4. FOOD CHEMISTRY & BIOTECHNOLOGY

4.1. Lectures

L01 STRATEGIES TO ENZYMATIC LIPOPHILIZATION OF SACCHARIDES

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Lipophilic derivatives of saccharides, mainly aliphatic glycosides and sugar esters find use as surface active substances in food, cosmetic, pharmaceutic, polymer, textile and laundry industries. Besides they biodegradability, they can be prepared by ecologically friendly procedures, especially if biocatalysis is introduced as the key reaction step. Apart from mentioned facts, preparation of sugar surfactants from renewable agricultural side-products turns them to a very attractive for chemical industry of 21st century.

Besides use of whole microorganisms, ester hydrolases and glycosidases proved to be the only two enzyme classes, which can be employed in effective enzymatic synthesis of sugar-lipid conjugates. Various approaches may be used to overcome problems arising from the nature of both biocatalyst and the substrate. This work offers comparation of such techniques for preparation of surface active sugar derivatives in our laboratory.

Chemical production of alkyl glycosides involves several synthetic steps and leads often to mixtures of two anomers in both possible cyclic forms of the glycoside, which means lower chemical yield of the target substance and complication of its purification. For this reason, enzymatic preparation of alkyl glycosides gains rising interest of researchers and technologists. Glycosidases are used with advantage in such preparations in far cheaper and simplier way comparing to glycosyl transferases. Chemical yields of syntheses depend on combination of used glycosidase, substrates and reaction conditions. Generally, two methods can be employed in one-step preparation of alkyl glycosides catalyzed by glycosidases – transglycosylation and reverse hydrolysis[1].

Reverse hydrolysis represents a thermodynamic approach where equilibrium of glycoside hydrolysis is strongly shifted towards left side of the reaction in environment comprising low water activity and high concentrations of reactants (theoretical product of hydrolysis) – free monosaccharide and alcohol. Yields in these reactions are generally low and long reaction times are required. Transglycosylation is a kinetically controlled approach employing transferring activity of glycosidases. As a substrate, an oligosaccharide or a preformed glycoside is used, giving a complex of enzyme and saccharide moiety transferred from non-reducing end of the substrate. This moiety is then transferred to nuclephile acceptor present in reaction medium – water in case of hydrolysis and alcohol when transglycosylation is performed. The main advantage is short time of reaction together with high yields. To be profitable, cheap substrate must be used in the process.

Alkyl β-galactosides

Transgalactosidation starting from lactose is preferred in preparation of alkyl β-galactosides due to the lactose price, high product yields and short times of reaction (usually 2 or 3 hours). β-Galactosidases are commercially available as preparations of various origin and purity especially for food industry. According to our experience, fungal lactases are the most suitable for synthesis of alkyl β-galactosides due to high transgalactosidase ability enabling us to prepare range of products with alkyl chain from 1 to 8 carbons[2,3].

Alkyl β-glucosides

Cellobiose as a possible sugar donor for transglycosylation is an expensive material, therefore preparation of β-glucosides by reverse hydrolysis of glucose is much more realistic solution. Despite low product yield, this reaction offers an advantage of use of various plant materials as a source of β-glucosidase. Since glucose is the only sugar present in starting reaction solution, side activities comprised in the biocatalyst (with exception of a-glucosidase) do not intrigue the synthesis. Defatted meals of several plant seeds may be used, among them almond, apricot, peach and evening primrose[4]. Due to stability and low cost of the latter three materials (being wastes from food and pharmaceutical industries), no immobilisation is necessary. Higher yields may be obtained by simple replacing the alcohol layer after reaching the reaction equilibrium. Almond glucosidase is also available commercially as a relatively expensive pure enzyme, use of defatted almond meal for practical synthesis is however satisfactory.

Alkyl α-glucosides

Since amylases are commercially distributed in huge extent and number of variants especially for food and beverage industry, they seem to be an ideal candidate for cheap synthesis of α-glucosides from starch. Only a few attempts are however mentioned in literature on glycosylation of aliphatic and aromatic alcohols and phenols[5–7]. The tranglycosylation activity of amylases very variates case to case and combination with glucoamylase may be necessary to avoid creation of mixtures of oligoglucosides.

Baker's yeasts seem to be another promissing catalyst for cheap preparation of α-glucosides from sucrose. Howe-

ver, production of ethanol during reaction as well as variation of quality of fresh yeast may hamper its wider use.

Comparing to glycosides, sugar esters have a wider scale of applications especially in food and cosmetic products since their are non-toxic, easily degradable in human organism and no odor or flavor. Moreover, they are preparable in a very large interval of HLB. Lipases, esterases and proteases are routinely used as biocatalysts for synthesis of sugar esters. Although their enzymatic preparation has a great advantage over chemical process in high selectivity and thus preparation of highly defined products, this approach brings several new problems arising from low solubility of sugars in the reaction media. Besides sporadic exceptions[7], such reaction require limited or almost zero content of water, which means that toxic or hardly removable solvents as pyridine, dimethylformamide or dimethylsulfoxide serve as the best rection media or their components. To overcome this problem, several approaches as derivatisation of saccharide (glycosylation[8], complexation with phenyl borates[9], glycolation[10]), reaction in the melt of acylation aliphatic acid[10], reaction in solid phase with adjuvant additive[11], or solubilisation of enzyme instead of sugar[12] may be used. As the acylation agent, free aliphatic acids, their esters or activated esters (vinyl-, isopropenyl- or haloalkyl-) shifting the equilibrium to the right side of the reaction are eployied. Comparing to standard chemical procedures, enzymatic esterifications with free acids or non-activated esters give only low to moderate yields. The main advantage lies in regioselectivity of saccharides, mild reaction conditions and possibity to use various acyl donors giving new functional properties to the product as for example esterification with ferulic acid[13].

