polyvinylalcohol, lens-shaped capsules called LentiKats® on pilot plant equipment in Mega, a. s. (Czech Republic, www.mega.cz).

The experiments were carried out in a 5 l glass bioreactor containing 2.6 dm^3 of production medium (in grams per litre) – glucose or sucrose 80, yeast extract (Oxoid) 10, $MgSO_4 . 7H_2O$ 0.2, $(NH_4)_2HPO_4$ 1, $MnSO_4 . 4H_2O$ 0.05, $FeSO_4 . 7H_2O$ 0.01. Culture was incubated at 50–52 °C at agitation 200 rpm min^{-1}, the pH was maintained between 6.3–6.5 by addition of 26% NH_4OH.

The medium was boiled for 1 min, cooled on 51 °C. After that the 10 ml of spore suspension or immobilized bacteria cells were added to the medium. During repeated batch experiments, when the substrate was converted to 95–99 %, the LentiKats® were filtered, washed in distillate water and used in the next batch cycle.

Biomass concentration of free cells in medium was estimated using a correlation between optical density measurement at 620 nm and cell dry weight. Biomass concentration in immobilizates was determined as cell dry weight within melted LentiKats®. Glucose, sucrose, and lactic acid were analysed by HPLC using an IEX H form column (Watrex, Czech Republic). Analysis was done at 50 °C with 9 mM sulfuric acid as the eluent at a flow rate of 0.7 ml min^{-1}.

Results and discussion

Fermentation on glucose medium for free spore inoculum shows typical pattern of microorganism growth, consumption of substrate and lactic acid production. Lag phase of fermentations took 7.5 hours. The yield of lactic acid was 97.5 % of theoretical amount after 28 hours of fermentation and the specific rate of lactic acid production (Q_s) was 0.578 $g_{LA}/g_{cell}/h$ (Fig. 1.).
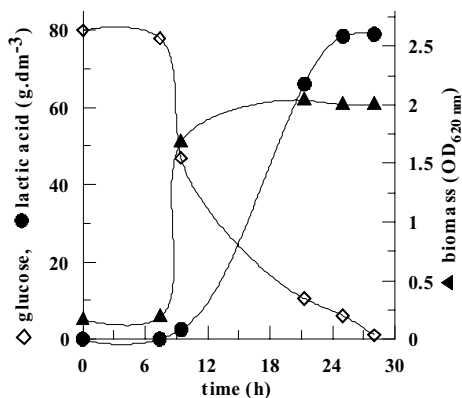
Fig. 1. **Lactic acid production by *Bacillus coagulans* CCM 4318 on glucose medium. Conditions: 2.6 dm^3 of production medium inoculated with 10 ml of spore suspension, temperature 50 °C, agitation 200 rpm min^{-1}, neutralization with 26% NH_4OH**

Fermentation with immobilized spores in glucose medium was much more effectively. The concentration of cell biomass immobilized to PVA gel increased from 1.45 to 27.5 mg cell dry mass per gram of gel. The increase in biomass concentration inside the LentiKats® significantly influenced the fermentation time (Fig. 2.). The microscopic analysis of immobilizates shows the overgrowing of the cells out from the matrix after the fifth batch fermentation (Fig. 3.). Free cells experiments confirm the inhibition effect of lactic acid on biomass growth and consequently on lactic acid production (data not shown). This negative effect can be eliminate by immobilization, because the growth of the cells within LentiKats® does not require maximal μ_{max}, but only consecutive change of lysed cells. Therefore, during the sixth conversion, after 4 hours of cultivation (lactic acid concentration 40 g l^{-1}) the continual regime of fermentation was initialled. The lactic acid concentration in the broth was kept at 40–50 g l^{-1} during the steady state (300 h). The biomass concentration gradually increased from 27.5 to 114.4 mg cell dry mass per gram of gel, and reached plateau when the volumetric productivity of lactic acid was 15.5 g_{LA} l h^{-1} (Fig. 4.).

Fermentation on sucrose medium with free microorganism was similar to the glucose medium experiment – lag phase of fermentations took 7.5 hours, the yield of lactic acid was 96.9 % of theoretical amount after 28 hours of fermentation. Second step of ours experiments was immobilization of Bacillus coagulans spores and their testing in repeated batch fermentations. After each batch cycle, immobilizates
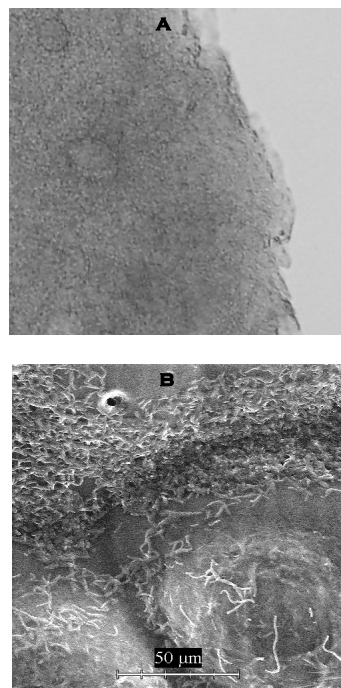
Fig. 3. **Microscopic view of LentiKats® with immobilized cells *Bacillus coagulans* CCM 4318 after fifth repeated batch fermentation in glucose medium. A – optical microscopy (extension 100×), B – electron microscopy**

have been separated and used for next conversion. Specific production rate (q) was between 23–29 mg of lactic acid/g of LentiKats® /h after the second conversion (after accumula-

tion of biomass within immobilizates). Each batch cycle took from 6 to 10 hours, and system was stable for 20 fermentations without any significant changes (Fig. 5.).
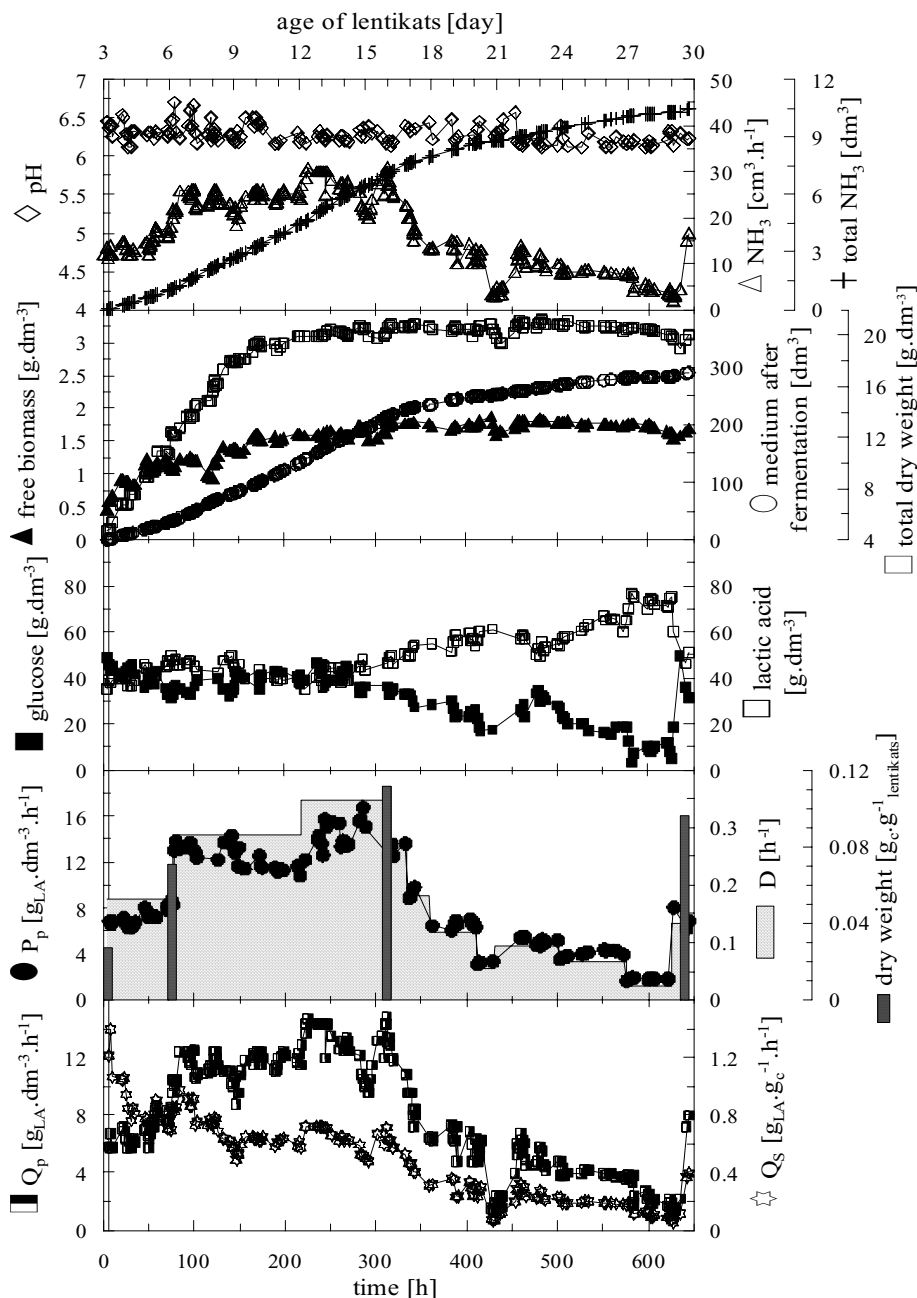
Fig. 4. **Continual production of lactic acid by immobilized cells *Bacillus coagulans* CCM 4318. Conditions: 420 g wet LentiKats®, 2.6 dm³ production medium, 50 °C, 200 rpm min⁻¹, neutralization with 26% NH₄OH**
D – **dilution rate**, P_P – **volumetric productivity**, Q_P – **rate of lactic acid production** Q_S – **specific rate of lactic acid production**
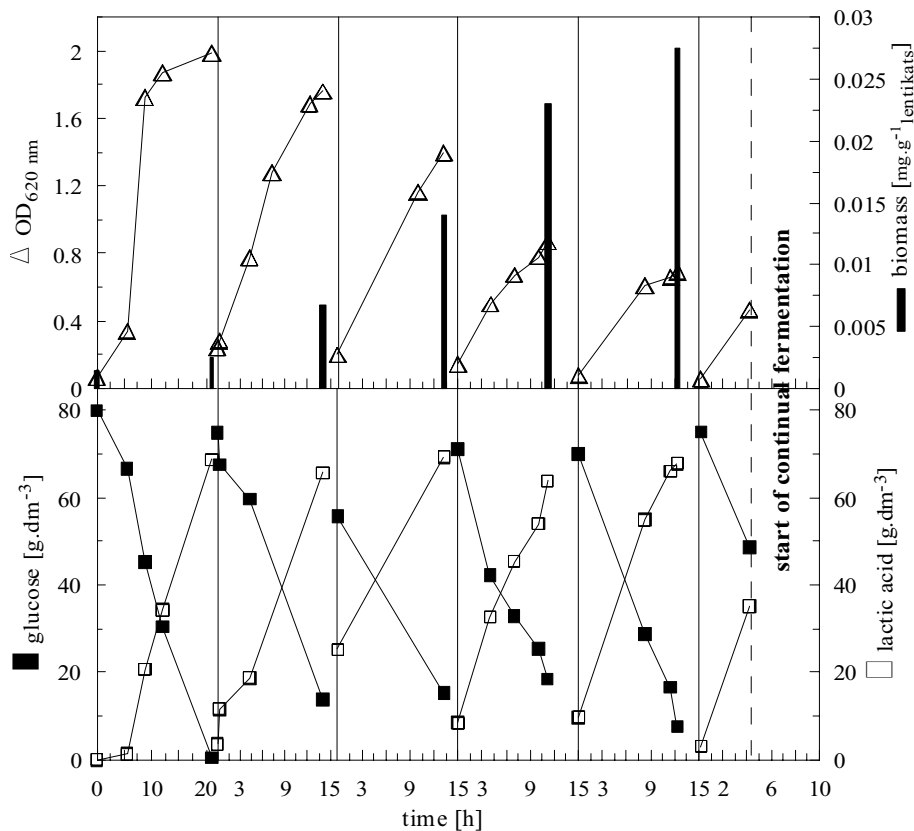
Fig. 2.　**Repeated batch fermentation by immobilized cells of *Bacillus coagulans* CCM 4318. Conditions: 635 g wet LentiKats® in 2.6 dm³ production medium, temperature 50 °C, agitation 200 rpm min⁻¹, neutralization with 26% NH₄OH**
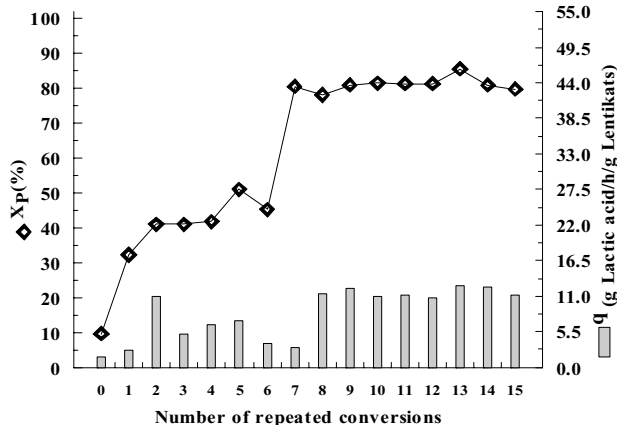
Fig. 5.　**Specific rate of lactic acid production (q) and a ratio between immobilized and total biomass of *Bacillus coagulans* CCM 4318 in bioreactor (Xₚ) during repeated batch fermentations on sucrose medium**

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P44 INFLUENCE OF TWO STERILIZATION WAYS ON THE VOLATILES OF BLACK PEPPER (*PIPER NIGRUM L.*)

JANA SÁDECKÁ, EMIL KOLEK, JÁN PEŤKA
and MILAN ŠUHAJ

Food Research Institute, Priemyselná 4, 824 75 Bratislava, Slovak Republic, sadecka@vup.sk

Introduction

Black pepper (*Piper nigrum L.*) is the most widely used of all condiments and is among the most highly contaminated spices [1], containing viable counts reaching levels of 10^7–10^8 g^{-1}. Radiation pasteurization with low doses of gamma-rays, X-rays and electrons effectively control foodborne pathogens, and can protect the public from diseases such as salmonellosis, hemorrhagic diarrhea caused by *Escherichia coli*, and gastroenteritis from *Vibrio vulnificus*[2]. Moreover, this sterilization method is replacing the use of highly toxic and carcinogenic fumigants (ethylene oxide, methyl bromide), is less harmful to the spice than heat sterilization, which may implicates the loss of thermolabile aromatic volatiles, and irradiation does not leave chemical residues on product. Toxicological and nutritional tests have confirmed the safety of foods irradiated at doses below 10 kGy[3,4]. However, ionizing radiation treatment may alter chemical composition and flavour of spices. The aim of present study was to investigate and compare the effects of ionizing irradiation with different recommended doses (5 kGy, 10 kGy), exceed dose (30 kGy) of γ-rays, to the heat sterilization on the composition and organoleptic quality of black pepper essential oil, employing GC, GC/MS and aroma extract dilution analysis (AEDA)[5].

Experimental

Material. A sample of dried spice marked as Vietnamese powdered black pepper 550 ($\rho = 550$ g dm^{-3}) was obtained from the supplier: Mäspoma, s. r. o., Zvolen, SK. The spice moisture content was 11.87 %, the samples were dried at 100 °C during 6 hours (by the STN 580110 standard, article 32). Portions of 80 g were stored in polyethylene and paper bags (simulation of retail packing) at ambient temperature on dry place.

Heat sterilization. Black pepper berries were treated by dry steam at temperature 130 °C during 3 minutes, inner temperature of berries were 98 °C max. Berries were subsequently ground.

Irradiation. The packed samples of powdered spice were irradiated with 5 kGy, 10 kGy and 30 kGy at a rate 2 kGy h^{-1} using γ-rays Co-60 irradiator at ARTIM, s. r. o., Praha, CZ.

Microbiological analysis. Elementary microbiological investigation of spice samples untreated (control), heat treated and γ-irradiated at doses of 5 kGy, 10 kGy and 30 kGy was carried out under STN ISO 4833, STN ISO 4832, STN ISO 7954 immediately after irradiation and after 3 months of storage.

Extracts. Black pepper essential oils for GC/MS, GC/FID and GC-olfactometry were isolated from powdered spice untreated, heat treated and treated at stated doses of γ-irradiation by simultaneous distillation extraction using Likens-Nickerson apparatus and diethyl ether as extraction solvent. Total essential oils contents from samples of powdered stated spice were determined by a method European Pharmacopoea 4 with xylene as an extraction solvent.

Gas chromatography/Mass spectrometry (GC/MS). GC/MS analyses were performed on Hewlett-Packard HP 5971A mass-selective detector directly coupled to HP 5890II gas chromatograph. Fused silica capillary column Ultra 1 (HP), 50 m × 0.20 mm × 0.33 μm was employed with helium as a carrier gas. The samples were injected by split technique at 250 °C. The column temperature was programmed from 35 °C to 250 °C with gradient 1.7 °C min^{-1}. The ionizing voltage (EI) was 70 eV.

Gas chromatography (GC). Hewlett-Packard HP 5890II gas chromatograph with FID was used for determination of relative percentage composition of volatile compounds and of their linear temperature programmed retention indices. The samples of extracts were analyzed on Ultra 1 (HP), fused silica capillary column 50 m × 0.32 mm × 0.50 μm, at the temperature programmed from 35 °C up to 250 °C with gradient of 2 °C min^{-1}.

Linear velocity of the carrier gas hydrogen was 36 cm min^{-1} (measured at column temperature 143 °C). The linear retention indices (RI) were calculated after Van den Dool and Kratz[6] equation. n-Alkanes C_8–C_{18} were used as the reference standards.