References

1. Amalric Ch., Lecocu-Michel N.: Pat. US 5670471 (1997).
2. Bilaničová D., Garajová E., Schmidt Š., Mastihubová M., Mastihuba V.: *Enzýmové modifikácie laktózy*. In.: Proceedings "Milk and Cheeses" 2003, Prague, 107.
3. Bilaničová D., Mastihuba V., Kaufmanová J., Mastihubová M., Schmidt Š.: Chemické listy *97*, 784 (2003).
4. Bilaničová D., Mastihuba V., Plencnerová S., Poláková L., Schmidt Š.: *Rastlinné izoláty na syntézu povrchovoaktívnych glykozidov*. In: Proceedings of XLII. International conference of fat technology and analysis, Stachy, 2004.
5. Santamaría R. I., Del Río G., Saab G., Rodríguez M. E., Soberón X., López-Munguía A.: FEBS Letters *452*, 346 (1999).
6. Nishimura T. , Kometani T., Takii H., Terada Y., Okada S.: J. Ferment. Bioeng. *78*, 31 (1994).
7. Kremnický Ľ., Mastihuba V., Côté G. L.: J. Mol. Catal. B.: Enzymatic *30*, 229 (2004).
8. Adelhorst K., Bjőrkling F., Godtfredsen S. E., Kirk O.: *Enzyme catalysed preparation of 6-O-acylpyranosides*. Synthesis, 112–115 (1990).
9. Castillo E., Marty A., Combes D., Condoret J. S.: Biotechnology Letters *16*, 169 (1994).
10. Fregapane G., Sarney D. B., Vulfson E. N.: Enzyme and Microbial Technology *13*, 796 (1991).
11. Cao L.: *Lipase-catalyzed solid phase synthesis of sugar fatty acid esters*. Dissertation. University Stuttgart, Stuttgart, Germany (1997).
12. Bruno F. F., Dordick J. S., Kaplan D. L., Akkara J. A.: Regioselective enzymatic transesterification of polysaccharides in organic solvents. ACS SYMPOSIUM SERIES *684*, 167 (1998).
13. Mastihubová M., Mastihuba V., Bilaničová D., Dudíková J.: *Enzymatic preparation of carbohydrate ferulates: scopes and limitations*. 6th Carbohydrate Bioengineering Meeting, CosmoCaixa, Barcelona, 2005, P49.

L02 PERSPECTIVES OF LENTIKATS® IMMOBILIZATION SYSTEM IN ETHANOL FERMENTATION PROCESS

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Introduction

Zymomonas mobilis, a gram-negative facultative bacterium, has been considered as an alternative to yeast in the synthesis of ethanol in industrial. These facultative anaerobic bacteria metabolize glucose and fructose via glycolitic Entner-Doudoroff pathway, which is usually present in aerobic microorganism[1]. Within this metabolic pathway each mole of glucose consumed produces one mole of ATP. This mechanism of glucose uptake minimizes the amount of glucose converted into biomass and increases the ethanol productivity.

During last three decades there is increased interest in the research of immobilized living cell systems which has several benefits compared to conventional fermentation processes. Very effective and useful matrices for microorganisms entrapment is polyvinylalcohol (PVA). The gelation of the immobilizates is based on partial drying at room temperature and therefore it is very gentle to microorganisms[2]. Compared with another gels, lens-shaped hydrogel capsules, called LentiKats® (Fig. 1.) offer several advantages: low cost for matrix and production, easy preparation, excellent mechanical stability, easy separation from reaction mixture (diameter: 3–4 mm), low diffusion limitation (thickness: 200–400 μm) and no degradation of the matrix[3]. This type of immobilizates were successfully investigated in several biological processes such as cider fermentation[4], nitrifiers immobilization[2], amiloglucosidase entrapment[3] and bioconversion of glycerol into 1,3-propanediol (ref.[5]).

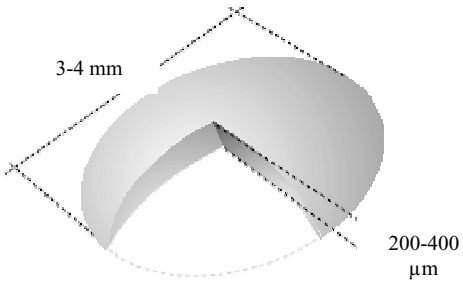

Fig. 1. **Lens shaped PVA immobilizates – LentiKats®**

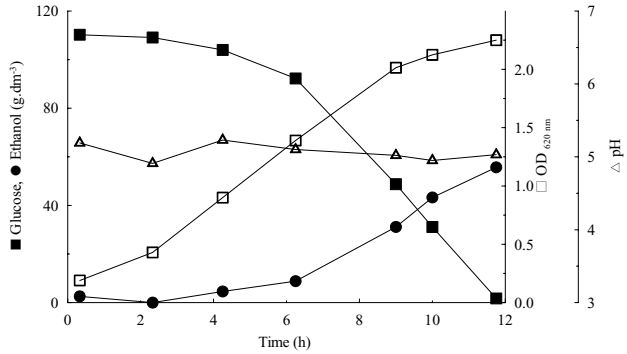

Fig. 2. **Batch fermentation with free cells Zymomonas mobilis**

In this work we have studied the application of LentiKats® for immobilization of *Zymomonas mobilis*.

Material and methods

Zymomonas mobilis CCM 2770 (obtained from Czech Collection of Microorganisms) was used in all experiments.

The medium composition was the following: glucose 150 g dm^{-3}; yeast extract 5 g dm^{-3}; $(NH_4)_2SO_4$ 1 g dm^{-3}; $MgSO_4.7H_2O$ 0.5 g dm^{-3}; KH_2PO_4 1 g dm^{-3}. For all fermentation experiments, pH of 5.0 and a temperature of 30 °C were maintained in 1.3 dm^3, gently stirred (200 rpm) fermentor. Biomass for immobilization was prepared by batch cultivation and cells were harvested in mid-exponential phase of growth by centrifugation. Immobilizates were prepared on pilot scale equipment in MEGA a. s. according to manufacturer (Czech Republic, www.mega.cz).

Batch fermentations were performed in fermentor containing 1 l of medium, inoculated with 10 % v/v of seed culture (for the free-cell system) or 180 g of propagated LentiKats®. In repeated batch fermentations immobilizates were used for 50 fermentations. After each run, the medium was separated, fresh medium was added and the next batch fermentation was initiated.

Glucose and ethanol were analyzed by HPLC using a 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}. 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®.

Results and discussion

Zymomonas mobilis is considered as an alternative to the yeast in the ethanol production on industrial scale[6]. Batch fermentation is classical alternative for ethanol production in industrial scale. *Zymomonas mobilis* at optimal fermentation conditions (temperature 30 °C, pH = 5), is able to convert 110 g dm^{-3} glucose to ethanol within 12 h with yield of ethanol 98 % of theoretical amount. The final ethanol concentration in the medium of 55.7 g dm^{-3}. (Fig. 2.). The concentration of biomass rise up from 0.18 g dm^{-3} to 3.1 g dm^{-3}l. Compared to the yeast it is almost 2 times higher production rate. As we mentioned above, *Z. mobilis* employ the Entner-Doudoroff pathway to catabolise glucose yielding 1 mol of ATP, whereas *S. cerevisiae* employing the Embden-Meyerhof pathway produces 2 mol of ATP per mol of glucose. It means that *Z. mobilis* forms low biomass[1]. It is only 2–5 % of glucose, which is converted into cell mass in this microorganism[7]. Moreover the enzymes of Entner-Doudoroff pathway are tolerant to ethanol, as cell-free system can rapidly consume glucose and produce ethanol more than 15% w/v (ref.[1]). Next advantage associated with metabolism is an ethanol yield. For the production of the same volume of pure ethanol we need significantly less bacterial biomass than yeast biomass. The bacteria biomass need for 1 dm^3 of 100% ethanol production is from 2 times (glucose medium 85.5 g dm^{-3}) to 3–4 times (glucose medium 241 g dm^{-3}) less than in yeasts[7]. Using medium with the amount of sugars normally present (136 g dm^{-3}) in industrial mashes, the yield for both types of microorganisms starts to differentiate and the use of glucose in the amount close to 250 g in 1 dm^3 medium proved that under high osmotic pressure bacteria producing ethanol with significantly higher yield[7]. Advantage of immobilized system is that the same cells can be used in repeated batch fermentations for many times. Therefore this microorganism was successfully immobilized into PVA hydrogel capsules – LentiKats®. Increase of biomass concentration influenced the fermentation time of process. Fermentation time of the fifth repeated batch fermentation was reduced to 2.2 h (Fig. 3.). The fermentation with cells immobilized in LentiKats® has shown almost the same growth and fermentation pattern but no lag phase was observed. The volumetric production of batch fermentation system increased almost 9 times without any changes in theoretical ethanol yield. Moreover the batch system was stable during 50 batch fermentations. Particular advantage of LentiKats® immobilizates is the shape and the material which minimizes and eliminates diffusion limitation inside the hydrogel[2]. Compared to immobilized *Zymomonas mobilis* cells in polyurethane foams, the obtained effectiveness was three times higher than reported for repeated-batch fermentation[8]. This developed ethanol fermentation system is considered to be used in industrial scale (MEGA a. s., www.mega.cz).

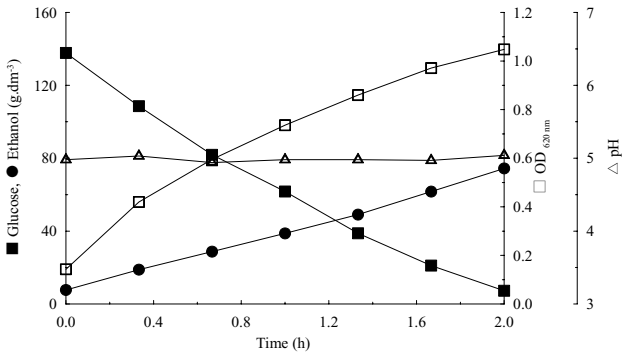

Fig. 3. **Batch fermentation with Zymomonas mobilis immobilized into LentiKats®**

Conclusion

Zymomonas mobilis immobilized in LentiKats® shows excellent fermentation stability in consecutive batch fermentation system. Repeated batch fermentation system offers several advantages: reduction of fermentation time, inutility of inoculum preparation, reduction of lag phase of the process and possibility of repeated use of immobilizates.