Statistical analyses. Influence of spice treatments on relative percentage composition of volatiles were compared using Analysis of variance (one-way, repeated measurements). Holm–Sidak test was used for pairwise comparison if the data passed test of normality and equal variance. ANOVA on ranks (repeated measurements) was calculated and Tukey test was used for pairwise comparisons.

Gas chromatography/olfactometry – aroma extract dilution analysis (AEDA). For the AEDA Hewlett-Packard HP 5980II gas chromatograph equipped with FID, Ultra1 (HP), the fused silica capillary column 50 m × 0.32 mm × 0.50 μm and the column effluent splitter 1:1 and sniffing port were used. The temperature was programmed from 35 °C up to 250 °C with a gradient of 2 °C min^{-1}. The flavour dilution (FD) factors[5] and odor descriptions were determined by sniffing of compounds eluting from the capillary column. The extracts for AEDA were diluted with diethyl ether stepwise 1:10, 1:100, 1:200. Sensory evaluations were performed by a panel of 3 trained judges.

Results and discussion

Decrease of microbial contamination level up to the total elimination of present microorganisms (MO) is the primary aim of spice heat or irradiation treatment. The microbiological results confirmed that the total count of MO 10^6 colonies in the untreated (control) sample of black pepper decreased

Table I
Microbiological analysis of untreated, heat treated and irradiated black pepper immediately after irradiation and after 3 months of storage

Irrad. dose (kGy)	Total MO [KTJ g^{-1}]		Coliforms [KTJ g^{-1}]		Yeasts [KTJ g^{-1}]		Moulds [KTJ g^{-1}]	
Storage	without	3 months	without	3 months	without	3 months	without	3 months
0	$1.0 \cdot 10^6$	$2.5 \cdot 10^6$	$1.0 \cdot 10^1$	2	<10	<10	$1.0 \cdot 10^1$	<10
5	<1	<1	<1	<1	<10	<10	<10	<10
10	<1	<1	<1	<1	<10	<10	<10	<10
30	<1	<1	<1	<1	<10	<10	<10	<10
heat treated*	$1.6 \cdot 10^5$	$1.6 \cdot 10^6$	<1	<1	<10	<10	<10	<10

*heat sterilization of black pepper berries by dry steam at 130 °C and subsequent grinding; KTJ g^{-1} count of colonies per gram

using 5 kGy irradiation dose to less than 1 KTJ g^{-1} (Table I). It was found out by the heat treatment of black pepper berries, the total MO count was lower by one order as the control sample. In the control sample number of MO has increased 2.5-times during 3 months storage. In the heat treated sample during 3 months the count of MO multiplied by one order. The status in γ-irradiated samples at all stated doses was unchanged within 3 months storage. Qualitative composition of volatile oils obtained from control, heat treated sample and from irradiated samples of spice at various doses, was identical. The steam-volatile black pepper oil consisted primarily of monoterpene and sesquiterpene hydrocarbons, and oxygenated compounds. The GC/FID and GC/MS analyses revealed more than sixty compounds from which we were able to identify more than fifty ones using the mass spectra and published retention indices. α- and β-pinene, sabinene, car-3-ene,

Table II
Influence of heat treatment and radiation treatment on potent odorants of black pepper

No.	Compound	RI[a] Ultra 1	FD factor					Aroma character	Identification[b]
			0 kGy	5 kGy	10 kGy	30 kGy	heat steril.		
1	3-methylthiopropanal	861.3	100	100	100	100	100	cooked potato-like	RI, ST, A
2	unknown	900.7	100	100	10	10	10	musty, burnt, mousy	–
3	oct-1-en-3-one	954.0	100	100	100	100	100	mushroom-like	RI, ST, A
4	myrcene	981.9	10	10	10	10	10	hop oil-like, herbaceous	MS, RI, ST, A
5	1,8-cineole	1016.0	10	10	10	10	10	peppermint, cool, fresh	MS, RI, ST, A
6	unknown	1031.2	10	10	10	10	10	herbaceous, earthy, bitter	–
7	unknown	1054.7	100	100	100	10	10	smoke, terpeny	–
8	α-terpinolene[t]	1076.0	10	10	10	10	10	vegetable, bitter, green	MS
9	linalool	1083.1	200	200	200	200	10	flowery	MS, RI, ST, A
10	unknown	1142.0	100	100	100	100	10	thiamin, meat broth	–
11	p-cymen-8-ol	1156.2	10	10	10	10	10	phenolic, bitter, fuel-like	MS, RI, A
12	cis-sabinol[t]	1177.1	10	10	10	10	10	earthy, muddy, musty	MS
13	unknown	1191.4	10	10	10	10	1	rancid fat-like	–
14	piperitone	1220.8	100	100	100	100	10	balsamic, sweet, anise	MS, RI
15	unknown	1288.6	10	10	100	100	10	terpeny, almond	–
16	unknown	1292.4	10	10	100	100	10	rancid fat-like	–
17	β-damascenone	1375.5	100	100	100	100	10	fruity, prune-like	RI, A
18	β-farnesene	1446.0	10	10	10	10	10	terpeny, spicy	MS, RI, ST, A
19	germacrene D[t]	1469.6	10	10	10	10	1	flowery	MS
20	β-bisabolene[t]	1497.8	100	100	100	100	10	terpeny, earthy, celery	MS
21	δ-cadinene	1509.9	100	100	100	100	10	thyme, sweet, terpeny	MS, A
22	unknown	1716.9	200	200	200	200	10	spicy, black pepper-like	–

[a]linear retention index; [b]means of the identification: MS-EI – mass spectrum, RI – retention index, ST – sniffing of standard compounds, A – known character, [t]tentative identification

limonene are the important components of the monoterpene fraction. β-Caryophyllene is the major sesquiterpene and it is the main compound of volatile black pepper oil according to the quantity. Most of the compounds were affected by the heat treatment only. Heat treatment caused a significant increase (1% significance level) of some monoterpenes (α- and β-pinene, camphene, sabinene, myrcene, α-phellandrene, 3-carene, α-terpinene, p-cymene, 1-8-cineole, limonene, γ-terpinene) proportional share towards the control. Both heat treatment and exceed dose of 30 kGy caused a significant decrease some volatiles, comparing with control. Control had significantly higher level of β-elemene, α-guaiene, α-humulene, β-farnesene. No significant changes were observed in the volatile oil compound content at radiation doses 5 kGy and 10 kGy (toxicologically and nutritionally confirmed as safe maximal dose). The most important change was possible to observe at ionizing of 30 kGy (3-times exceed authorized dose) resulting in triple increase of caryophyllene oxide concentration in compare with the control. This effect was observed in 30 kGy irradiated spice and also in 30 kGy irradiated essential oil on neutral carrier (Na_2SO_4). Gas chromatography-olfactometry analyses (AEDA) of volatile extracts revealed 22 potent odorants with FD factors in the range from 10 to 200. It was found out that 3-methylthiopropanal, oct-1-en-3-one, myrcene, 1,8-cineole, linalool, β-damascenone, β-farnesene, δ-cadinene, α-terpinolene, p-cymen-8-ol, piperitone, germacrene D, β-bisabolene and eight unknown compounds are responsible for characteristic flavour of black pepper essential oils untreated, heat treated and irradiated at the stated doses (Table II). In the majority of individual compounds was effect of irradiation on the FD factors not proved. The most potent odorants are linalool (9) with flowery aroma character and unknown compound (22) with spicy, typical black pepper-like aroma, either of them with FD = 200. Influence of the irradiation on components (2, 7, 15, 16) marked as unknowns, is not significant. Differences of obtained FD factors are in one dilution step, however, no important changes in overall aroma of stated black pepper volatile oils were perceived. In connection with decrease of some thermolabile volatiles caused by spice heat treatment, their FD factors were changed in compare with both control and stated radiation doses. The biggest alterations arise in context with linalool (FD = 10), piperitone (FD = 10), β-damascenone (FD = 10) and unknown compound (22) with FD = 10. Noticeable decrease of FD factors given aroma-active compounds produce significant effect on overall aroma of heat treated black pepper.

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P45   STUDY OF GAMMA IRRADIATION EFFECT ON BLACK PEPPER

ZUZANA SALKOVÁ, JANA SADECKÁ, LÍVIA JURÁŠOVÁ and MILAN KOVÁČ
Food Research Institute, Priemyselná 4, 824 75 Bratislava, Slovak Republic, salkova@.vup.sk

Introduction

Food irradiation is increasingly recognized as a method for reducing post harvest food losses, ensuring hygienic quality, and facilitating wider trade in foodstuffs. Codex General Standard for Irradiated Foods was adopted in 1983, and concluded that irradiation of any food commodity up to an overall average dose of 10 kGy introduces no toxicological hazard[1]. Directive 1999/3/EC of the European Parliament and of the Council of 22. 2. 1999 establishes a Community initial positive list of food and food ingredients that may be treated with ionising radiation, with the maximum doses 10 kGy. There are dried aromatic herbs, spices and vegetable seasonings authorised for irradiation treatment because, they are frequently contaminated and infested with organisms and their metabolites which are harmful to public health. However, progress in commercialisation of the food irradiation process, and consumer demand for clear labelling of irradiated food highlighted the need for tests and development of detection methods.

Black and white pepper is a major food item treated with ionising radiation because they in its natural state is highly contaminated with molds, yeasts and bacteria[2]. The starch is significant component of spices. The radio-depolymerization of starch in the irradiated spices decreases of dispersion viscosity of their heat gelatinized suspensions as compared to that of unirradiated samples[3]. This may provide a relatively simple detection method of irradiation treatment of pepper.

In this paper we present the results of viscosity and starch content changes of black pepper, which was irradiated at doses from 2.5 kGy to 10 kGy and 30 kGy.

Materials and methods

Black pepper harvested in Vietnam was received from fy Mäspoma s. r. o., Slovakia, was finaly ground, packed in polyethylene bags and irradiated with 2.5 kGy, 5 kGy, 7.5 kGy, 10 kGy and 30 kGy using Co-60 irradiator, fy *Artim* s. r. o., Prague, Czech Republik. The dry matter and starch content of black pepper were 89.45 (0.00)% and 41.41 (0.22)%.

Viscosity. Viscosity of irradiated ground black pepper (particles < 0.5 mm) was measured according to the method

of Formanek at al.[4]. Suspensions of pepper (10%, w/v) was homogenized and pH was adjusted to 12.5 with 33 % NaOH. The suspensions were heated at 100 °C and at 94–95 °C in water bath for 30 min, followed by cooling 1 hr at 25–26 °C in water bath. Viscosity was measured at 25 °C ±1 °C with RHEOTEST 2, VEB MLW Prufgeräte-Werk Medingen and coaxial cylinder in the velocity gradient range of 9–1312 s⁻¹ in tree replicates.

Starch. Starch content was measured according to slovak standard STN 57 0157. Black pepper sample was heated with HCl (1 mol dm⁻³) for 2.5 hr in boiling water, and formed glucose was then determined after added of Luff-Schoorl agent and cupric sulphate surplus was iodometric determined.

Results and discussion

Shear stress, viscosity. The viscosity changes were determined after irradiation treatment and after 12 month of storage in dark room at 20–25 °C. Shear-stress curves on the first days after irradiation of pepper samples are shown in Fig. 1. The shear stress-values increased at increasing of velocity gradient (s⁻¹). Significant differences between shear-stress values were find only from 145.8 s⁻¹ to 1312 s⁻¹. Fig. 2. shows apparent viscosity degreasing of pepper samples suspension heated at 100 °C and 94 °C with increasing of irradiated doses. Irradiated treatment caused significant changes in the apparent viscosity at 2.5 kGy dose already. The difference between the sample irradiated with 10 kGy doses and the control was more than 50 % t velocity gradient 437.4 s⁻¹. After 12 month of storage the same measurements showed similar data for the shear-stress and viscosity values.

The viscosity of pepper suspension depends on a starch content. Starch is degraded by ionising radiation what resulting in a decrease of viscosity. Comparing results (Table I) of the starch content of the irradiated and control samples there are no significant differences as a function of irradiation until to 10 kGy. The starch content decreased at 30 kGy dose about 2 %.

Fig. 2.   **Viscosity (mPa s⁻¹) of the gelatinized irradiated black pepper suspension at velocity gradient of 437.4 s⁻¹, heated at 100 °C and 94 °C**

Table I
Starch content (%) of irradiated black pepper

Time (month)	Irradiation doses [kGy]					
	0	2.5	5	7.5	10	30
0	41.41 (0.22)	41.02 (0.89)	41.26 (0.16)	40.44 (0.00)	40.18 (0.08)	39.09 (0.42)
8	40.90 (0.57)	–	40.54 (0.00)	–	40.37 (0.28)	38.75 (0.00)

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Fig. 1.   **Shear-stress of gelatinised irradiated black pepper suspension, heated at 100 °C**

P46　EFFECT OF INOCULATION AND CULTIVATION TECHNIQUES ON GAMMA-LINOLENIC ACID PRODUCTION DURING FUNGAL SOLID-STATE FERMENTATION

LUCIA SLÁVIKOVÁ, MARCELA ANGELOVIČOVÁ, LINDA LENGYELOVÁ and MILAN ČERTÍK

Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak technical University, Radlinského 9, 812 37 Bratislava, Slovak Republic,
lucia.slavikova@stuba.sk

Introduction

Effort for development of suitable alternatives for poly-unsatured fatty acids (PUFA) production compared with their traditional sources ended in progress in fermentation technology of oleaginous microorganisms. Particularly active in the synthesis of fatty acids are species of oleaginous lower filamentous fungi belonging to *Zygomycetes*[1,2]. Slovak group has pioneered solid-state fermentation process, in which microorganisms belonging to *Mucoraceae* easily and efficiently utilized cereals containing starch, proteins and low lipid amount and accumulated lipid with dietetic valuable PUFAs[3]. Thus, the connection of oleaginous lower filamentous fungi with solid-state fermentation (SSF) technique has shown perspectives in development of promising bioprocess providing new bioproducts enriched with PUFAs. γ-Linolenic acid (GLA) as biologically active PUFA is of great medical, pharmaceutical and food interest because of its unique attributes in the treatment of various diseases[4]. One of alternative sources is the production of GLA by fungal SSF where the bioproduct can directly serve as food or feed supplement[3].

The objective of this work was to study effect of inoculation and cultivation techniques on GLA formation by *Thamnidium elegans* grown on mixture of wheat bran and spent malt grains (WB : SMG; 3 : 1, w/w).

Materials and methods

Method of inoculation. Mixture of wheat bran and spent malt grains (WB: SMG) in ratio 3 : 1 (w/w) was used as solid substrate for GLA production by fungal SSF. Autoclavable microporous polypropylene bags were filled with 10 g of dry substrate, moistened by the addition of 10 ml of distilled water, soaked for 2 h at laboratory temperature and sterilized in autoclave (120 kPa, 120 °C, 20 min). The substrate was inoculated with culture of *Thamnidium elegans* prepared by three different ways: a) the spore suspension (SP) was prepared from 2-week-old modified Czapek-Dox agar slants by washing the mycelium with a sterile distilled water in order to achieve the final concentration of $1–2.10^6$ spores per ml; b) the bioproduct (BP) was obtained after solid-state fermentation of WB: SMG (3:1) for 3 days; and c) the vegetative mycelium (VM) was grown in modified Czapek-Dox medium with or without 0.1% (w/v) linoleic acid (LA) for 3 days. The cereal substrate was inoculated with 2 ml of SP,

2 g of BP or 2 ml of VM. The inoculated substrate was spread in bags to get substrate layer of about 1 cm and fermented statically at 23 °C for 4 days.

Cultivation techniques. Autoclavable microporous polypropylene bag was filled with 20 g of dry spent malt grains, moistened by the addition of 20 ml distilled water, soaked for 2 h at laboratory temperature and sterilized in autoclave (120 kPa, 120 °C, 20 min). The substrate was inoculated and gently mixed with 4 ml of *Thamnidium elegans* spore suspension prepared as described above. This prepared mixture of amount about 5 to 6 g was consequently inserted into Erlenmayer flasks, Petri dishes or Petri dishes covered with plastic bag foil. The inoculated substrate was spread to obtain substrate layer of about 1 cm and incubated statically at 23 °C for 4 days.

Fatty acids analysis. Bioproduct gained after cultivation was gently dried at 65 °C for 10 h, weighted, homogenized with sea sand and used for fatty methyl esteres (FAME) preparation. FAMEs were obtained using methanolic HCl and dichlomethane with heptadecanoic acid as internal standard (ISTD) in screwed glass tubes at 50 °C for 3 hours. Then FAMEs were pre-extracted into hexane and analyzed by gas chromatography (GC-6890 N, Agilent Technologies) equipped with DB-23 column according to Certik et al[5].

Results and discussion

Selection of suitable inoculum is one of the key moments of each fermentation process. The most used techniques are inoculation with spore suspension or vegetative mycelium. In the case of SSF also inoculation with pre-fermented solid material containing microbial culture and residual substrate can be employed. Properties of microorganism could be influenced by many agents, e. g. by culture conditions, aeration, pH or by the presence of various compounds, which can serve as growth factors, vitamins, detergents or precursors. In these experiment, LA served as a precursor of GLA. Therefore the effect of LA addition to inoculum on GLA production was tested. It is surprising that the highest yield of 7.2 g GLA/kg bioproduct was obtained by VM without LA (Fig. 1.). Thus LA concentration in inoculation medium will be optimized in the future experiments. Inoculation of substrate with SP provided higher GLA content in the final bioproduct than inoculation with BP. It might be caused by more massive inoculation and better substrate utilization by the fungus using SP than BP.