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REFERENCES

1. Gunasekaran P., Raj K. Ch.: Curr. Sci. *77*, 56 (1999).
2. Jekel M., Buhr A., Willke T., Vorlop K.-D.: Chem. Eng. Technol. *21*, 275 (1998).
3. Czichocki G., Dautzenberg H., Capan E., Vorlop K.-D.: Biotech. Lett. *23*, 1303 (2001).
4. Durieux A., Nicolay X., Simon J.-P.: Biotech. Lett. *22*, 1679 (2000).
5. Wittlich P., Lutz J., Reimann C., Wilke T., Vorlop K.-D.: *Proceedings of the 6th symposium on Renewable Resources,* p. 524. Bonn 1999.
6. Rebroš M., Rosenberg M., Krištofíková Ľ., Stloukal R.: Chem. Listy *99*, in press (2005).
7. Nowak J.: Electron. J. Polish Agricultural Universities *4–2,* (2001).
8. Amin G., Doelle H. W.: Enzyme Microb. Technol. *12*, 443 (1990).

L03 INFLUENCE OF CONTAMINANTS ON QUALITY OF FOODSTUFFS

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Introduction

Within the assigned competencies the Czech Agriculture and Food Inspection Authority (CAFIA) inspects foodstuffs, raw materials for the production of foodstuffs, agricultural products, soap products and tobacco products[1]. The priority of inspections are inspections of food safety and quality with regard to disclosing adulteration. Food safety controls are aimed at inspection of foreign substances in foodstuffs and at microbiological inspection, inspections of purity and labelling of foodstuffs are carried out in order to disclose adulteration. The aim of inspections is based on various information sources, with the integration of the Czech Republic to the European structures possibilities are expanding, mainly by EC Recommendations, contacts with organizations abroad, RASFF[2].

Food quality from the viewpoint of foreign substances inspection

As to the content of foreign substances, 4.9 % of samples did not comply with requirements of Decree No. 53/2004 Coll. (ref.[3]) and Decree No. 465/2002 Coll. (ref.[4]) in 2003. The majority of analyses were performed for pesticide residues, colourings and preservatives.

The pesticide residues exceeding the limit were found in 8 samples of citrus fruit, cabbages and fruit vegetables, which represented only 0.01 % of analyses performed. The pesticides found were fenpropathrin, tebuconazole, metalaxyle and dithiocarbamates. As to the inspection of colourings, the highest number of unsatisfactory samples was identified in egg-free pasta, table wine and jams. Unauthorised synthetic colourings or their limit exceeding amount were identified, this particularly applied to azorubin, brillant black, ponceau 4R, and tartrazin. From the group of preservatives, sulphur dioxide and sorbic acid were checked the most frequently, followed by benzoic acid mainly in wine. With regard to sweeteners, inspections focused on saccharine and sorbitol, deficiencies were found mainly in jams and marmalades, or in fermented vegetable mixtures. As for other additives (e. g. acetates, glutamates, quinine or caffeine), the majority of limit exceeding levels were proven in case of hesperidin.

During the detection of chemical elements 5.1 % of performed analyses failed due to exceeded hygiene limits, mainly in case of phosphorus, cadmium, mercury and manganese in poppy seeds, cocoa, jams, and marmalades.

As to organic contaminants, positive findings were proven for phthalates in spirits (1 %). In the group of aromatic hydrocarbons (toluene, xylene, styrene, ethylbenzene), natural mineral water and rice were positive (2.5 %). Polyaroma-

tic hydrocarbons, polychlorinated biphenyls and polychlorinated dibenzo-p-dioxines were not proven, nor were proven other organic contaminants such as amines, nitrosamines or alkaloids. On the contrary, positive findings were ascertained in case of mycotoxines, aflatoxin B1 exceeding the limit was found in 2 pistachio samples. As for other natural toxins (ethylcarbametes, enterotoxins), 2 % of samples were assessed as non-complying.

However, the highest percentage of unsatisfactory results (7.8 %) gave analyses for nitrates in cabbages and root vegetables and for antibiotic residues (13.5 %) in foodstuffs of animal origin.

Compared to results from previous years' inspections, on the one hand a smaller number of unsatisfactory samples was identified (e. g. 9.8 % of samples failed in 2000), on the other, some analytes were not proven at all. For example, arsine in rice, which represented more than a half of all unsatisfactory samples in case of chemical elements, was not proven in 2003, and similarly, limit exceeding content of polyaromatic hydrocarbons in oils and tea or biogenic amines in fish and cheeses was not proven. The inspection results were certainly affected by changes in the legislation, which should ensure compliance of Czech legislation with EU legislation, but mainly they were affected by changes in the field of food industry, such as introduction of new products, technology in production and storage, and implementation of quality management principles.

Food quality from the viewpoint of monitoring foreign substances

Results obtained during inspections are supported by findings acquired during planned inspections (monitoring) of foreign substances for the presence of contaminants or pesticide residues in foodstuffs of plant and animal origin. Out of more than 2300 samples, only 57 samples exceeded the hygiene limit or the maximum limit for pesticide residue, which means only 2.5 % of unsatisfactory samples.

The unsatisfactory samples were proven only in foodstuffs of plant origin. It is the results of monitoring that give greater evidence of burdening the food chain with contaminants, rather than results obtained during inspections, since the latter is burdened with the influence of targeted examination of samples, where the assumption of positive finding is bigger.

The objective of monitoring is also monitoring of analytes that are not subject of inspections. Studies on their presence in different commodities are under way, their results will then be taken as one of the bases for fixing hygiene limits, or they can be used for inspections.

Within the monitoring carried out in 2003, mycotoxins were monitored, particularly aflatoxin B1, aflatoxin M1, ochratoxin A, deoxinivalenol, and patuline. Aflatoxins were monitored in spices, confectionery products and dry nuts. Although that positive finding of mycotoxins was ascertained in 12.5 %, the hygiene limit was exceeded only in one case (aflatoxin B1 in pistachios). Deoxinivalenol was monitored in bear and maize products; 2 products were positive. Ochratoxin A was ascertained in 20.5 % of samples of spices, coffee, bear, and dried fruit, however, cereal baby food was mycotoxin-free. Patuline was monitored in fruit juices and baby food, all samples were negative.

Other analytes that are not usually inspected were also monitored. What is important is the presence of ethylcarbamate in fruit distillates, which was registered in 75 % of samples, although the limits are exceeded rarely. The presence of methanol in distillates was also monitored with the same conclusions as for ethylcarbamate. Results of monitoring 3-monochloropropane 1,2-diol in products containing protein hydrolysate and in seasonings are also important. Positive findings were ascertained in 68 % of samples, whereas limits were exceed in 16 %. The levels of bisphenol-A-diglycidyl ether or its derivates, which is released into foodstuffs from varnishes used for coating internal sides of tin surfaces, are also important. Positive findings ranging up to a half of the limit were ascertained in more than 80 % of samples examined, however, the limit has not been exceeded. A study on acrylamide levels in foodstuffs put on the market in the Czech Republic was carried out. This study focused on products with a high content of starch. The highest levels were found in crisps, where the levels reached up to 1550 μg kg^{-1}; relatively high levels were ascertained in long-life bakery products (crackers, bars) – they reached up to 746 μg kg^{-1}. The results obtained are comparable to values of foreign studies.

Food inspection from the viewpoint of rapid alert system

The Rapid Alert System for Food and Feed in the Czech Republic is based on the Regulation of the European Parliament and of the Council (EC) No. 178/2002 (ref.[5]), laying down the general principles and requirements of food law and procedures in matters of food safety. The system is operated as a network for notifying direct and indirect risk for human health posed by foodstuffs. The purpose of exchanging information between RASFF participants is to prevent foodstuffs that were exported outside the territory of individual country and that pose a health risk from being placed on the market, or to withdraw them. The exchange of information on products threatening human health between the Czech Republic and the European Commission is ensured by national contact point, which is, since 2002, the CAFIA. Notifications to RASFF are only used if the foodstuff poses a serious risk and it is present on the market of more Member States.

The most important notifications or notifications whose number has noticeably increased for given analyte within the EC during the year[6] are the following:

a) aflatoxins in nuts

In 2003, the number of notifications concerning aflatoxins has increased more than twice (to 763). The notifications concerned mainly pistachios from Iran, followed by peanuts and hazelnuts. In case of pistachios, the problem is connected with unsuitable conditions during packaging and transportation. As to the nuts from China, long-term monitoring was recommended.

b) dioxines

Out of 26 notifications only a minority concerned foodstuffs (6 samples), this particularly applied to limit exceeding levels in oils.

c) polycyclic aromatic hydrocarbons (PAH)

PAH were found in sprats from Baltic countries, even in high concentrations (up to 1160 μg kg^{-1}). The occurrence of these carcinogens has been explained by technological procedures based on using direct flame for drying and smoking. The limit exceeding PAHs were found in fish meat and oil.

d) 3-monochloropropane 1,2-diol (3-MCPD)

3-MCPD was found in 20 samples of soya sauce from Asia; the concentration reached up to 1015 mg kg^{-1}, whereas the maximum limit according to the Regulation (EC) No. 466/2001 is only 0.02 mg kg^{-1}.

e) cadmium and copper

The limit exceeding levels of cadmium were proven in swordfish meat in 63 cases, followed by cuttlefish and octopuses. High levels of copper were found in swordfish.

f) residues of veterinary medicinal products

The presence of residues of veterinary medicinal products concerned chloramphenicol in honey and milk products from Baltic countries. Furthermore, nitrofuranes were proven in fish, poultry and meat products. The occurrence of fungicidal dye malachite green in salmons from Chile was interesting.

g) Sudan Red

Potentially carcinogenic Sudan Red 1 was used for adulterating chilli powder. This dye was proven in 119 cases, not only in paprika, but also in other spice mixtures and products in which the spices or seasoning were used.

Food quality from the viewpoint of European organizations

With regard to involvement of the Czech Republic in the European structures, a number of activities related to the EC leading to greater efficiency of food safety are under way: a number of CAFIA staff take part in working groups or EC and Council groups meetings, new legislation and its transposition to the Czech regulations is monitored, missions concerning pesticides and contaminants are carried out in order to assess the ability of food control authorities to comply with requirements determined by EC legislation, as well as missions aimed at evaluation of critical control points systems in food production and training inspectors in this field.

Conclusions of commissions and working groups are transposed to activities in the field that ensures food safety. The Codex Committee on Food Additives and Contaminants[7] begun to prepare specific measures concerning changes in assessing maximum limits for various analytes and commodities and adopted proposals for new approaches for selected groups of contaminants and additives. At the meeting of the Working Group for Codex Alimentarius (Food Additives and Contaminants)[8] 4 main objectives were discussed, these will be further talked over at the CCFAC meeting in April 2005 in the Hague.

The first objective is to formulate the policy of evaluating contaminants and toxins in foodstuffs and groups of foodstuffs, based on 2 pillars of consumer health protection: the first pillar is prevention and reduction of contamination in the food chain, the second pillar is effective application of limits.