Cultivation in plastic bags offers good fermentation conditions for laboratory scale experiments. It provides spreading the substrate in the bags to obtain thick layer that supports appropriate heat removing, oxygen transfer and possibility of gently homogenization. Nevertheless, the bags handling is quite difficult and the bags are susceptible to rupture in autoclave. Thus, further possible systems, including Erlenmayer flasks and plastic bags covered by cotton plugs and also Petri dishes and Petri dishes covered with plastic bag foil were tested. The highest content of GLA in TFA (14.0 %) was observed in Erlenmayer flasks. It was probably caused

by adequate air supply above the substrate layer, because the oxygen availability is limiting factor in the GLA biosynthesis. It should be noted that although GLA concentration in TFA decreases in order Erlenmayer flasks, Petri dishes and plastic bags, respectively, the final yield of GLA in bioproduct remains approximately the same. This may indicate enhanced lipid production with lower GLA content. The best GLA yield of 7.2 g kg^{-1} BP was observed on the Petri dishes covered with plastic bag foil, however, this system from the view of handling is not very suitable. Therefore, application of Erlenmayer flasks or Petri dishes could be potential alternative of plastic bags.
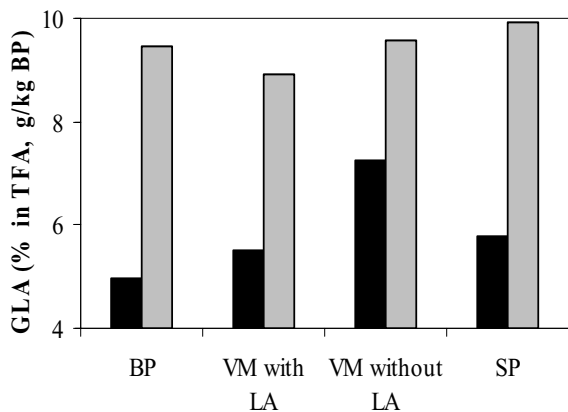

Fig. 1. **The effect of inoculation technique on GLA content in total fatty acids (▢) and bioproduct (■) during solid-state fermentation of wheat bran and spent malt grains (3 : 1, w/w). The moistened solid substrate was inoculated with spore suspension (SP), bioproduct (BP) or with vegetative mycelium (VM) grown with or without linoleic acid (LA). Fermentation was carried out statically in plastic bags at 23 °C for 4 days**
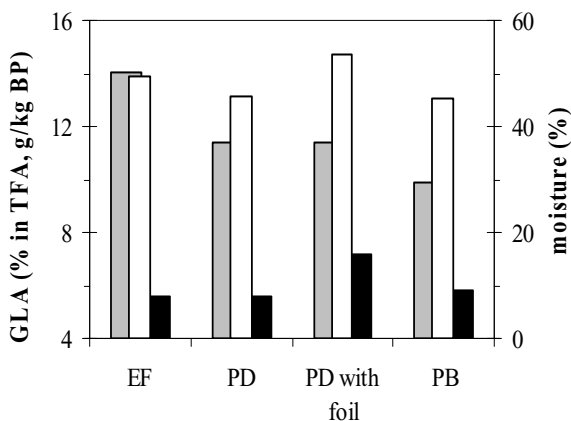

Fig. 2. **The effect of cultivation technique on moisture (▢) and GLA content in total fatty acids - TFA (▨) and bioproduct – BP (■) during solid-state fermentation of wheat bran and spent malt grains (3 : 1, w/w). The moistened solid substrate was inoculated with spore suspension and inserted into Erlenmayer flasks (EF), Petri dishes (PD) or Petri dishes covered with plastic bag foil (PD with foil). Fermentation in plastic bags (PB) served as control. Culture conditions: statically at 23 °C for 4 days**

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P47 EFFECT OF SELECTED ADDITIVES ON CHANGES OF AW AND PH DURING PRODUCTION OF DRIED FERMENTED HAMS

LADISLAV STARUCH, ĽUBOMÍR BREZOVICKÝ, ZUZANA SIROTNÁ, KATARÍNA FAŠIANGOVÁ, PETR PIPEK and IVANA POUSTKOVÁ
Department of Food Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic, ladislav.staruch@stuba.sk

Introduction

Fermented whole meat products are preserved by salting and drying. Their typical aroma is developed during the process of maturing of the product. Unlike fermented sausages, lactic acid bacteria and drop of pH resulting from their presence do not affect considerably the durability of whole meat products. The hindrance of the growth of undesirable microorganisms during production of dried fermented hams is characterised by so-called „barrier effect". Decrease of a_w accomplished by curing, seasoning and drying is the crucial factor for durability of hams e.g. (Serrano ham, Parma ham).

The work deals with modified production of fermented meat products in Slovakia which is based on traditional production in Switzerland. Objective was to observe changes of two main physio-chemical parameters, water activity and pH value, during ten weeks of fermentation process and to determine whether obtained values are in range set by Food codex of Slovak republic. Drying and fermentation are considered to be the oldest ways to preserve meat products. During the history traditional procedures were formed (Italian Serrano, Spanish Parma, etc.). In Slovak region is such typical traditional practice a production of sausage. However, production of whole meat fermented dried products has no tradition.

Dried meats (fermented hams, raw hams) are products made of whole piece of meat, cured by salting and drying and typical flavor develops during maturation. Final

products don't need to be refrigerated and are consumed without previous heat treatment.

According Slovak Food codex dried meats are supposed to meet this criteria:

a)   water activity value (a_w) under 0.90,
b)   pH less than 5.5 for dried and fermented and between 5.5 and 6.2 for dried,
c)   salt content (as NaCl) no greater than $60\,000$ mg kg^{-1}.

Experimental

These parts were chosen for processing: pork loin, pork neck, ham with skin and beef ham. They were salted and spice following the method of Heinzer Metzgerei company, Switzerland, with certain modifications. Spices of Adivit company were used. The samples were further technologically processed. During the ten weeks of fermentation process pH and a_w values were periodically measured.

pH values were taken directly using needle pH meter OP 211 (Radelkis, Budapest). Measurement was performed by a direct incision of electrode into the sample. Five measurements were carried out for each sample.

Measurement of water activity value was held in State veterinary and food institute using aw meter NOVASINA AW SPRINT TH 500. Machine was calibrated after each switch-on. Sample was homogenized using laboratory meat-grinder ($\varnothing$ 2 mm), homogenizate was put into measuring bowl. Filled bowl was inserted into the measuring machine and sealed. After value steadied it was recorded into the table. Measuring chamber was tempered to 25 °C. Each sample was made into two parallels and each was measured three times. Final value was evaluated as an arithmetical average of particular measurements .

Chlorides were assessed according to Mohr.

Water content was reckoned from weight difference after sample drying at 105 °C to constant weight .

Fig. 1.   **Growth barriers of undesirable microorganisms and their development during processing of fermented meat products**

Results and discussion

Hams from Slovak and Swiss region were analyzed. Swiss samples were bought in Swiss market. Slovak samples were produced in company Tauris Rimavska Sobota and dried in Mojmirovce in air-conditioning.

Salting and drying are main factors determining preservation of dried fermented meats. These two factors were modified several times.

Whole technology follows the processing of Swiss hams in family company Heinzer Metzgerei. In Switzerland during
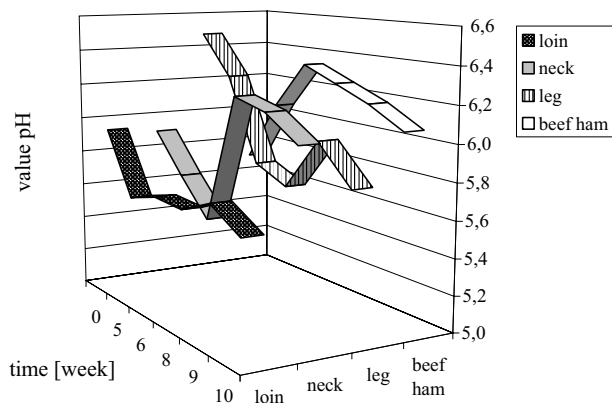
Fig. 2.   **Changes of pH value during ripening**
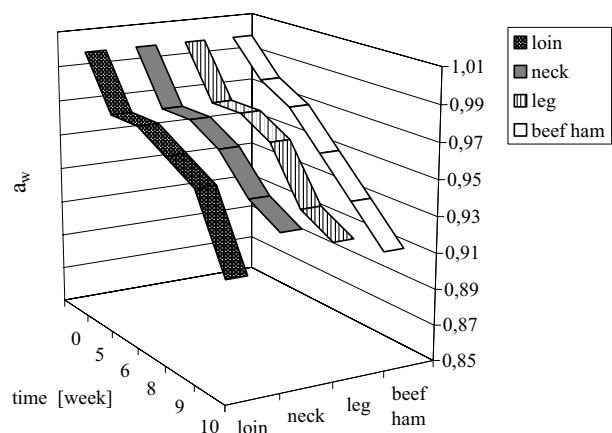
Fig. 3.   **Changes of a_w value during ripening**

the whole processing of dried hams the only parameters measured were water and salt content, pH value and water activity.

pH value of sample "Urwaldschinken" was 5.25 and represents the lowest value found during the whole observed period.

Water activity of Swiss samples ranged from between 0.88 and 0.91.

It is remarkable, that Swiss legislation doesn't designate certain limits for water activity value. Sodium chloride content differed from 6.2 to 4.2 %. Raw material selection significantly influences chemical parameters of final product (total solids, fat, protein). Final product protein content was 35–40 %. This high number witnesses of convenient selection of raw material.

Spice mixtures LAY and Adivit were used.

Water activity is a parameter, which plays the most important microbiologic role.

Conclusion

Objective of this work was to describe technological process of dried fermented meat production from raw ma-

terial to the final product and to examine, whether it meets Slovak Food Codex criteria.

Dried meats are defined as meat products made from whole piece of meat. They are allowed to be signed as dried ham, if product is made from whole pork ham with or without bone.

Samples were made from karé, neck, ham and sample originated from beef ham. Spice mixtures of three different producers were used (LAY Gewürze, ADIVIT Nitra, MOGUNTIA of Progast).

During the processing drying, pressing and air conditions were optimized according to determined parameters a_w, pH, % NaCl, total solids.

Sensorial test of meat products is also very important and it has outstanding significance in appraisal of technological process and can early point out possible defects caused by wrongly conducted drying process.

It is recommended to finalize production process of particular meat product in relevance with achieved physio-chemical (a_w, pH), microbiological and sensorial results and to choose certain spice mixture. For example karé salted in a conventional way with application of LAY spice mixture.

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P48   MONITORING OF MICROBIAL QUALITY OF FERMENTED WHOLE MEAT PRODUCTS

LADISLAV STARUCH, ZUZANA SIROTNÁ, ALEXANDRA HAVRANOVÁ, ĽUBOMÍR BREZOVICKÝ, KATARÍNA FAŠIANGOVÁ, PETR PIPEK, IVANA POUSTKOVÁ

Department of Food Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic, ladislav.staruch@stuba.sk

Introduction

Fermented whole meat products and dried meats belong to the category of uncooked meat products. Their durability and health safety is achieved either by fermentation and drying or solely by drying. Followingly, the production requires from producer to follow high hygienic standards. The microbial contamination of products can be suppres-sed by application of principles of good production praxis (HACCP), its risk may be however never negated. High propagation of microorganisms results in changes of organoleptic properies of the product. Hence, it is important to prevent growth of microorganisms by proper preserving operations.

Following parameters were monitored: total count of microorganisms, number of coliforms and *Escherichia coli*, count of mesophilic sporulating anaerobes, *Staphylococcus aureus*, *Samonella*, *Listeria monocytogenes*, *E. coli O 157:H7*.

Microbiology is one of important indicators of quality of meat and meat products. Meat samples were analysed according to ISO STN and EN standards and pathogens (*Samonella, Listeria monocytogenes, E. coli O 157:H7*) were detected by enzyme-linked fluorescent immunoassay – VIDAS system.

Codex Alimentarius of Slovak Republic requires maximal possible limits of microoosrganisms:

	n	c	m	M
Total count	5	3	$5 \cdot 10^5$	$5 \cdot 10^6$
Coliform bacteria	5	2	10^4	10^5
Escherichia coli	5	2	50	$5 \cdot 10^2$
Salmonella sp.	5	0	0/10	–
Mesophilic sporulating anaerobes	5	2	10	10^2
Staphylococcus aureus	5	2	10^2	$5 \cdot 10^3$

where

n is number of samples for microbiology analyses,

m is maximum allowed number of microorganisms within the interval n in a predefined amount of the sample,

M is maximum allowed number of microorganisms in predefined amount of sample which is allowed, but only in the amount of samples less than c or equal c,

c is number of samples from the interval n, in which is allowed maximum value M, but it is deal, in the number of samples n minus c is allowed only value m.

Experimental

These parts were chosen for processing: pork loin, pork neck, ham with skin and beef ham. They were salted and spice following the method of Heinzer Metzgerei company, Switzerland, with certain modifications. Spices of LAY company were used.

Microbiological analyses of sample LAY were realized from the final product. Total numbers of microorganisms varied from $2.5 \cdot 10^3$ to $3.0 \cdot 10^4$ cfu g^{-1}.

Neither coliforms nor pathogens were present in the samples. From among other isolated microorganisms, only *Staphylococcus epidermidis* and sporulating aerobes were found in LAY samples.

The results of analysed samples corresponded with microbiological requirements of Codex Alimentarius of Slovak Republic.

Table I   Results of microbial monitoring in sample LAY

Taking of samples	Sample	Total cfu g^{-1}	Coliform bacteria [cfu g^{-1}]	Mesophilic sporulating anaerobes [cfu g^{-1}]	Staphylococcus aureus [cfu g^{-1}]
1. week	Pork loin	$1.3 \cdot 10^3$	0	0	0
	Pork neck	90	60	0	0
	Pork ham	$2.6 \cdot 10^2$	0	0	0
	Pork belly	$4.0 \cdot 10^3$	0	0	0
4. week	Pork loin	$3.0 \cdot 105$	0	0	0
	Pork neck	$2.1 \cdot 10^6$	0	0	0
	Pork ham	$4.0 \cdot 10^5$	0	0	0
	Pork belly	$7.7 \cdot 10^4$	0	0	0
6. week	Pork loin	$3.0 \cdot 105$	0	0	0
	Pork neck	$3.0 \cdot 10^5$	0	0	0
	Pork ham.	$3.0 \cdot 10^6$	0	0	0
	Pork belly	$3.0 \cdot 10^5$	70	0	$1.5 \cdot 10^2$
8. week	Pork loin	$4.7 \cdot 105$	0	0	0
	Pork neck	$1.1 \cdot 10^6$	0	0	0
	Pork ham .	$1.4 \cdot 10^5$	0	0	0
	Pork belly	$3.4 \cdot 10^5$	0	0	0
9. week	Pork loin	$4.7 \cdot 105$	0	0	0
	Pork neck	$1.1 \cdot 10^6$	0	0	0
	Pork ham	$1.5 \cdot 10^4$	0	0	0
	Pork belly	$2.3 \cdot 10^5$	0	0	0

Table II   Results of microbial monitoring pathogenic microorganisms in sample LAY

Taking of samples LAY	Sample	Salmonella sp. in 25 g	Listeria monocytogenes in 25 g	E. coli O157:H7 in 25 g	E. coli [cfu g^{-1}]
1. week	Pork loin	negative	negative	negative	0
	Pork neck	negative	negative	negative	0
	Pork ham	negative	negative	negative	0
	Pork belly	negative	negative	negative	0
4. week	Pork loin	negative	negative	negative	0
	Pork neck	negative	negative	negative	0
	Pork ham	negative	negative	negative	0
	Pork belly	negative	negative	negative	0
6. week	Pork loin	negative	negative	negative	0
	Pork neck	negative	negative	negative	0
	Pork ham	negative	negative	negative	0
	Pork belly	negative	negative	negative	0
8. week	Pork loin	negative	negative	negative	0
	Pork neck	negative	negative	negative	0
	Pork ham	negative	negative	negative	0
	Pork belly	negative	negative	negative	0
9. week	Pork loin	negative	negative	negative	0
	Pork neck	negative	negative	negative	0
	Pork ham	negative	negative	negative	0
	Pork belly	negative	negative	negative	0

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P49 COMPARISON OF OLIGO-D-GALACTOSI DURONATE HYDROLASE PRODUCED BY MICROORGANISMS AND PLANTS

EVA STRATILOVÁ[a], MÁRIA DZÚROVÁ[a] and JIŘINA OMELKOVÁ[k]

[a]*Institute of Chemistry of Slovak Academy of Sciences, Dúbravská cesta 9, SK – 845 38 Bratislava, chemevi@savba.sk, [b]Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Technical University Brno, Purkyňova 118, CZ – 612 00 Brno*

Introduction

Exopolygalacturonases [poly(1,4-α-D-galacturonate) galacturonohydrolase, EC 3.2.1.67] are exo-hydrolases catalyzing the hydrolytic cleavage of glycosidic α-1,4-bonds of D-galacturonan at its nonreducing end under releasing D-galactopyranuronic acid as the sole reaction product. The particullar enzymes differ from each other by the range and rate of the effects on substrate in relation to the chain length. Oligo-D-galactosiduronate hydrolase (OGH) is other name of exopolygalacturonases preferring substrates with low degree of polymerization (DP). The aim of this work was a comparison of these enzymes produced by plants and microorganisms.

Methods

Purified OGHs produced in carrot roots and by yeast-like microorganism *Aureobasidium pullulans* were used for comparison. Extraction of proteins: juice from carrot roots and pectin medium after cultivation of *A. pullulans* precipitated with ammonium sulfate and 96% ethanol, desalted on a Sephadex G-25 column. Purification of OGHs: ion-exchange, affinity and gel-permeation chromatographies[1–3], followed for plant enzyme by hydrophobic interaction based chromatography and for microbial one with chromatofocusing and preparative isoelectrofocusing. Identification: activity assay according to Somogyi[4], viscosity measurements, SDS-PAGE, N-glycosidase F cleavage, IEF, TLC.