The second objective is the field of agricultural contaminants. Mycotoxins are nowadays the main problem: aflatoxins in almonds and nuts, deoxynivalenol in cereals, ochratoxin A, patulin and fusarium toxins.

The third objective is the field of industry contaminants. Levels of tin in canned foodstuffs, cadmium in rice and mollusc meat, 3-MCPD in soya sauces, lead and mercury in fish constitute a problem, as well as irradiated foodstuffs.

The fourth objective is to incorporate aromatic substances into the Codex Alimentarius system. The problem is lacking definition of aromatic substances fulfilling present view of aromatic substances and procedures how to include aromatic substances into the system of additives.

The CCFAC materials are one of the bases for creating Commission Recommendation on the official food control programme as a guideline for supervisory bodies which samples should be taken, what analyses should be performed and what objective should be followed.

Conclusions

The accession of the Czech Republic to the European Union has brought on the one hand, an increase in activities from growth of legislation to application of procedures for addressing individual issues, as well as change in thinking linked to the fact that the market has opened and food safety had to be ensured in different ways than before. Some negative expectations of worsening food quality were not fulfilled; on the contrary, the numbers of unsatisfactory samples are decreasing. Thanks to the link to the European structures the Czech Republic can be better informed about European issues concerning foodstuffs and can react faster to ensure safe foodstuffs.

REFERENCES

1. Zákon č. 110/1997 Sb., o potravinách a tabákových výrobcích a o změně a doplnění některých souvisejících zákonů, v platném znění
2. CAFIA annual report for 2003, CAFIA Brno, 2004, *67* p.
3. Vyhláška MZd. č. 53/2002 Sb., kterou se stanoví chemické požadavky na zdravotní nezávadnost jednotlivých druhů potravin a potravinových surovin, podmínky použití látek přídatných, pomocných a potravních doplňků
4. Vyhláška MZd. č. 465/2002 Sb., kterou se stanoví přípustné množství reziduí jednotlivých pesticidů v potravinách a potravinových surovinách, v platném znění
5. Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food

law, establishing the European Food Safety Authority and laying down procedures in matters of food safety
6. Annual Report on the Functioning of the RASFF, 16 February 2004, European Commission, 32 p.
7. Report of the 36th Session of the Codex Committee on Food Additives and Contaminants, Rotterdam, The Netherlands, 22–26 March 2004, 210 p. Ed. Codex Alimentarius Commission, Rome, April 2004, ALINORM 04/27/12
8. Codex Alimentarius Working Party (Food Additives and Contaminants), 7 October 2004, Brussels, 2 p. Ed. Council of the European Union, Brussels, 22 September 2004, CM 3353/04

L04 BIOTECHNOLOGICAL CONVERSION OF FATTY ACIDS TO THEIR CONJUGATED METABOLITES BY BACTERIA

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Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (C18:2) with conjugated double bonds in several positions and conformations. These isomers (especially c9, t11- and t10, c12- CLA isomers) have received considerable interest during the past decade because they are responsible for many biological properties relating to health, i.e. anticarcinogenic, antiadipogenic, antiatherogenic, bone modelling and immune modulating effects. CLA arises along the ruminal process which ends with full saturation of linoleic acid into stearic acid. This stepped pathway is carried out by rumen bacteria. These isomers are incorporated into the fat in beef and milk of ruminants before the saturation process has been completed. Thus, food products from ruminants, particularly dairy products, are the major dietary source of CLA for humans[1].

A great demand for conjugated isomers of linoleic acid motivates researchers to look for the possible biotechnological methods of their preparation. Since past decade, several microbial strains (*Lactobacillus, Propionibacterium, Bifidobacterium*) have been studied with the aim to transform linoleic acid into CLA metabolites. Although many studies on fatty acids conversion to CLA have been performed, the mechanism of CLA formation has not been elucidated in detail. OGAWA et al. indicated that the transformation of linoleic acid to CLA using washed cells of *Lactobacillus acidophilus* AKU 1137 is not a single one-step isomerization of non-conjugated diene to a conjugated diene[2]. The transformation involves the production of hydroxy fatty acids, namely 10-hydroxy-*trans*-octadecaenoic acid and 10-hydroxy-cis-octadecaenoic acid as intermediates.

We screened almost 100 bacterial strains for possible CLA production, mainly lactic acid bacteria, rumen bacteria and probiotic bacteria. Some of them produced CLA isomers, i. e., c9, t11-, t10, c12- and various t,t-CLA isomers. The 10-hydroxy-*trans*-octadecaenoic acid and 10-hydroxy-*cis*-octadecaenoic acid have also been detected. Four strains with the highest CLA productivity (*L. plantarum* AKU 1130, *L. plantarum* JCM 8341, *L. plantarum* DSMZ 2601 and one strain isolated from rumen) have been selected for further optimization of fermentation conditions with the aim to improve the yield of CLA.

Materials and methods

Chemicals. Linoleic acid (more than 99% purity) was produced from sunflower oil by the urea fractionation method developed in our laboratory[3]. All other chemicals were of analytical grade and are commercially available.