Table I
Properties of purified OGHs from carrot roots and *A. pullulans*

Properties	OGH from carrot	OGH from *A. pullulans*
Isoelectric point	4.5	7.9
pH optimum	3.8	4.0
Temperature optimum	70 °C	50 °C
Mr	54 kDa	associated (70–275 kDa)
Glycosylation	yes (deglyc. 31.5 kDa)	yes
Thermal stability (60°, 2 h)	90% of activity	50% of activity
Product	D-galactopyranuronic acid	
Action pattern	terminal	
Orientation of substrate cleavage	from nonreducing end	
Degradation of pectate	extremely slowly	
Pectate degraded with	exopolygalacturonase	endopolygalacturonase

Table II
The initial rates of OGHs on oligogalacturonides (GA)$_n$, where n is the degree of polymerization

Substrate	Activity [μmol min⁻¹ mg⁻¹]	
	OGH from carrot	OGH from *A. pullulans*
(GA)$_2$	0.061	0.048
(GA)$_3$	0.290	0.058
(GA)$_4$	0.426	0.053
(GA)$_5$	0.508	0.038
(GA)$_6$	0.571	0.032
(GA)$_7$	0.363	0.026

Results and discussion

Purified OGHs were characterized (Table I). The optimal substrate for enzyme from carrots was hexamer while for that produced by *A. pullulans* trimer (Table II). This seems to be related with other cooperating enzyme in pectolytic system and its end product, which is the optimal substrate for OGH – exopolygalacturonase in carrot roots and endopolygalacturonase in extracellular precipitate of *A. pullulans*[1] (Table I). From kinetic analysis of degradation of substrates with DP 2–8 (for carrot OGH shown in Table III) the affinity of this enzyme increased with increasing DP of substrate as

Table III
Kinetic analysis of degradation of $(GA)_{2-8}$ by OGH from carrot roots

Substrate $(GA)_n$	$10^5 \cdot K_M$ [mol l^{-1}]	V [μmol min^{-1}]	k_0 [s^{-1}]	log k_0	k_0/K_M	log k_0/K_M
$(GA)_2$	85.9	0.206	0.171	−0.766	199.5	2.30
$(GA)_3$	19.3	0.526	0.438	−0.358	2269.4	3.36
$(GA)_4$	10.2	0.625	0.521	−0.283	5105.9	3.71
$(GA)_5$	7.65	0.681	0.568	−0.246	7422.2	3.87
$(GA)_6$	5.70	0.703	0.586	−0.232	10280.7	4.01
$(GA)_7$	2.85	0.400	0.334	−0.477	11695.9	4.07
$(GA)_8$	2.04	0.367	0.306	−0.515	14990.2	4.18

was determined for both endo- and exo- polygalacturonases. The difference between these enzymes was observed in value of their maximal reaction rate on substrates with different DP, which increased with DP, reached the maximal value and than decreased.

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P50 ANTIOXIDANT ACTIVITY STUDY OF GAMMA-IRRADIATED OREGANO (*ORIGANUM VULGARE L.*)

MILAN SUHAJ and JANA RÁCOVÁ

Food Research Institute, Priemyselná 4, P. O. Box 25, SK-824 75 Bratislava, Slovak Republic, milan.suhaj@vup.sk

Introduction

Gamma-ray irradiation is now internationally recognized as an effective method to maintain the quality of spices for a long time. The Directive 1999/3/EC established a Community list of foods and food ingredients that may be treated with ionizing radiation and maximum overall average absorbed dose may be 10 kGy for dried aromatic herbs, spices and vegetable seasonings. Limitation of FDA for these foodstuffs has not to exceed 30 kGy.

Not many contributions concern to the study of influence of irradiation procedures on antioxidant activity of herbs and spices. Effects of γ-irradiation at 10 kGy on the free radical and antioxidant contents in nine aromatic herbs and spices (basil, bird pepper, black pepper, cinnamon, nutmeg, oregano, parsley, rosemary, and sage) were studied by Calucci et al.[3]. The effect of irradiation on antioxidant properties of seven dessert spices (anise, cinnamon, ginger, licorice, mint, nutmeg, and vanilla) was evaluated by Murcia et al.[4]. With respect to the non-irradiated samples, water extracts of irradiated spices at 1, 3, 5, and 10 kGy did not show significant differences in the antioxidant activity in the radical scavenging assays used. Sun-dried and dehydrated paprika (*Capsicum annuum* L.) samples were irradiated at doses from 2.5 to 10 kGy and capsaicinoid contents were analysed[5]. The increases of capsaicin, dihydrocapsaicin and homodihydrocapsaicin significantly increased about 10% with the dose of 10 kGy.

A variety of tests expressing antioxidant potency of food components has been suggested. These can be categorized into two groups: assays for radical scavenging ability and assays that test the ability to inhibit lipid oxidation under accelerated conditions[6]. Scavenging of the stable radical DPPH assay has been applied to characterize antioxidant activities extract oregano[6,7]. Antioxidant activity of oregano ethanol extract was determined according to inhibitory effect on oxidation model lipid system made up of refined bleached peanut oil[8].

Experimental

For the antioxidant activity study commercial oregano N. Bükey, A. S. from Camdibi – Izmir – Turkey, was used. The spice samples were irradiated using ^{60}Co source at doses of 5, 10, and 30 kGy according to commercial practices at Artim, s. r. o., Prague, Czech Republic. Determination of some antioxidant properties was made with extracts prepared from 2 g oregano extracted for 1 hour with 50 ml 80 % (v/v) water-methanol solution. DPPH radical scavenging assay was modified according to Bandoniené[9]. Thiobarbituric acid reactive substances were determined according to method by Zin[10]. Determination of reducing power was realized according to Chyau et al.[11]. Total phenolics were determined using the Folin-Ciocalteau modified method (Chaovanalikit and Wrolstad, 2004).
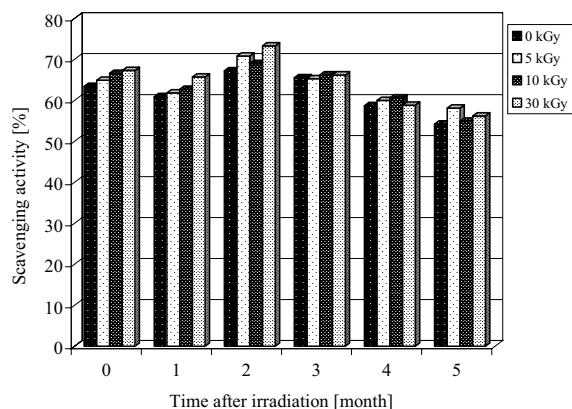
Fig. 1. **Effect of irradiation and storage time on DPPH radical--scavenging activity of oregano methanolic extracts**
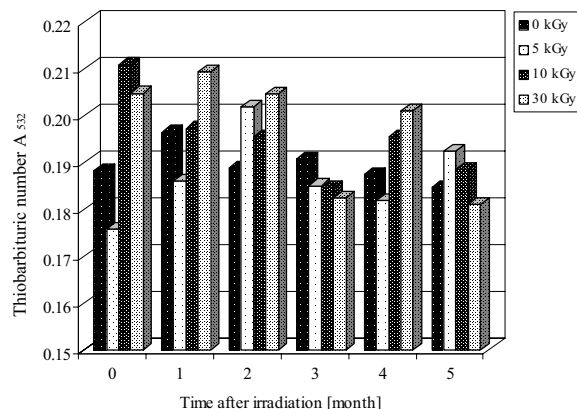

Fig. 2. **Effect of irradiation and storage time on thiobarbituric number of oregano methanolic extracts**
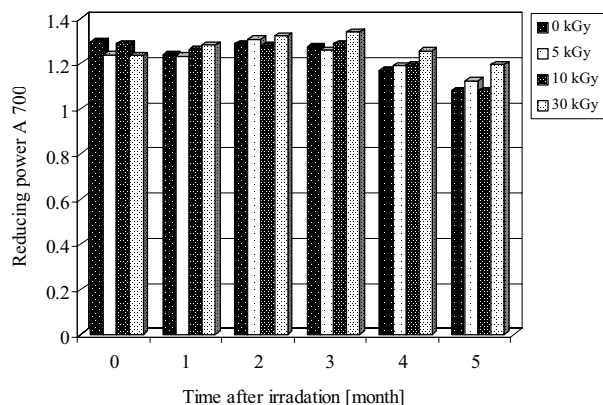

Fig. 3. **Effect of irradiation and storage time on reducing power of oregano methanolic extracts**
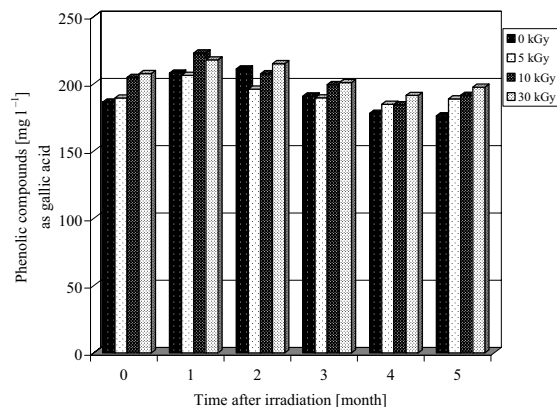

Fig. 4. **Effect of irradiation and storage time on total phenolics content of oregano methanolic extracts**

Results and discussion

Fig. 1. shows the results of DPPH scavenging activity of γ-irradiated oregano measured in methanolic extracts. Irradiation resulted in a statistically non-significant tendency of increasing DPPH scavenging activity immediately after oregano irradiation and after the first and second month of its storage. In comparison with effect of irradiation doses, storage caused more significant changes of DPPH activity.

Fig. 2. shows the effect of γ-irradiation doses and storage time on thiobarbituric acid number of methanolic extracts of oregano. The differences found, caused by the irradiation and storage time, were not statistically significant because of low precision of used analytical procedure for TBARS number determination.

Effect of irradiation and storage time on reducing power of oregano resulted in the similar changes as in the DPPH assay (Fig. 3.).

The most important changes were found in the case of total phenolic compounds determination (Fig. 4.). Statistically significant increase of content of these substances is proportional to the absorbed dose of gamma radiation. Storage of both non-irradiated and irradiated oregano samples

resulted in a moderate increase of the phenolics content in methanolic extracts.

Found significant and maximal antioxidant activity differences between non-irradiated and irradiated oregano at 30 kGy reached at irradiation effect 12 % measured by total phenolics increase. At storage conditions in all cases decrease of antioxidant activity was about 12 % measured by DPPH scavenging method, 16 % by reducing power, and 6 % by total phenolics content. Increase of oregano radical-scavenging activity may be due to the increase of dry matter content during the storage of this spice at laboratory conditions. This change in DPPH activity can be partially explained by increasing of yield from extraction of irradiated oregano, because of disruption of the cell wall structure and consequent higher oregano extrability.

Conclusions

Irradiation of oregano at the doses studied shows not so significant influences on the antioxidant activity as the storage conditions. With respect to non irradiated samples of oregano significant changes of antioxidant activity were observed only in the case of total content of phenolic com-

pounds, which were proportional to the doses of irradiation. Under the influence of storage were changes of antioxidant activities of oregano more markedly.

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P51 CARBONYL COMPOUNDS IN CONCENTRATES OF APPLE AROMA

PAVEL VALÁŠEK and OTAKAR ROP

Department of Food Engineering and Chemistry, Faculty of Technology, Tomas Bata University in Zlín, valasek@ft.utb.cz

Introduction

Carbonyl compounds belong to important components of apple aroma. A lot of them is very active sensorially and significantly participates in determination of generic, or variety aroma. They arise mainly as so called secondary aromatic substances in the time of maturing and during the processing of the fruits. After the separation of aromatic substances from the juice some components look like non-specific then, for example ethanal, but the others can be indicated as characteristic, for example *hexanal*, or *trans*-2-*hexanal*.

For the insulation of carbonyl compounds from concentrates of aromatic substances the condensation agents are frequently used, especially 2,4-dinitdophenylhydrasin, which creates with mono-carbonyl compounds the yellow to orange precipitate of 2,4-dinitrophenyl-hydrasons, or with dicarbonyl compounds the precipitate of appropriate osasons, respectively. These derivates can be very well determined by spectral photometric method, or, after the separation, by thin-layer chromatography method, or by the gas chromatography method, respectively. In this article the results are shown, which were obtained by separation of carbonyl compounds of apple aroma after their conversion on 2,4-dinitrophenyl-hydrasons by the thin-layer chromatography method. This method is relatively simple and does not require special laboratory or instrument equipment.

Experimental part

For the analysis, the industrially produced concentrates of aromatic substances from Linea Nivnice and Bajer Přerov companies were used.

From the samples, 10 ml of concentrate of apple aroma was pipetted and the same volume of the solution of 2% 2,4-dinitrophenylhydrazin in 35% perchloric acid. During the rule conditions, the appropriate 2,4-dinitrophenylhydrasons of carbonyl compounds were precipitated. After the precipitation was filtered off with help of the filter pot with frit, the precipitated 2,4-dinitrophenylhydrasons were transferred to the solution under the method conditions and the measurement of absorbance was performed at 360 nm in 1 cm cell.

The calibration line was constructed with help of *acetaldehyde* standard.

For the separation of carbonyl compounds the completed boards Silufol were used. Chromatograms were developed in the system of solvents – carbon tetrachloride, n-hexane, ethyl acetate, acetone in the ratio $100:20:10:5$ parts by volume. The separated 2,4-dinitrophenylhydrasons were compared with the standards from the point of view of elution data. The results were elaborated statistically and set to the tables.

Results and discussion

Reproducibility of determined R_f values

The obtained results of separated 2,4-dinitrophenylhydrasons on the thin layer and their statistical elaboration is shown in Table I. From the table it follows, that the separation of single spots is relatively good and the reproducibility of the separation shows the error mostly up to ± 2 %. Only in case of spots with low R_f values the rate of accuracy is lesser; 4; 4.5 and 8.4 %. It could be explained by the fact, that relative values are concerned, which value depends on *x*.

Identification and qualitative evaluation

The most frequently present 2,4-dinitrophenylhydrasons separated by chromatography on thin layers were identified with help of R_f values of the standards. They are shown in

Table I

R_f values of 2,4-dinitrophenylhydrasone spots separated by means of thin-layer chromatography

| Spot No. | Sample No. | | | | | Statistical data | |
| | 1 | 2 | 3 | 4 | x | $s_R\ 10^{-4}$ | M' [%] |
			R_f				
1	0.00	0.00	0.00	0.00	0.000		
2	0.11	0.12	0.12	0.11	0.115	49	8.4
3	0.21	0.21	0.22	0.22	0.215	49	4.5
4	0.48	0.48	0.47	0.49	0.480	97	4.0
5	0.83	0.82	0.82	0.83	0.825	49	1.2
6	1.01	1.00	1.00	0.99	1.000	97	1.9
7	1.16	1.16	1.15	1.17	1.160	97	1.7
8	1.47	1.48	1.48	1.46	1.473	97	1.3
9	1.75	1.76	1.75	1.76	1.755	49	0.6
10	1.93	1.94	1.94	1.96	1.943	146	1.5

Notation: x = arithmetic mean, s_R = standard deviation, M' = accuracy rate

Table II

Identification of 2,4-dinitrophenylhydrasones

Standard	Spot of thin-layer chromatography No.
acetaldehyde	6
benzaldehyde	8
hexanal	9
trans-2-hexenal	9
aceton	7
metyletylketon	8
cyklohexanon	8–9

Table II. From this table it follows, that some components were separated one from the other relatively hardly. For example, *hexanale* and *trans-2-hexanal* were separated as one spot.

Cyclohexanone showed an oblong spot, which ran from *methylethylketone* up to *hexanal*. The other components were not identified in more detail.

From the chromatograms there was calculated the percentage representation of single spots relatively to their total area. From these data, there was possible to calculate the quantitative representation of single components and to express them as acetaldehyde in mg l^{-1}. Table III shows these values. From them it is obvious, that the total volume of carbonyl components in single samples is different to a great extent, what reflects the different level of concentration of aromatic substances.

In the sample No. 1 the volume of carbonyl compounds was extremely high – 11594 mg l^{-1}, in the sample No. 4 was the lowest one – 212 mg l^{-1} only. Further, in this sample the presence of sugars and pectin substances was found, what reflects the fact, that the sample was polluted by the apple juice.

From the volume components, the *acetaldehyde* (40–60 %) is mostly represented and the spot, which had the

Table III

Representation of carbonyl compounds separated by means of thin-layer chromatography (expressed as acetaldehyde in mg l^{-1})

| Compound No. | Sample No. | | | |
	1	2	3	4
1	14	23	12	12
2	40	20	19	6
3	14	12	13	9
4	34	16	16	9
5	71	32	66	23
6	1054	505	364	80
7	163	30	30	18
8	49	29	20	12
9	105	64	92	18
10	50	30	39	24

same elution data as *acetone* (4–35 %) had. From the other components, mainly *trans-2-hexenal* (7–15 %), (except of the sample No. 4–0.7 % only), and *hexanal* are interesting. Remaining components are presents in small volumes only.

From the above-mentioned values it is evident, that from the volume of carbonyl components in concentrates of aromatic substances is possible to deduce on the total quality of these products (what directly correlates with the sensorial evaluation). The thin-layer chromatography method is suitable for the rough investigation of carbonyl compounds only, nevertheless, it is fully adequate for the current laboratory checks.

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P52　PHOTOCATALYTIC DISINFECTION OF WATER IN FLOW-REACTOR

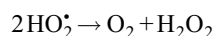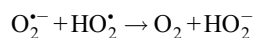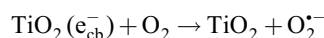MÁRIA VESELÁ, MARCELA HOŠÁKOVÁ, MICHAL VESELÝ, JANA CHOVANCOVÁ and JANA CHOMOUCKÁ

Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, vesela@fch.vutbr.cz
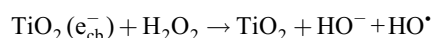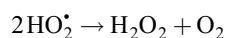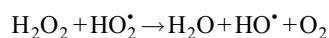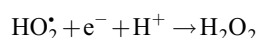
Introduction

Titanium dioxide photocatalysts attract a great attention as materials for photocatalytic sterilization, especially in the food and environmental industry.

Photocatalytic reactions with TiO_2 are used for organic compounds oxidation, metal reduction, disinfection of various surfaces and waters. As the photogenerated active oxygen species are formed at the irradiated TiO_2 surface, this system can be utilized for microbes deactivation instead of the conventional methods such as ultraviolet irradiation, heat treatment or chemical disinfectant dosage[1].

In the presence of O_2 the main way of the $HO^{\bullet}$ formation on TiO_2 surface is expressed by equations:

$$TiO_2\,(e_{cb}^-) + O_2 \rightarrow TiO_2 + O_2^{\bullet-}$$

$$O_2^{\bullet-} + H^+ \rightarrow HO_2^{\bullet}$$

$$O_2^{\bullet-} + HO_2^{\bullet} \rightarrow O_2 + HO_2^-$$

$$2\,HO_2^{\bullet} \rightarrow O_2 + H_2O_2$$

Superoxide anion and its protonated form subsequently dismute to yield hydrogen peroxide or peroxide anion. Those species can form hydroxyl radical.

$$HO_2^{\bullet} + e^- + H^+ \rightarrow H_2O_2$$

$$H_2O_2 + HO_2^{\bullet} \rightarrow H_2O + HO^{\bullet} + O_2$$

$$2\,HO_2^{\bullet} \rightarrow H_2O_2 + O_2$$

$$TiO_2\,(e_{cb}^-) + H_2O_2 \rightarrow TiO_2 + HO^- + HO^{\bullet}$$

In water solution, microorganisms in contact with irradiated TiO_2 surface are damaged by the action of hydroxyl radicals and active oxygen species generated on such surface. This process can lead to the changes of outer membrane permeability, which enables the reactive species to easily reach the cytoplasmic membrane. The structural and functional disorders of cytoplasmic membrane lead to the loss of cell viability and cell death.

The photocatalytic flow reactors usage is reported, especially for organic compound degradation and water sterilization. This process is used not only in laboratory scale[2], but in solar pilot-plants, too[3,4,5]. A typical photocatalytic flow reactor is designed as a system of parallel glass tubes, connected together by knee fittings permitting the studied suspension to circulate through the whole system.

A successful photocatalytic reaction requires approaching of microorganism cells to irradiated surface at very close distance. Only under these conditions the photogenerated species can react with the cells. Therefore, optimal flow parameters, determining an effective adsorption on the irradiated surface were studied. Under these conditions the photocatalytic process of yeast killing was performed.

Experimental

Materials

TiO_2 thin layer inside of glass tubes was prepared by sol-gel process using titanium tetraisopropoxide. The sol was applied on roughened inner surface of glass tubes. The created gel was calcinated at 450 °C to obtain titanium dioxide layer, predominantly in anatase form.

The yeast strains used were *Hansenula anomala* and *Candida tropicalis*, both supplied by Slovak Collection of Yeasts, Bratislava (Slovakia). The strains were inoculated into malt extract and cultivated for 24 hours.

Photochemical experiments

Aliquots of *Hansenula anomala* or *Candida tropicalis* cultures were diluted by sterilized water to obtain a suspension containing from 500 to 2000 CFU ml^{-1}, checked by Bürker chamber calculation. A 500 ml of the suspension were pumped through flow reactor using a peristaltic pump. The flow reactor was irradiated by medium pressure mercury lamp HPLN 125 W (Philips). Light intensity was measured by UV-meter (UV 340, Lutron) and was set to 0.55 mW cm^{-2} by the lamp distance adjustment. The samples were irradiated up to 240 min. In the course of the reaction samples were collected and the number of live cell was found out by cultivation on malt agar. Also an alternative method for cells calculation was used at the beginning and at end of reaction. 250 ml of reaction suspension was filtered through membrane filter and the 150 μl of $1.8 \cdot 10^{-4}$ M Acridine Orange was spread over the membrane filter. The solution of Acridine Orange was prepared by dissolution of 10 mg of Acridine Orange in 50 ml of water and filled with 100 ml of phosphate buffer with pH 6. Epifluorescence microscope Nikon was used for dead and live cell resolution and calculation. A 470–490 nm excitation filter, 520 nm barrier filter and 510 nm dichroic mirror were used.
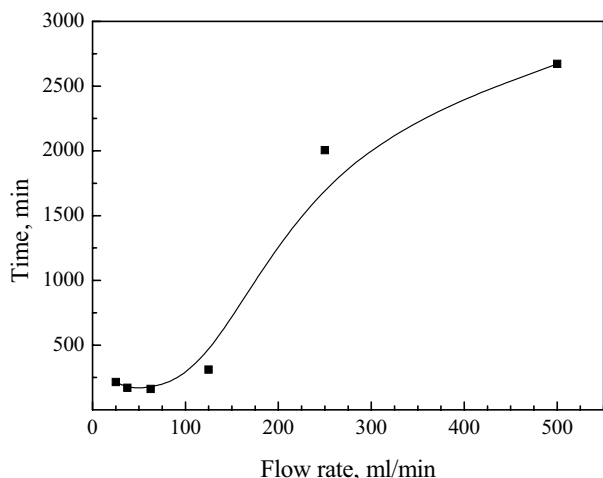
Fig. 1. **A required time of irradiation calculated for 50% cell killing, depending on flow rate in photocatalytic flow reactor**
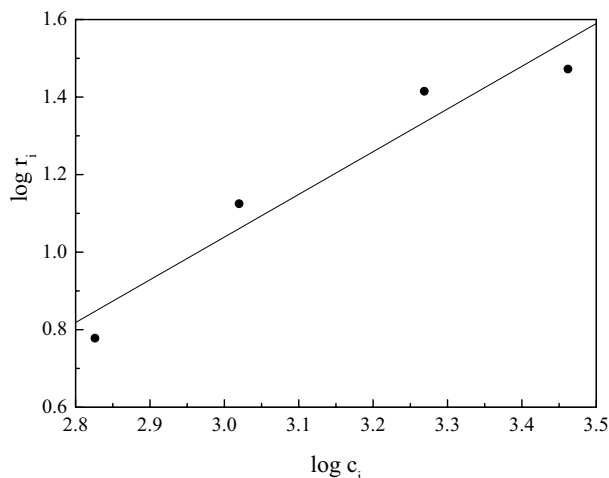
Fig. 2. **A plot of initial reaction rate on initial cell concentration**
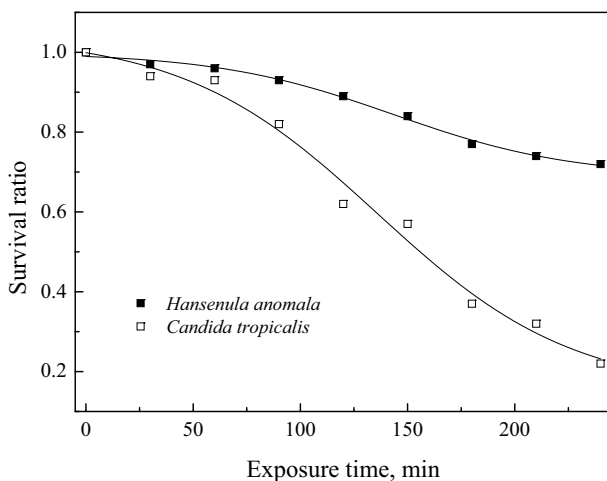
Fig. 3. **A survival ratio of yeasts in photocatalytic flow reactor. The incident light intensity 0.4 mW cm^{-2}**

Results and discussion

The flow rate significantly influences the photocatalytic yeast killing process. At a high flow rate the probability of both cell and reactive oxide species collision is very small. On the other hand, at a low flow rate, the cells tend to adsorb to the TiO$_2$ layer and are hold in the tubes. This leads to an effective killing process but the overall efficiency is very low. In Fig. 1., the time required for killing of 50 % yeast cells as a function of flow rate is plotted. According to these results, the flow rate 37.5 ml min^{-1} was chosen for further experiments.

The initial concentration of yeast cells is an important parameter. In Fig. 2., the dependence between initial concentration and initial rate, which is increasing, is shown. Initial rate could be expressed in the following form:

$$r_i = k \cdot c_i^n \ ,$$

where r_i is the initial rate of cell inactivation, c_i is the initial cell concentration, k is the rate constant and n is the reaction order.

The slope of a plot log r_i versus log c_i yields a reaction order value n. We obtained a value of 1.101 ± 0.212 and we can assume the first order kinetics consistency (Fig. 2.).

It was found that at the flow rate of 37.5 ml min^{-1} and light intensity of 0.4 mW cm^{-2} the cells of *Candida tropicalis* were more sensitive to oxidative attack than cells of *Hansenula anomala* (Fig. 3.). The similar results were obtained when these cells were subjected to higher light intensity.

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P53   YEASTS ON PHOTOCATALYTICALY ACTIVE SURFACE

MÁRIA VESELÁ, BLANKA KREMLÁČKOVÁ, MICHAL VESELÝ, JANA CHOVANCOVÁ and JANA CHOMOUCKÁ

Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, vesela@fch.vutbr.cz

Introduction

Particularly in microbiological laboratories and areas of intensive medical use, regular and thorough disinfection of surfaces is required in order to reduce the numbers of bacteria and to p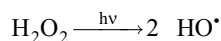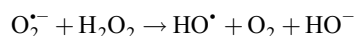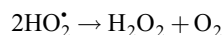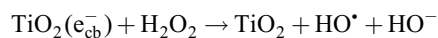revent bacterial transmission. Conventional methods of manual disinfection with wiping are not effective in the longer term, cannot be standardized, and are time-intensive and staff-intensive. In addition, there are problems associated with the use of aggressive chemicals[1].

Using thin and transparent TiO_2 layers coated on glass areas, ceramics and on other base materials could help to solve this problem. Titanium dioxide, as a wide band-gap semiconductor, can act as a sensitizer for light-induced redox processes due to its electronic structure, which is characterized by a filled valence band and an empty conduction band[2]. Irradiation of titanium dioxide particles leads to electron excitation to the conduction band. Both hole in the valence band and the electron in conduction band migrate to the particle surface. If no recombination occurs, this charge carriers can react with adsorbed molecules, e. g. with water, hydroxyl anion, oxygen or organic compounds. The hydroxyl radical generation can be expressed by the following equations.

$$TiO_2 \xrightarrow{h\nu} TiO_2\,(e^-_{cb}, h^+_{vb}) \rightarrow recombination$$

$$TiO_2\ (h^+_{vb}) + H_2O_{ads} \rightarrow TiO_2 + HO^\bullet_{ads} + H^+$$

$$TiO_2\ (h^+_{vb}) + HO^-_{ads} \rightarrow TiO_2 + HO^\bullet_{ads}$$

$$TiO_2\ (e^-_{cb}) + O_{2ads} + H^+ \rightarrow TiO_2 + HO^\bullet_2 \rightleftarrows O^{\bullet-}_2 + H^+$$

$$TiO_2(e^-_{cb}) + HO^\bullet_2 + H^+ \rightarrow TiO_2 + H_2O_2$$

$$TiO_2(e^-_{cb}) + H_2O_2 \rightarrow TiO_2 + HO^\bullet + HO^-$$

$$2HO^\bullet_2 \rightarrow H_2O_2 + O_2$$

$$O^{\bullet-}_2 + H_2O_2 \rightarrow HO^\bullet + O_2 + HO^-$$

$$H_2O_2 \xrightarrow{h\nu} 2\ HO^\bullet$$
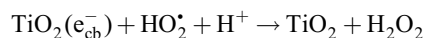
Furthermore, an organic molecule in close contact to TiO_2 undergoes oxidation, which can be described schematically:

$$HO^\bullet + D_{ads} \rightarrow D_{ox}$$

The hydroxyl radicals ($HO^\bullet$) generated by the TiO_2 photocatalyst are very potent oxidants and are nonselective in reactivity. When irradiated TiO_2 particles are in a direct contact with or close to microbes, an oxidative attack on the cell membranes can occur. Different mechanisms involved in the bactericidal action of TiO_2 photocatalysis have been proposed[3,4,5]. Results from the above cited studies suggest that the cell membrane is the primary site of the reactive photogenerated oxygen species attack. The oxidative attack of the cell membrane leads to lipid peroxidation. The combination of cell membrane damage and further oxidative attacks on internal cellular components ultimately results in the cell death.

We have studied the process of cell killing on irradiated titanium dioxide surface. As suitable cells we have chosen yeasts. The first strain was *Candida tropicalis*, harmful yeast in food industry, the second strain was *Saccharomyces cerevisiae*, the production yeast widely used in food industry. *Saccharomyces cerevisiae was chosen for comparing.*
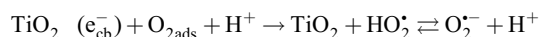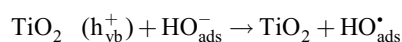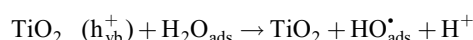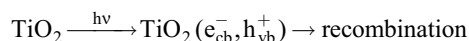
Experimental

Materials

TiO_2 thin layer on a glass plate was prepared by sol-gel process using titanium tetraisopropoxide and spin coating method. According to the rotation speed various layer thicknesses were obtained. The yeast strains used were *Saccharomyces cerevisiae* and *Candida tropicalis*, both supplied by Slovak Collection of Yeasts, Bratislava (Slovakia). The strains were inoculated into malt extract and cultivated for 24 hours. Acridine Orange was supplied from Biotech.

Photochemical experiments

Aliquots of cultures were diluted 10^4 times by saline solution and dropped onto the TiO_2 layer deposited on glass plate (150 µl). The plate was placed on the bottom of glass box with a constant humidity and irradiated by a medium pressure mercury lamp HPLN 125 W (Philips). Light intensity was measured by UV-meter (UV 340, Lutron) and was set to 0.265 mW cm^{-2} by lamp distance adjustment. The
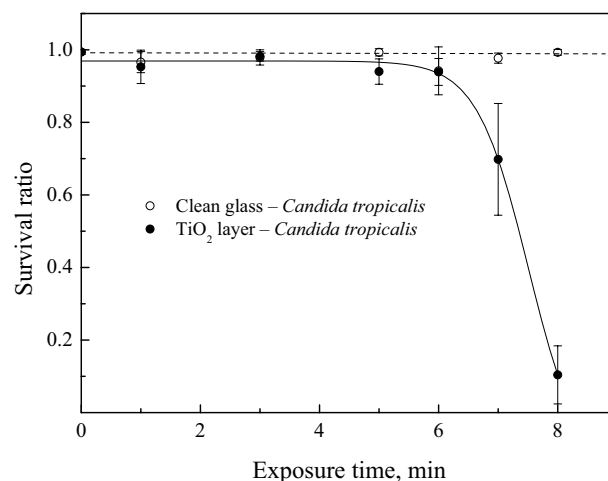

Fig. 1.   **Survival ratio of *Candida tropicalis* during irradiation on TiO_2 surface (solid circles) and on the clean glass (open circles)**
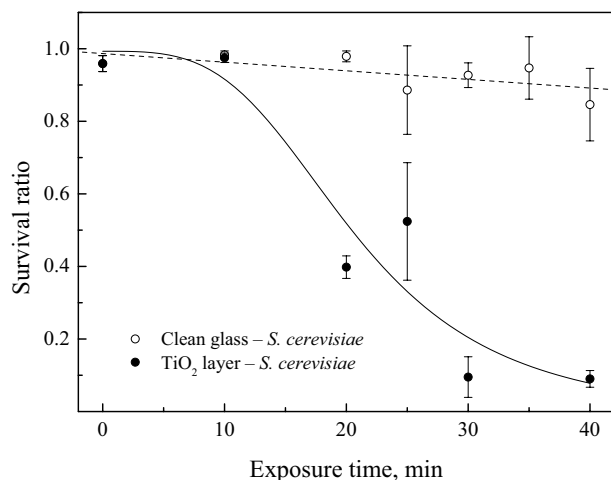
Fig. 2. **Survival ratio of *Saccharomyces cerevisiae* during irradiation on TiO$_2$ surface (solid circles) and on the clean glass (open circles)**

samples were irradiated up to 60 min. After exposure 100 μl of $1.8 \cdot 10^{-4}$ M Acridine Orange was added to the drop of irradiated sample. The solution of Acridine Orange was prepared by dissolution of 10 mg of Acridine Orange in 50 ml of water and filled with 100 ml of phosphate buffer with pH 6. Epifluorescence microscope (Nikon Eclipse 200 with epifluorescence adapter) was used for dead and live cell resolution and calculation. The 470–490 nm excitation filter, 520 nm barrier filter and 510 nm dichroic mirror were used. The images were recorded by Pixelink PL–A662 CCD camera (Pixelink Canada) and processed by Lucia software.

Results and discussion

As the cell calculation and live/dead cell resolution was not possible by the usual way in Bürker chamber, another procedure was proposed. The Acridine Orange dye is capable to bind to DNA in dead cell and in epifluorescence microscope this complex emits red light. Then the dead cells appear as red and live cell as green in colour. A random selection of 20 places on a sample was made and images were saved. On each image the number of live and dead cells was calculated and expressed the survival ratio – number of live cells to total number of cells in each image. The obtained survival ratio was averaged and processed by statistical methods.