Microorganisms, cultivation and washed cells preparation. Microorganisms were obtained from the LAKTOFLORA (Czech Collection of Lactic Cultures, Praha), AKU (Faculty of Agriculture, Kyoto University, Kyoto), JCM (Japan Collection of Microorganisms, Wako) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig). Strains were cultivated in MRS medium (tryptone 10 g, meat extract 10 g, yeast extract 5 g, glucose 20 g, Tween 80 1 ml, K_2HPO_4 2 g, sodium acetate 5 g, diammonium citrate 2 g, $MgSO_4.7H_2O$ 0.2 g, $MnSO_4.H_2O$ 0.05 g, distilled water, 1 dm^{-3}) supplemented with 0.06% (w/v) linoleic acid. The cells grown at 30 °C for 24 h were harvested by centrifugation (4000 × g, 20 min), washed twice with 0.85% (w/v) NaCl, centrifuged again and then used as the washed cells for bioconversion.

Bioconversion. Reactions were carried out in anaerobic conditions at 30 °C and shaking (120 strokes/min) in screw-cap tubes. Reaction mixture contained washed cells (approximately 20 mg dry weight) and 0.44% (w/v) linoleic acid dispersed in 1 ml of 0.1M potassium phosphate buffer (pH 6.5).

Effect of linoleic acid on biomass viability and biotransformation. The cells for bioconversion were prepared by two methods: A) Cultivation of cells was performed in MRS medium for 96 h where linoleic acid (0.2%; w/v) was added at 0 h, 12 h, 24 h, 48 h and 72 h of cell growth. Washed cells were then used for subsequent bioconversion at 30 °C for 48 h. Cell viability was observed during whole experiment and was calculated as amount of colony forming units (CFU)/ml. B) Cultivation of cells was carried out in MRS medium with 0%, 0.06%, 0.1% and 0.2% LA (w/v) for 24 h. Washed cells were then used for subsequent bioconversion at 30 °C for 12–96 h. Biotransformation was realized as described previously.

Effect of detergents. Biomass was cultivated in MRS medium with 0.06% LA for 24h. Washed cells were used for further bioconversion with 0.2 % or 0.1 % detergent

(Tween 80, Struktol, sodium taurocholate and Span 80) as described previously.

Lipid analyses. After reaction the cells were harvested by centrifugation (4000 × *g*, 20 min), washed twice with 0.85% (w/v) NaCl and centrifuged again. 1 ml of dichlormethane with 2 ml of methanolic HCl were added to resuspended sediment and incubated at 50 °C for 20 min. Then 1ml of distilled water and 4 ml of hexane were added, stirred again and centrifuged (3000 × *g*, 5 min). The upper hexane layer containing fatty acid methyl esters was used for their detection by gas chromatography. Gas chromatograph (GC-6890 N, Agilent Technologies) was equipped with a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies) and a FID detector (constant flow, hydrogen 35 ml min^{-1}, air 350 ml min^{-1}, 250 °C). Analyses were carried out under a temperature gradient (130 °C for 1 min; 130–170 °C at program rate 6.5 °C min^{-1}; 170–215 °C at program rate 2.7 °C min^{-1}; 220–240 °C at program rate 2 °C min^{-1}) with hydrogen as a carrier gas (flow 2.1 ml min^{-1}, velocity 49 cm s^{-1}, pressure 174 kPa) and a split ratio of 1/50 (inlets: heater 230 °C, total hydrogen flow 114 ml min^{-1}, pressure 174 kPa). The fatty acid methyl ester peaks were identified by authentic standards of C_4–C_{24} fatty acid methyl esters mixture (Supelco, USA) and quantified by an internal standard of heptadecanoic acid (C17:0, Supelco, USA). Fatty acid concentration was evaluated with ChemStation software (Agilent technologies, USA).

Results

Screening of bacterial CLA producers. Almost 100 bacterial strains (used in food industry, from various Collections, bacteria isolated from the digestive tract of ruminants and probiotic bacteria) from the genera *Lactobacillus* and *Enterococcus* were tested for production of CLA. From the numerous bacteria, only six strains produced more than 3 mg total CLA/g dry cell (Table I). Of various CLA isomers, these strains produced considerable amounts of t, t-CLA (CLA3) isomer and small amount of both c9, t11-CLA (CLA1) and t10, c12-CLA (CLA2). The presence of 10-hydroxy-*trans*-octadecaenoic acid (HY1) and 10-hydroxy-cis-octadecaenoic acid (HY2) was also detected in all of these strains. Maximal ability to transform linoleic acid to CLA was found for *L. plantarum* JCM 8341 with yield of 16.2 mg CLA/g dry cell. *L. plantarum* JCM 8341 was then used for further optimization of reaction conditions.

Effect of linoleic acid on biomass viability and biotransformation. Biotransformation of linoleic acid to CLA is two-step process. The first step involves preparation of biomass with suitable activity of the key enzyme (linoleic acid isomerase). The second step is transformation of linoleic acid to CLA isomers, which proceeds in non-growing conditions. To induce the activity of linoleic acid isomerase, we tried to find optimal linoleic acid (LA) concentration during the biomass preparation. Therefore, the first effort was focused on the study of the optimal time for addition of linoleic acid (as inductor of CLA formation) to cultivation medium. The highest amount of viable cells was determined after 24 h cultivation for all LA additions. However, the cells grown on the medium with LA addition from the beginning of cultivation displayed the highest capacity to transform linoleic acid to CLA. The second attempt was aimed to investigation of various linoleic acid amounts in the cultivation medium. The result showed that 0.06 % of LA concentration in the cultivation medium was optimal for subsequent LA conversion to final yield of 35.4 mg CLA/g dry cell (Fig. 1.).