It was found that *Candida tropicalis* (Fig. 1.) is more sensitive to oxidative attack by the hydroxyl radical and the highly reactive oxygen species on irradiated TiO$_2$ layer compared to *Sacharomyces cerevisiae* (Fig. 2.). After 10 minutes, all *Candida tropicalis* cells were killed while the *Sacharomyces cerevisiae* survided and were completely killed after 40 minutes.

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P54 INORGANIC AND ORGANIC SELENIUM SPECIATION USING HPLC-HEATING-UV-HG-AFS

EVA VITOULOVÁ, MARCELA DREXLEROVÁ, MARTINA KŘEČKOVÁ and MIROSLAV FIŠERA
Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, xcvitoulova@fch.vutbr.cz

Introduction

Selenium is an essential nutrient at low concentrations, but is toxic for humans and animals at high doses. It is a component of the enzyme glutathione peroxidase, which is one of the antioxidant defence systems of the body, catalyses intermediate metabolic reactions, and inhibits the toxicity of some heavy metals[1].

The effect of the element on human health is highly dependent on the chemical species under which it is consumed. Selenium exists in different chemical forms, as inorganic (selenite and selenate) and as organic species (selenoaminoacids, selenoproteins), in environmental and biological matrices. The nutritional bioavailability and cancer chemoprotective activity of selenium depend on the concentration and the chemical form in which it is present[2,3].

The availability of analytical techniques for the separation and determination of the compounds of an element at trace level has gained considerable importance. In this context, hyphenated techniques are those most frequently used. For selenium, speciation is necessary because of the differing mobilities, toxicities and bioavailabilities of its compounds[4].

Analytical systems developed for the speciation of selenium species employ a powerful high-performance liquid chromatography (HPLC) coupled to a specific atomic detector with a high efficiency sample introduction system[5]. Spectrometry methods are those most widely used as a detection system. Atomic fluorescence spectrometry (AFS) and inductively coupled plasma – mass spectrometry (ICP-MS) have been incorporated with very good results. Atomic spectrometry methods are the most widely used because of their high selectivity and sensitivity[4].

Experimental

I n s t r u m e n t a t i o n

The HPLC system consisted of an HPLC pump (Pye Unicam PU 4011, Philips) equipped with a six-port sample

injection valve (C & D, Ecom) and a 20 µl loop for sample introduction. The separation of the selenium species occurred in column Hamilton PRP X-100 (250×4.1 mm, 10 µm). Atomic fluorescence spectrometry (AFS) detection was achieved with the Excalibur detector (PS Analytical, Orpington, Kent, UK) using a boosted-discharge hollow cathode Se lamp (Fig. 1.).
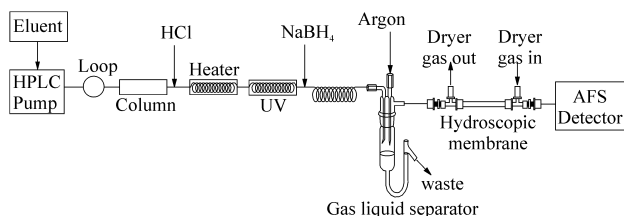

Fig. 1.   **Scheme of the HPLC-Heating-UV-HG-AFS**

Results and discussion

The concentrations of hydrochloric acid and sodium tetrahydroborate solutions were optimised. The best results were obtained with 32 % HCl and 1 % $NaBH_4$ in 0.1 M sodium hydroxide solution.

For the separation of the four selenium species we used a polystyrene-divinylbenzene-based anion-exchange column – Hamilton PRP X-100. Firstly we studied the dependence of the retention time of the four species on the pH of the phosphate solution used as mobile phase. The flow of the mobile phase was 1 ml min^{-1}. Inorganic species selenite and selenate required the use of an alkaline solution as mobile phase to obtain low retention times. Selenoaminoacids Se-(methyl)-selenocysteine and L-(+)-selenomethionine showed very high retention times at alkaline pH, whereas at lower pH values retention times were shortened. By working at pH 6.5 we were able to separate all mentioned selenium species, but peaks for SeCys and selenite were joined in part. The species were eluted in the following order: SeCys, selenite, SeMet and selenate. Secondly we varied the concentration of the mobile phase. Several concentrations of the mobile phase were tested, ranging from the 20 mmol l^{-1} to the 80 mmol l^{-1}. The concentration 40 mmol l^{-1} was chosen as the best, because this concentration gave sufficient resolution. Thirdly we tried to change the composition of the mobile phase, by adding of the methanol to the mixture. This addition of methanol had no significant influence on the separation. Forthcoming work will be directed to another optimisation of parameters and testing of another mobile phase, column etc. When we will be able to separate all species in a good quality, the procedure will be applied to determination of biological samples.

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P55   CHANGES OF FATTY ACIDS IN STERILIZED PROCESSED CHEESE

EVA VÍTOVÁ[a], BLANKA LOUPANCOVÁ[a], FRANTIŠEK BUŇKA[b], LENKA HROZOVÁ[a] and JANA ZEMANOVÁ[a]

[a]*Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, evavitova@post.cz,* [b]*Faculty of Technology, University of Tomas Baťa, T. G. Masaryk square 588, 760 01 Zlín, Czech Republic.*

Introduction

Processed cheese are produced by comminuting, blending and melting one ore more natural cheeses and optional ingredients into a smooth homogeneous blend with the aid of heat, mechanical shear and emulsifying salts. Optional ingredients, which are determined by the product type, include dairy ingredients, vegetables, meats, stabilizers, emulsifying salts, flavours, colours, preservatives and water.

The most important part of processed cheese manufacture is heat treatment of the blend, while constantly agitating. It has two main functions:
– to kill any potential pathogenic and spoilage microorganisms, and thereby extend the shelf-life of the product
– to facilitate the physicochemical and microstructural changes which transform the blend to an end product with the desired characteristics.

Processing is performed in batch cookers. The temperature-time treatment varies (70–95 °C for 4–15 min), depending on the formulation, extent of agitation, the desired product texture and shelf-life characteristics.

Today, global production of processed cheese increase steadily, factors contributing to this fact include versatility of processed cheese, their nutritive value (as a source of calcium and protein), relative low cost compared to most natural cheeses, convenient portion size and packaging, excellent preservation, functionality etc. There are various types of processed cheese depending on the particular region where they are produced[1].

Attempts to increase the shelf-life of processed cheese were inspired by the possibility of increased cheese trade, via the production of more stable transportable products. Thermosterilization is practically the only method how to elongate the durability of processed cheese. However, if processed cheese is sterilized in order to achieve a longer shelf life, sterilization temperature influences all the cheese components[2]. The aim of this work was to monitor changes of fatty acids in sterilized and non sterilized processed cheeses.

Experimental

S a m p l e s

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 was 5 minutes. Non sterilized cheeses were consequently cooled down to 10 °C in 2 hours. Sterilized cheeses were heated in the sterilizer at 117 °C for 20 minutes and than cooled.

M e t h o d s

Cheese lipids were extracted by the mixture of diethyl ether and petrol ether. The fatty acids were analyzed as methyl esters by gas chromatography; methanol esterification using potassium hydroxide catalysis was used for preparing of the fatty acids methyl esters. The methods are described in a previous paper[3].

Results and discussion

Chemical composition of processed cheese depends on a processed cheese type and raw materials used[2,4]. Milk fat contained in processed cheese is mainly composed of saturated fatty acids with up to 20 °C chain length that make up almost 70 % total fatty acids. The most important saturated fatty acids are myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). Unsaturated fatty acids are represented mainly by oleic acid (C18:1), linoleic (C18:2) and linolenic (C18:3) acids[1,2].

If processed cheese is sterilized, sterilization temperatures used influence all the cheese components including protein and fat. At temperatures exceeding 100 °C both unsaturated and saturated fatty acids are exposed to oxidation processes. Primary products of autooxidation are hydroperoxides of fatty acids that are very unstable and enter other reactions producing numerous substances such as aldehydes, cyclic peroxides, epoxyacids, hydroxyacids, hydrocarbons, etc. The most important process affecting proteins during the sterilization is the Maillard reaction. Intermediate products or final products of the Maillard reaction can influence a number of other processes including lipid oxidation[2].

Fatty acids identified in processed cheese samples are listed in Table I, the chromatograms of fatty acids in sterilized and non sterilized processed cheeses are compared in Fig. 1. As can be seen, the sterilization process probably has no significant effect on total amount and composition of fatty acids present in processed cheese.

Table I
The fatty acids identified in processed cheese

| Fatty acid		Retention time [min]
Caproic	C6:0	14.31
Caprylic	C8:0	16.29
Capric	C10:0	18.25
Undecanoic	C11:0	19.11
Lauric	C12:0	20.10
Tridecanoic	C13:0	20.93
Myristic	C14:0	21.93
Myristoleic	C14:1n9c	22.62
Pentadecanoic	C15:0	22.71
Palmitic	C16:0	23.82
Palmitoleic	C16:1n9c	24.31
Heptadecanoic	C17:0	24.57
cis-10-Heptadecenoic	C17:1	25.28
Stearic	C18:0	25.48
Oleic	C18:1n9c	26.45
Linolelaidic	C18:2n6t	26.80
Linoleic	C18:2n6c	27.49
γ-Linolenic	C18:3n6	28.55
cis-11-Eicosenoic	C20:1	28.66
Linolenic	C18:3n3	29.02
Heneicosanoic	C21:0	29.11
cis-11,14-Eicosadienoic	C20:2	30.27
Behenic	C22:0	30.85
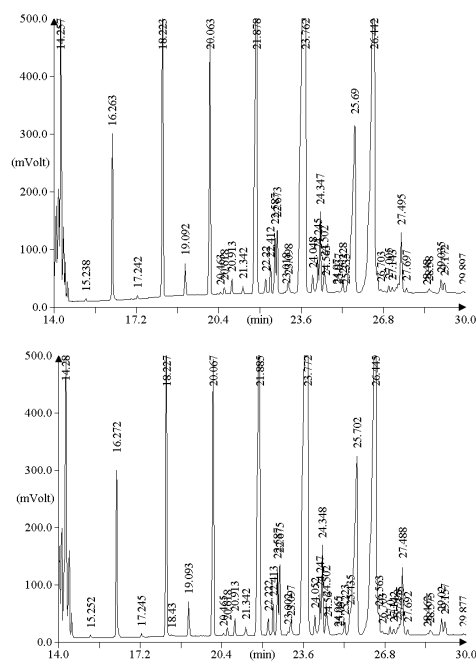
Fig. 1. **Chromatograms of fatty acids identified in non sterilized (upper) and sterilized (bottom) processed cheeses. GC column SP^{TM} 2560 (100 m × 0.25 mm × 0.2 μm), temperature programme 60 °C 2 min, 10 °C min^{-1} up to 220 °C, 20 min. Carrier gas N$_2$ 1.2 ml min^{-1}**

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P56 PCR-RFLP METHOD FOR IDENTIFICATION OF SCCHAROMYCES SPECIES

DANA VRÁNOVÁ[a] and RENATA VADKERTIOVÁ[b]

[a]*Faculty of Chemistry, Brno University of Technology, Purkyňova 118, Brno, Czech republic, vranova@fch.vutbr.cz,* [b]*Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava, Slovakia, Renata.Vadkertiova@savba.sk*

Introduction

Yeasts are important microorganism for the food industry, and contribute in a positive way in the processing and/or rippening of wine, beer, bread, cheese.

For identification, typing and detection of spoilage yeasts, to trace rotes and sources of contamination in the food production chain, it is necessary to have adequate tools.

Traditional methods do not allow fine typing of yeasts at the subspeciales level, witch is essential for tracing routes of contamination. DNA-based methods have advantages over the traditional phenotypic methods, since they are not influenced by enviromental conditions are allow diferentiation at various levels ranging from species to strain.

The aim of this study was potential using RFLP-PCR method to identify of Saccharomyces species (17 strains, for the time being). This method proved useful to classify of the strains which could not be assigned previously by genetic methods and should be readilly extendable to the autentification of all strains of this genus.

Experimental part

C h e m i c a l s

Agarose, Taq DNA polymerase, 10× Taq DNA buffer, primers, Ultra Clean 20 bp Ladder and Ultra Clean Microbial DNA Isolation Kit were supplied by Elisabeth Pharmacon (CZ). Ethidium bromide, EDTA, boric acid, ethanol, 2-mercaptoethanol, ammonium persulfate, bromphenol blue dye and other chemicals were purchased from Serva (analytical and/or research grade). dNTP mix, DNA size standards: Lambda DNA/EcoRI, HindIII, GeneRuler™DNA Ladder Mix, restriction enzymes: AluI, TaqI, BshNI, EcoRI, DraI (AhaIII), HinfI, Eco881 (AvaI), Hin61 (HhaI), BsuRI (HaeIII), TruI (MseI) were purchased from Fermentas.

Y e a s t   s t r e i n s

Streins used in this study were obtained from the Culture Collection of Yeasts, Bratislava, Slovakia. They are listed in Table I. Cells were directly collected from a fresh yeast colony using a microbiological loop.

Table I
Strains studied

Number		Strains
1	21-9-1	Saccharomycesexiguus
2	21-53-2	Saccharomycesparadoxus
3	21-31-10	Saccharomycesparadoxus
4	21-4-96	Saccharomycescerevisiae
5	21-4-93	Saccharomycescerevisiae
6	21-31-6	Saccharomycesbayanus
7	21-22-5	Saccharomycesunisporus
8	3	Saccharomycescerevisiae
9	21-15-5	Saccharomycesbayanus/cerevisiae
10	231	Saccharomycescerevisiae
11	21-13-4	Saccharomycesbayanus
12	21-58-1	Saccharomycesmikatae
13	21-13-1	Saccharomycesbayanus
14	21-57-1	Saccharomyceskudriavzevii
15	21-6-7	Saccharomycespastorianus
16	21-56-1	Saccharomycescariocanus
17	21-5-1	Saccharomyceskluyveri

D N A   i s o l a t i o n ,   P C R   r e a c t i o n ,   r e s t r i c t i o n   d i g e s t s

DNA was extracted and purified by Ultra Clean Microbial DNA Isolation Kit. 3–10 ng of DNA in 1–2 μl of TBE buffrer were used in a 50 μl amplification reaction. DNA was checked on a 0.7% agarose gel. PCR micture contained in 50 μl Taq polymerase buffer, 0.02 μM dNTP, 0.02 μM of each primer (Lower: 5′TGT CTT CAA CTG CTT T 3′; Upper: 5′AAC GGT GCT TTC TGG TAG 3′), 1 unit of Taq polymerase. PCR amplification was carried out in a PTC-100™ thermocycler (MJ. Research, Inc.). PCR conditions were as followes: initial denaturation at 94 °C for 4 min; 25 cycles of amplification: denaturation at 94 °C for 1 min, annealing 48 °C for 30 sec, extension at 72 °C for 1 min, and final extension was at 72 °C for 10 min. Amplification products were analysed on a 0.7% agarose gel in 0.5 × TBE buffer.

The PCR products were cleared by ethanol precipitation and about 250 ng (1 μl of the reaction micture) amplified DNA was digested at 16 hours/37 °C with 5–10 units of various restriction enzymes. The fragments were resolved on a 2% agarose gel in 0.5 × TBE. DNA was stained with ethidium bromide and gels were analysed on Scion Image PC programme.

Results and discussion

This method was applied to test the possibility of identification of various strains of the genus *Saccharomyces*. We

tested ancient type strains and new strains which were isolated from different environments (Table I). A 1320 pb nucleotide fragments were expected after amplification of template DNA. They were discriminated on agarose gel electroforesis (Fig. 1.) Afterwards each amplification product was digested by warious restriction enzymes (Fig. 2.) The restriction pattern was analysed.
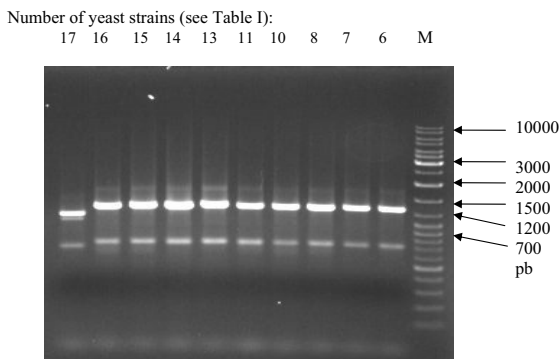
Number of yeast strains (see Table I):

Fig. 1. **PCR products from studied streins of genus Saccharomyces. M: Size marker λ/Eco RI/Hind III**
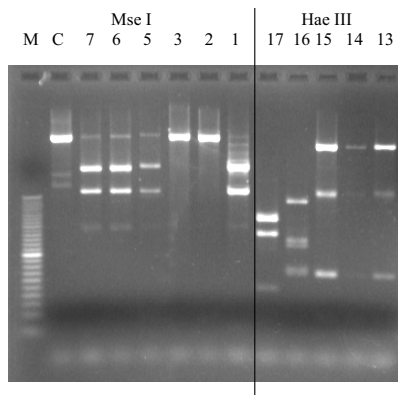

Fig. 2. **Example of digestion patterns of PCR products generated by MseI and Hae III. M: 20 pb size marker, C: control sample of DNA (PCR product)**

Conclusion

The size of the PCR products and their restriction patterns obtained with endonucleases yield a unique profile for each of species. The use of this approach is proposed as a new rapid and easy method of yeasts identification.

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P57 ACETYLCHOLINESTERASE INHIBITORS FROM MICROALGAE

PETR ZELÍK[a], JIŘÍ KOPECKÝ[b] and ALEXANDER ČEGAN[c]

[a]*Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic, zelik@fch.vutbr.cz,* [b]*Department of Autotrophic Microorganisms, Institute of Microbiology, Academy of Sciences of the Czech Republic, Opatovický mlýn, 379 01 Třeboň, Czech Republic, kopecky@alga.cz,* [c]*Department of Biological and Biochemical Sciences, Faculty of Technical Chemistry, University of Pardubice, Štrossova 239, 530 03 Pardubice, alexander.cegan@upce.cz*

Introduction

Alzheimer's disease (AD) is neurodegenerative disorder characterized by a gradual impairment of memory and cognitive function. Decrease in levels of cholinergic neurotransmission of central nervous system is associated with memory deficits and other cognitive disorders. Therefore several possibilities were proposed to enhance cholinergic deficit in AD patient. Acetylcholinesterase (AChE) inhibitors seem to be the most effective way to improve cognitive symptoms. Different types of AChE inhibitors have been studied but only several of them (eg. donepezil, rivastigmine, galanthamine) have been approved for the treatment of AD. There is requirement for the new AChE inhibitors because no drug of choice for the treatment of this disease has been decided till now. Searching for new AChE inhibitors is especially focused on higher plants or modification of chemical structures of known AChE inhibitors through the organic synthesis. It is astonishing that no screening of autotrophic microorganisms – algae and cyanobacteria – wasn't still done.

Natural compounds are an important source of pharmaceutical products. Number of active compounds have been derived from streptomycetes and fungi. A major problem in the search for new, biologically active molecules, is the rediscovery of previously known natural products. One way to overcome this problem is to develop selective bioassay directed on specific therapeutic target[1]. Another approach is to look for new and different source organisms of natural products. Cyanobacteria undoubtedly represent such a source[2–4]. Great variability of cyanobacteria (there are known more than 30 000 species in the nature) represent almost inexhaustible source of natural compounds. In contrast to streptomycetes, fungi and higher plants as a classical source of bioactiteve compounds, the cyanobacteria have been largely ignored so far.

In an attempt to find new AChE inhibitors of microalgal origin, approximatelly 200 species of algae and cyanobacteria have been screened for AChE inhibitory activity. The crude extracts of active strains were analyzed using HPLC-MS[n]. The fraction with anti-AChE activity was determined in the crude extract of the most active strain Nostoc sliz. kol.

(*SV-mol, ISB 93, tok Soj, 5/97, DE*). Next, isolation method for AChE inhibitor from this strain, *Nostoc sliz. kol.*, was developed.

Methods

Determination of AChE activity: Methanolic-tetrahydrofuran extracts and dichloromethane extracts were evaporated to dryness and redissolved in the same volume of methanol. Ellman's method has been optimized for microplate assay and used for determination of AChE inhibition activity. The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using pure methanol instead of methanolic extract. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. Every experiment was done in triplicate.

HPLC- MS analysis: The active extracts were analysed by HPLC/ESI-MS/MS on Agilent 1100 MSD SL-Ion Trap mass spectrometer. The ZORBAX Eclipse XDB-C8 column (150×4.6 mm, 5 μm) was used for HPLC analyses. Elution was realized in gradient mode of methanol – water with addition of formic acid.

Determination of AChE inhibitor from biomass of Nostoc sliz. kol.: The active fraction has been determined through the fractionation of the crude methanolic extract using HPLC and testing of these fractions for AChE inhibitory activity (analysis condition mentioned before).

Isolation AChE inhibitor from biomass of Nostoc sliz. kol.: The crude methanolic extract of freeze-dried biomass was evaporated to dryness and dissolved in the mixture of acetone-hexane ($3:7$, V/V). This solution was chromatographed in the system of acetone-hexane ($3:7$, V/V) on silicagel in gradient mode. Back fraction containing mainly AChE inhibitor was evaporated to dryness. Evaporation residue (AChE inhibitor concentrate) was disolved in minimum volume of methanol and preparative HPLC technique was used to collect the active fraction. WATREX Reprosil C$_8$ column (250×10 mm, 5 μm) and mobile phase of methanol – water in gradient mode were used in preparative HPLC.

Results and discussion

The crude biomass methanolic-tetrahydrofuran extracts, biomass methanolic extracts and dichloromethane extracts of cultivation medium of more than 200 microalgae species have been tested for AChE inhibitory activity using Ellman's method which was optimized for microplate assay. The results of AChE inhibitory activity of the tested crude biomass extracts is summarized in Table I. Any of the cultivation medium extracts proved AChE inhibitory activity.

The crude extracts of active strains were analyzed by HPLC-MS[n]. The crude extract of the most active strain *Nostoc sliz. kol.* (*SV-mol, ISB 93, tok Soj, 5/97, DE*) was fractionated to determine the active fraction. The only fraction with anti-AChE activity was the the peak with retention time of 23.6 min (Fig. 1.) and prominent molecular ion at m/z 799.5.

The molecular weight was estimate by altering of positive and negative ionisation to 798.

Isolation method for AChE inhibitor from this strain, Nostoc sliz. kol. was also developed (mentioned in chapter Methods) because of its future kinetics study (inhibition type, IC$_{50}$) and structure elucidation by NMR.

Table I
AChE inhibitory activity of the tested crude biomass extracts of algae and cyanobacteria species.

Algae/cyanobacteria specie	Inhibitory activity [%]
Monodus subteratus	99.7
Nostoc sliz. kol. (*SV*-mol, *ISB 93, tok Soj, 5/97, DE*)	98.4
Nostoc ellipsosporum (*2, GM,* Štěbal)	96.5
Nostoc clipsespor	84.9
Geminella terricola	58.8
Monodopsis subterranea	51.8
rest of the tested species	< 50
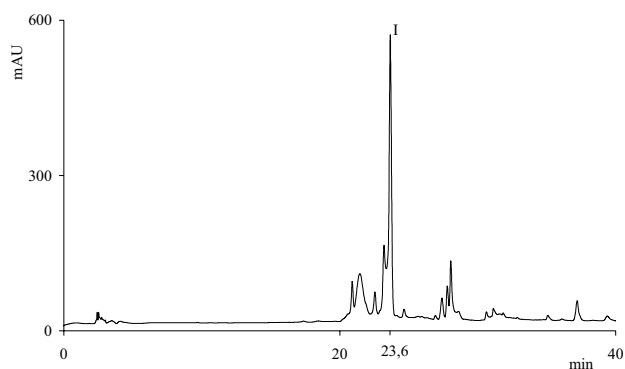
Fig. 1. **HPLC chromatogram of crude methanolic extract from biomas of Nostoc sliz. kol.; I – fraction with AChE inhibitory activity**

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P58 MONITORING OF LOW MOLECULAR MASS PEPTIDES IN MOULD CHEESE

JANA ZEMANOVÁ, ANEŽKA SEDLÁKOVÁ, MONIKA DUNAJOVÁ and EVA VÍTOVÁ

Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic,
zemanova@fch.vutbr.cz

Introduction

Cheese, milk and a variety of other products containing milk proteins are an integral part of the diet for much of the world's population. The overwhelming majority of these products currently are derived from bovine milk. The fresh cow's milk typically contains 3–3.5 % protein, of which approximately 80 % is casein, 15 % whey proteins, and the remainder a variety of small molecules collectively termed nonprotein nitrogen. The casein fraction exists almost exclusively in spherical particles termed micelles, and is comprised of four major proteins, α_{S1}-, α_{S2}-, β- and κ-casein[1].

Peptides in cheeses arise from the proteolysis of milk casein and its fractions in the process of ripening of cheeses.

The ability to assess the relative composition and integrity of proteins and peptides in cheeses and other dairy foods is important because these molecules have a profound effect on product functionality and quality[2].

Proteolysis is a major event in cheese ripening and contributes critically to the sensory properties of the finished product. Therefore, study of the nitrogenous compounds is necessary for the chemical characterization of the maturation process. The water soluble extract (WSE) of ripened cheese, which consist mainly of peptides and aminoacids, has a significant relationship to be intensity of flavour[3].

Experimental

We have used a Czech blue-veined cheese Niva for the analysis and we have prepared a water soluble extract (WSE). It was prepared by an aqueous extraction by *Kuchroo and Fox*[4,5] to isolate nitrogen compounds. Hundred grams of cheese (54 days old) were grated, mixed with deionized water and homogenized for 5 minutes at 40 °C by using a stomacher. The homogenate was then held for 1 hour at 40 °C. The samples were centrifuged at 3000 g for 30 minutes at 4 °C. The suspension was finally filtered through glasswool. WSN (water soluble nitrogen) extract was added to a 500 ml beaker. The pH value was measured and, if necessary (>5.5), adjusted with 1 mol l^{-1} hydrochloric acid solution to 5.5. The obtained extract was further fractionated and recleaned. First, high molecular peptides and proteins had to be precipitated by ethanol. So it was prepared an ethanol soluble extract (ESE) in 30% ethanol, 70% ethanol and 80% ethanol.

30% ethanol soluble extract (ESE):
3 ml of 960 g/kg ethanol was added to the 7 ml of WSN extract.

70% ethanol soluble extract (ESE):
9.5 ml of 960 g/kg ethanol was added to the 3.5 ml of WSN extract.

80% ethanol soluble extract (ESE):
10 ml of 960 g/kg ethanol was added to the 2 ml of WSN extract.

All suspensions were held at 25 °C overnight and then filtered through glasswool.

Then the ethanol from ethanol soluble fractions was evaporated and the samples were dissolved in 2 ml of sample buffer. The fractions obtained by this procedures were further analysed by the capillary zone electrophoresis.

Results and discussion

It was prepared a water soluble extract (WSE) and then an ethanol soluble extract (ESE) by the precipitation of water soluble fraction (WSE) by ethanol. After evaporating of solvent were this 3 samples dissolved in sample buffer (the mixture of deionized water and background elektrolyte in ratio 1:1) and analysed by capillary zone electrophoresis at optimalized conditions (for scheme see Fig. 1.). The CZE conditions were found by the analysis of the mixture of 5 oligopeptides (glycylglycine, glycylglycylglycine, leucylglycylglycine, glycylasparagine and glycyltyrosine). The optimi-
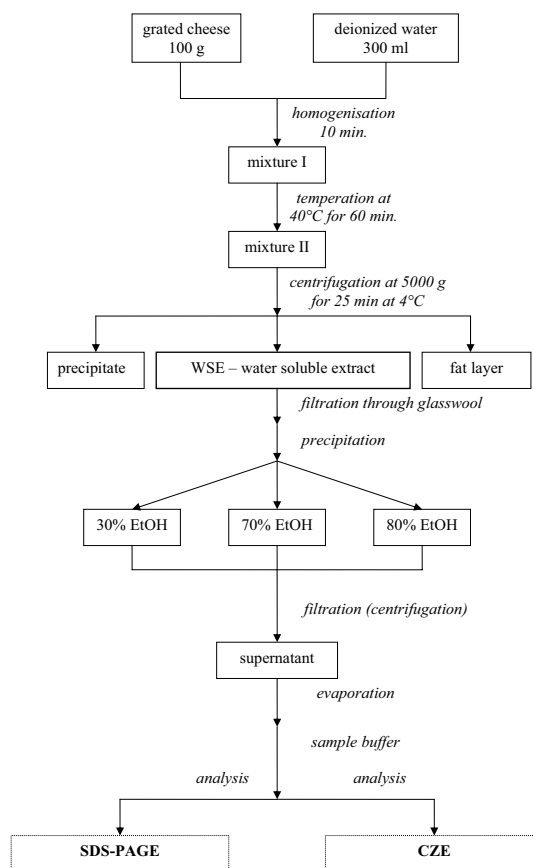
Fig. 1. **Scheme of preparation of samples**
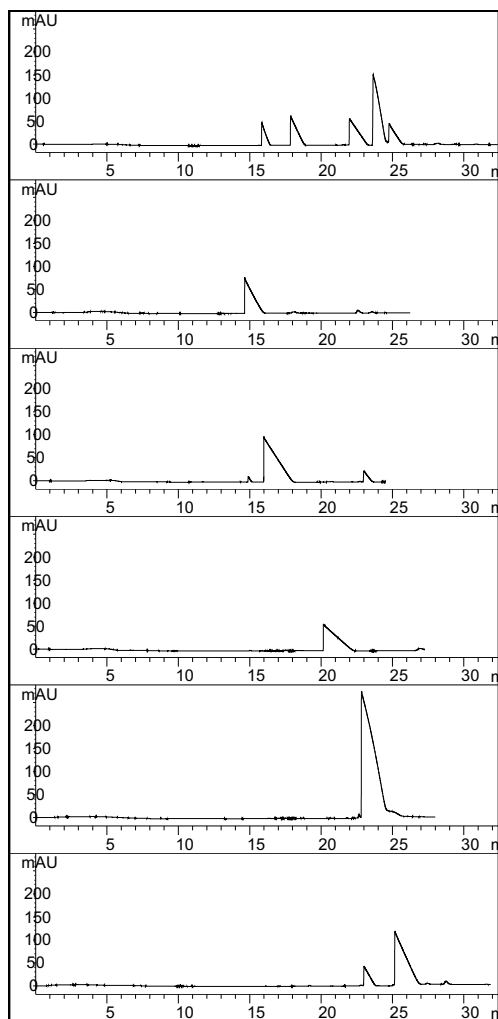
Fig. 2.   **CZE of a mixture of 5 oligopeptides and of the individual oligopeptides – peak No.1 is glycylglycine (GG), peak No.2 is glycylglycylglycine (GGG), peak No.3 is leucylglycylglycine (LGG), peak No.4 is glycylasparagine (GA) and peak No.5 is glycyltyrosine (GT). Concentration of each peptide was 2 mg ml^{-1} and they was dissolved in the mixture of deionized water and background elektrolyte in ratio 1:1**
Conditions of separation: background elektrolyte 0.05 mol l^{-1} phosphate with 0.1% HEC at pH 2.5 (adjusted with 1 mol l^{-1} sodium hydroxide solution), polyethylenoxide (PEO) coated capillary, ID 100 μm, effective length 70 cm, total length 77 cm, temperature 35°C, voltage 23 kV, injection 3 sec. at pressure 50 mBar

Fig. 3.   **CZE of ethanol soluble extract of cheese peptides – 30% ethanol soluble extract (1st graph), 70% ethanol soluble extract (2nd graph), 80% ethanol soluble extract (3rd graph). 10 ml of each ESE were after evaporation of ethanol dissolved in the mixture of deionized water and background elektrolyte in ratio 1:1.** Conditions of separation: background elektrolyte 0.05 mol l^{-1} phosphate with 0.1% HEC at pH 2.5 (adjusted with 1 mol l^{-1} sodium hydroxide solution), polyethylenoxide (PEO) coated capillary, ID 100 μm, effective length 70 cm, total length 77 cm, temperature 35°C, voltage 23 kV, injection 3 sec. at pressure 50 mBar

zed conditions of separation were as follows: background elektrolyte 0.05 mol l^{-1} phosphate with 0.1 % HEC at pH 2.5 (adjusted with 1 mol l^{-1} sodium hydroxide solution), polyethylenoxide (PEO) coated capillary, ID 100 μm, effective length 70 cm, total length 77 cm, temperature 35°C, voltage 23 kV, injection 3 sec. at pressure 50 mBar (Fig. 2.).

According to the graphs on Fig. 3. it was found the best extraction procedure with 70% ethanol solution. Generally using ethanol solutions as an extraction agens for cheese

peptides is very suitable then this solvent is very well removeable by evaporation before next analysis.

Conclusion

Ethanol (in the first place 70% solution) was found as suitable extraction agens for following separation of a large number of low molecular mass cheese peptides by capillary zone electrophoresis. It gives good resolution, good possibility of the fractionation and so these peptides should be identified, e. g. by mass spectrometry.

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P59　THE MONITORING OF DIETARY EXPOSURE OF THE CZECH POPULATION TO SELENIUM IN 1994–2003

JANA ŘEHÁKOVÁ, JAN MIKOLÁŠ, IRENA ŘEHŮŘKOVÁ and JIŘÍ RUPRICH

National Institute of Public Health – Centre for the Hygiene of Food Chains, Palackého 3a, 612 42 Brno, Czech Republic, *rehakova@chpr.szu.cz*

Introduction

Selenium is a trace element that is essential to good health but required only in small amounts[1,2]. Selenium is incorporated into proteins to make selenoproteins, which are important antioxidant enzymes. The antioxidant properties of selenoproteins help prevent cellular damage from free radicals thus preventing the development of chronic diseases such as cancer and heart disease[2,3]. Other selenoproteins help regulate thyroid function and play a role in the immune system[4–7].

Selenium deficiency can make the body more liable to illnesses due to other causes such as infections or biochemical disorders. This may result in development of a heart disease, hypothyroidism and weakened immune system.

On the other hand, excessive selenium concentrations in blood may lead to selenosis the symptoms of which are gastrointestinal upsets, hair loss, white blotchy nails, garlic breath odor, fatigue, irritability, and mild nerve damage.

Due to the potential health risks it is necessary to monitor the selenium content in foodstuffs. Many studies have been published on the determination of selenium in foods[8,9]. Selected food sources of selenium can be found, for example, on specialized internet sites[10]. To assess the health risk properly it is necessary to know the dietary exposure to selenium. Its estimate requires a systematic monitoring of the selenium content in the food basket of the Czech population as well as the knowledge of the consumption of the corresponding commodities. Hence, Centre for the Hygiene of Food Chains in Brno has been involved in the monitoring of the content of selenium in foods forming a part of "The Project on Dietary Exposure of the Czech Population to Selected Chemical Substances"[11,12].

This work summarizes the selenium contents found in individual foods in our laboratory during the past 10 years. Also, their influence on the dietary exposure is estimated.

Methodology

The analyzed food samples representative of the so called food basket were selected based on the knowledge of their average consumption by the average population of the Czech Republic[12,13,14]. On their delivery to Centre of Hygiene of Food Chains in Brno, the samples undergo the culinary treatment so that they are analyzed in the same state as they are consumed. The samples are then homogenized in mixers and sent to the laboratory for further treatment and analyses.

In the laboratory the samples undergo microwave wet digestion in closed system using nitric acid and hydrogen peroxide. The content of selenium in thus treated samples is measured using atomic absorption spectrometry coupled with volatile hydride generation, which provides adequate sensitivity for the determination of the selenium in foods. This method is accredited according to EN ISO IEC 17025. The limit of quantification is 5 μg kg^{-1} for a sample weight of 1 g. The degree of recovery ranged from 95 to 105 %. The RSD of repeatability does not exceed 10 %. To ensure accuracy of the results matrix reference materials as well as various internal test materials are used. In addition, our laboratory takes part in proficiency testing (FAPAS, IMEP etc.).

Results

The content of selenium was monitored in 108 matrix types, which represent 432 composite samples measured annually. Hence, only selected samples containing significant amount of selenium together with the corresponding selenium contents are included in Fig. 1. This figure shows the relationship between content (μg kg^{-1}) and time (years).
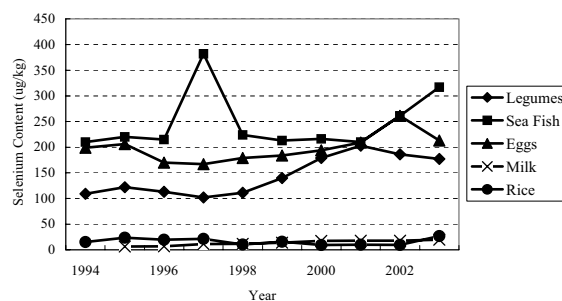
Fig. 1.　**Content of Selenium in Selected Foods vs. Time**
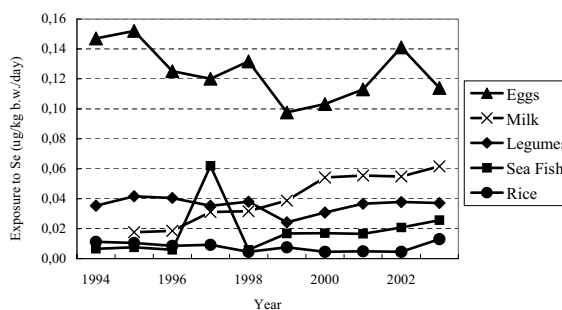
Fig. 2.　**Dietary Exposure to Selenium from Selected Foods vs. Time**

The dietary exposure is determined not only by the content of selenium in the given food, but also by the food consumption. To calculate the dietary exposure it is 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[12]. The exposure to selenium from selected foods is plotted against time in Fig. 2.

In Fig. 3., fifteen composite samples of the highest selenium content ($\mu g\ kg^{-1}$) are given.

The fifteen most significant sources of the total dietary exposure together with the corresponding exposure values are given in Fig. 4.
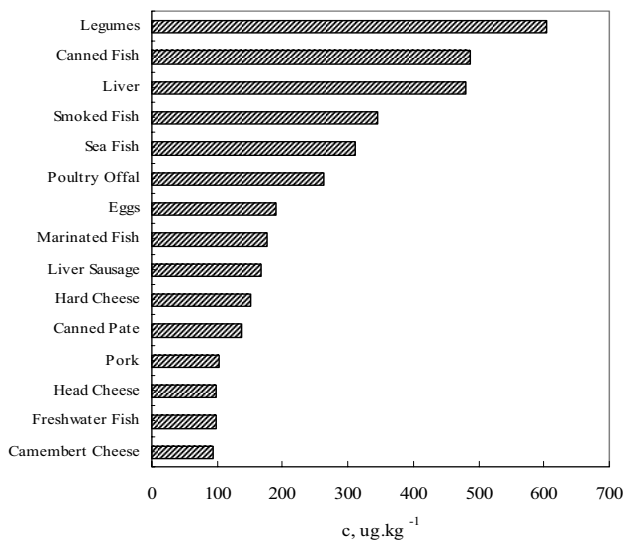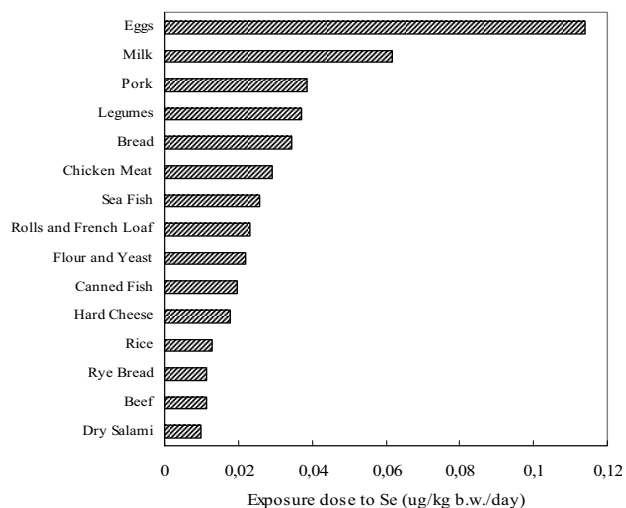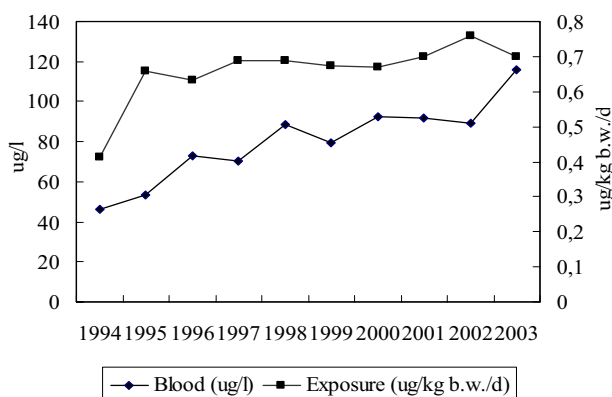
Fig. 5. **Comparison of the Total Dietary Exposure to Se with the Levels of Se in Blood**

In Fig. 5., the total dietary dose is shown for the period 1994–2003, and compared with levels of selenium in blood samples from the Czech population.

Discussion

The measured data showed that foods containing the highest amounts of selenium are legumes, fish, liver, poultry, eggs, pork meat, cheese and rice (Fig. 3.) When considering the significance of foods as sources of dietary exposure to selenium the most important foods are, on the other hand, eggs, milk, pork, legumes, bread, chicken meat, sea-fish etc. (Fig. 4.).

In general, the measured contents of selenium in food samples slightly fluctuated with a tendency to increase. Examples of five selected foods are given in Fig. 1. Also, the estimates of the dietary exposure tend to increase in the majority of samples. The same five significant sources of exposure are given in Fig. 2. This tendency to increase may be due to the trend of adding selenium to feeding mixtures and fertilizers [15]. In addition, the import of cereals from countries with soil rich in selenium may contribute to this trend.

Tendency of the total dietary exposure values is in agreement with the measured selenium levels in human blood throughout the period 1994–2003 (Fig. 5.)[16,17].

Conclusion

The content of selenium in the samples of the food basket of the Czech Republic has been monitored since 1994. The project of the monitoring of the dietary exposure has provided the data on the content of the selenium in individual foods. These data enabled the calculation of both the contribution to the dietary exposure from individual samples and the total dietary exposure. The values of the total dietary exposure in the period 1994–2003 ranged from 0.41 to 0.76 $\mu g\ kg^{-1}$ b.w./day. None of the found values lies beyond the interval determined by the value of population basal minimum (0.32 $\mu g\ kg^{-1}$ b.w./day)[18] and the safe dose for human health (6.15 $\mu g\ kg^{-1}$ b.w./day)[18]. However, the values of total dietary exposure lie below the recommended daily dose of

Fig. 3. **Content of Selenium in Selected Foods in the Czech Republic in 2003**

Fig. 4. **Dietary Exposure Sources of Selenium in the Czech Republic in 2003 (contribution of doses from individual food sources)**

selenium (0.92 µg kg⁻¹ b.w./day)[19]. This indicates that the daily need of selenium may not be fully covered for some population groups. The monitoring also showed an increasing trend for the dietary exposure to selenium which is in agreement with the levels of selenium in human blood in the same period.

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P60 FINDINGS AND INDICATION IN THE PAGE-ANALYSIS OF SWEET PROTEIN THAUMATIN

MILENA VESPALCOVÁ[a], RADANA HOLIŠOVÁ[a], ZDENĚK TŮMA[a,b] and DANA VRÁNOVÁ[a]

[a]*Institute of Food Science and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkynova 118, 612 00 Brno, Czech Republic, vespalcova@fch.vutbr.cz,* [b]*Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveri 97, 611 47 Brno, Czech Republic*

Introduction

Thaumatin (E 957) is a low-caloric alternative sweetener produced from fruits of the tropical plant Thaumatococcus danielli Benth.. It is absolutely safe and suitable for use. Thaumatin, the sweetest known substance, is approximately 3000 times sweeter than sucrose and acts as a taste modifier, too. Chemically, thaumatin is a basic protein, reportedly of extremely high pI = 11.7–12.0 (ref.[1]) and of relative molecular mass 22 kDa (ref.[1]). Sequence of 207 aminoacids in its chain is known. Commercially available thaumatin preparations consists of two main fractions, thaumatin I and thaumatin II, that differ only by five aminoacids in their chains, and several minor proteinic admixtures. Complete separation of constituents in commercially available thaumatins is very difficult. Well documented analytical technique for the purpose is missing yet. We decided therefore to start systematic research on the thaumatin analysis by exploration on analytical potential of PAGE techniques whose utilization for the purpose is the most frequently reported.

Experimental

The flat-bed gel electrophoresis instrument Major Science MP-500P (Major Science, USA) served for the development of polyacrylamide gels. Applied Biosystems 4700 Proteomic analyzer (Applied Biosystems, USA) served for obtaining of MALDI-TOF mass spectra of protein chains.

Thaumatin was from Sigma, USA. Acrylamide for preparation of electrophoretic beds, standard kits as well as all other chemicals were from SERVA Electrophoresis GmbH, Germany.

Gel electrophoresis at both native and denaturation conditions was carried out according to (ref.[2]) using gels of 0.3 mm thickness. Gels prepared from reaction mixtures containing either 7.5 or 8.0 % of acrylamide served for analyses at native conditions. Concentration of sampled thaumatin, dissolved in non-reducing sample buffer[2], ranged from 1 to 4 mg ml⁻¹. 5 µl sample has been applied for analysis. Electrophoresis took place in TRIS-glycine run buffer of pH 8.8 at 500 V driving voltage.

Gels for SDS-PAGE contained 8.0, 10.0, 17.5 and 20.0 % of acrylamide. Volume of thaumatin samples, dissolved in reducing sample buffer[2] in concentrations 1, 4 and 8 mg ml⁻¹,

was either 5.0 or 12.5 μl. SDS-PAGE has been carried out in TRIS-glycine run buffer of pH 9.0 with 0.1 % of SDS. Coomassie Blue R 250 served for visualization of separated bands.

Results

Non-denaturating (native) conditions

Electrophoretic migration of a dissolved ion at a chosen pH depends mainly on its isoelectric point and molecular mass. Migration speed of a protein at non-denaturating conditions allows obtaining of information on its isolelectric point. Gels of mean acrylamide concentrations, 7.5 and 8.0 %, have been chosen for experiments at non-denaturating conditions considering reported size of thaumatin molecule[3]. Thaumatin bears pronounced positive electrophoretic charge at the used run buffer pH 8.8 if its pI = 11.7–12.0 determined from experiments on starch gel[1] is correct. We found that thaumatin migrates slower than implies this commonly used high pI value (Fig. 1.). Its location in electrophoregram corresponds merely to pI = 8.46 calculated from pI values of aminoacids found in the thaumatin chain[4]. This finding casts doubt on commonly reported and generally accepted value of the basic thaumatin constant.
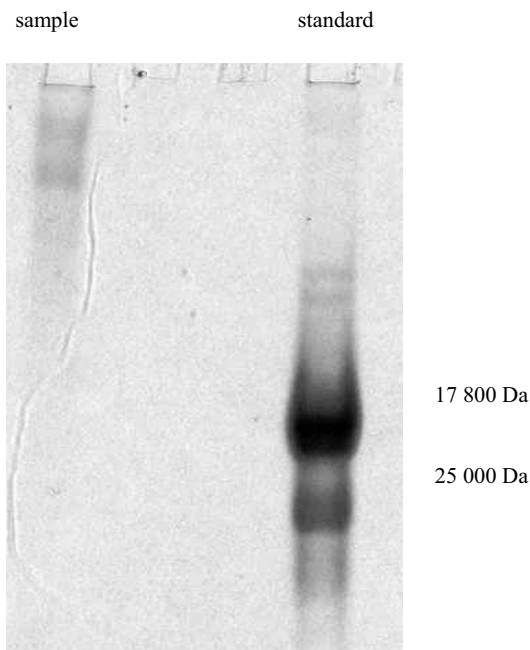

Fig. 1. **PAGE of commercially available thaumatin at a native conditions: 8.0% acrylamide gel, U = 500 V, I = 60 mA, t = 20 mins., sample volume 5 μl, thaumatin concentration 4 mg ml^{-1}**

Denaturating conditions

SDS-PAGE system, which informs on the protein relative molecular mass, was developed for neutral and weakly acid proteins. Thaumatin is a basic protein. We aimed therefore at verification of the method works for it.
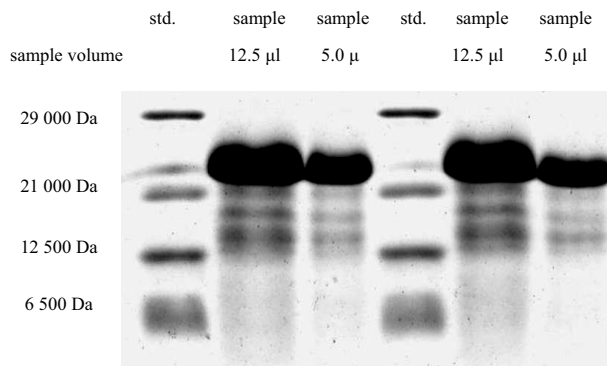

Fig. 2a **SDS-PAGE of commercially available thaumatin: 17.5% acrylamide gel, U = 500 V, I = 5 mA, t = 270 mins., thaumatin concentration 4 mg ml^{-1}**

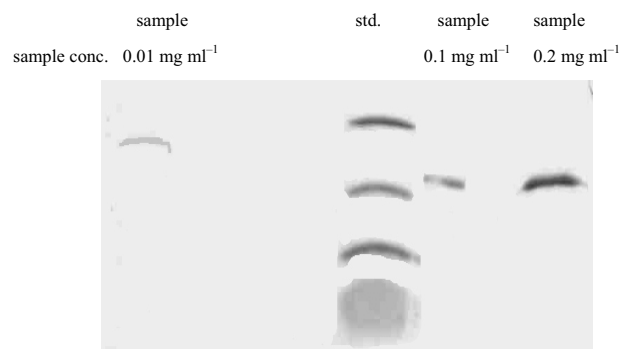Fig. 2b **SDS-PAGE of commercially available thaumatin: 17.5% acrylamide gel, U = 500 V, I = 5 mA, t = 180 mins., sample volume 4 μl**

Thaumatin was treated with SDS in standard way[2] and than analysed together with set of mass standards 6.5 kDa, 12.5 kDa, 21.0 kDa and 29.0 kDa. Various amounts of thaumatin have been applied. Analyses results were reproducible and dependent on the amount of applied thaumatin (Fig. 2a, 2b). Single thaumatin band was obtained in contrast to expectation based on its previous electrophoretic[5] and mass spectrometric analyses. Moreover, the mass spectrometric analyses of individual separated bands revealed that thaumartin is present in all zones created during a run disregarding concentration and volume of the applied thaumatin sample (Fig. 3.). This result strongly indicates that the standard denaturation method does not work properly if thaumatin is the analyte.

Conclusion

Experiments at both non-denaturating and denaturating conditions show that neither thaumatin pI derived from flat bed gel analyses nor efficacy of the SDS-PAGE method for thaumatin, respectively, are reliable. It casts doubt on the capability of flat bed gel analyses to give credible analytical results for thaumatin as the analyte.
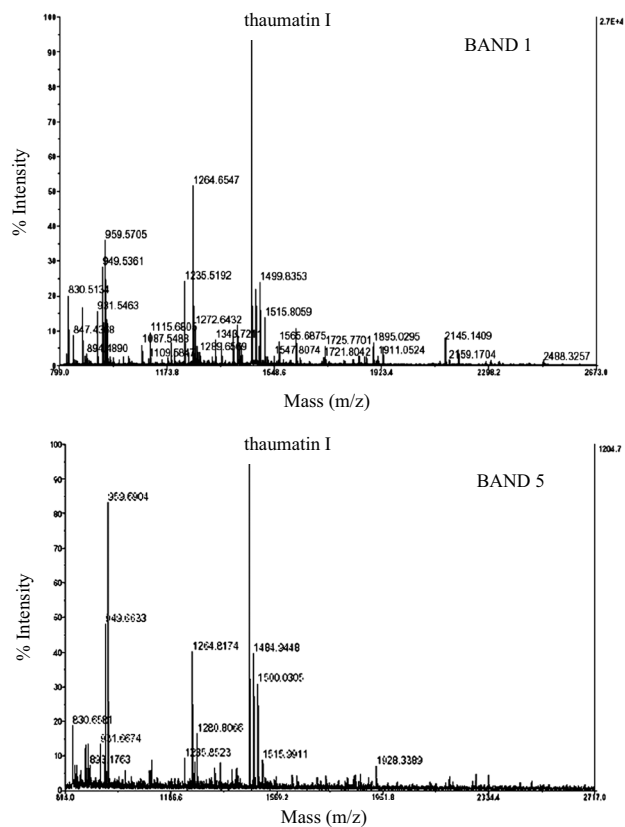
Fig. 3. **Typical mass spectrum of separated bands of SDS-
-PAGE thaumatin (Fig. 2a)**

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