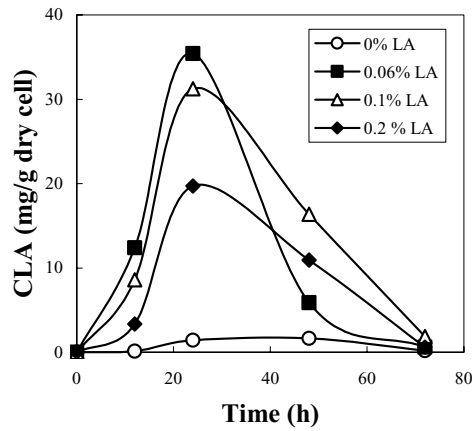

Fig. 1. **Effect of linoleic acid concentration in cultivation medium on CLA production by *L. plantarum* JCM 8341. Time indicates when biomass was taken from cultivation medium for bioconversion reaction**

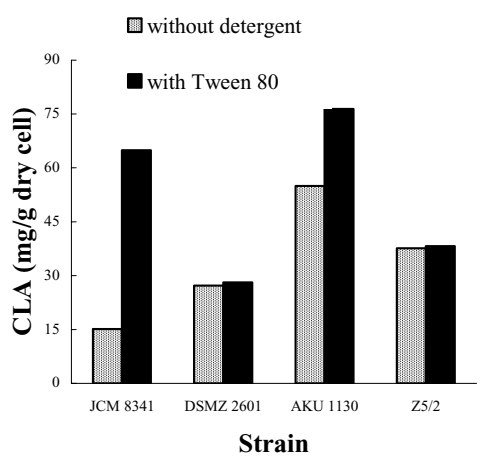

Fig. 2. **Effect of Tween 80 on LA conversion to CLA by various strains**

Effect of detergents. To increase the production of CLA, various detergent (Tween 80, sodium taurocholate, Span 80, Struktol) additions to biotransformation reaction were tested. It should be noted that only detergents presented at their concentration 0.2 % increased CLA formation markedly. Of them, maximal effect for CLA enhancement was observed in the reaction mixture with Tween 80. Under this condition, the yields of CLA produced by *L. plantarum* AKU 1130

Table I
Potential strains for conjugated linoleic acid (CLA) production

Strain[a]	Fatty acids[b] [mg/g dry cell]									
	16:0	18: 1	18:2	c-19:0	CLA1	CLA2	CLA3	HY1	HY2	Total CLA
L. p. AKU 1130	2.2	15.4	56.4	9.9	0.2	1.1	1.8	13.3	147.8	3.1
L. p. AKU 1138	5.8	9.2	40.7	13.3	–	0.6	9.8	18.5	14.2	10.4
L. p. DSMZ 2601	1.4	3.1	37.1	0.9	0.2	0.7	10.2	6.4	39.9	11.1
L. p. JCM 8341	0.9	5.7	57.8	7.5	2.2	0.5	13.5	68.5	3.5	16.2
L. p. 184/ 01	1.8	1.6	21.4	4.1	1.0	0.2	5.2	26.0	4.8	6.4
Z5	1.3	4.1	43.4	4.6	1.6	0.6	7.9	72.5	9.6	10.3

[a]Strains: *L. p. – Lactobacillus plantarum*, Z5 – culture isolated from rumen; [b]Fatty acids: 16:0 – palmitic acid; 18:1 – oleic acid; 18:2 – linoleic acid; c-19:0 – cyclo-nonadecanoic acid; CLA1 – c9, t11-CLA; CLA2 – t10, c12-CLA; CLA3 – t,t-CLA; HY1 – 10-hydroxy-*trans*-octadecaenoic acid; HY2 – 10-hydroxy-*cis*-octadecaenoic acid

and L. plantarum JCM 8341 were elevated up to 76.4 and 64.9 mg g^{-1} dry cells, respectively (Fig. 2.). CLA production by other strains was not significantly improved by tested detergents.

Discussion

Biotechnological techniques are promising alternative to chemical synthesis of CLA due to selective production of CLA isomers by various microorganisms. The fact that CLA is presented in very small amounts in ruminant meat and milk and in their products[4,5,6], it led to searching for suitable microbial producer which would increase CLA level in these products. Our results show that *L. plantarum* AKU 1130 or *L. plantarum* JCM 8341 could be promising CLA producers as they formed almost 35 % CLA of entire fatty acids present in the cells. However, further optimization of CLA overproduction is still required.

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REFERENCES

1. Khanal R. C., Dhiman T. R.: Pakistan J. Nutr. *3*, 72 (2004).
2. Ogawa J., Matsumura K., Kishino S., Omura Y., Shimizu S.: Appl. Environ. Microbiol. *67*, 1246 (2001).
3. Šajbidor J., Lamačka M., Čistá J., Čertík M.: Biotechnol. Techniques *8*, 561 (1994).
4. Bauman D. E., Baumgard L. H., Corl B. A., Griinari J. M.: Proc. Am. Soc. Anim. Sci. 1–15, (1999).
5. Lin H., Boylston T. D., Chang M. J., Luedecke L. O., Shultz T. D.: J. Food Sci. *64*, 874 (1999).
6. Zlatanos S., Laskaridis K., Feist C., Sagredos A.: Food Chem. *78*, 471 (2002).

L05 PURPOSE AND INDUSTRIAL APPLICATIONS OF PECTOLYTIC ENZYMES

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Introduction

Pectins represent a complex family of heterogeneous branched polysaccharides that arise from the primary cell walls and intercellular regions of higher plants. The terms pectins or pectic substances describe a group of polysaccharides in which the presence of partly methyl-esterified galacturonic acid and rhamnose is a distinctive feature[1] .

Pectic substances can undergo two main types of degradation, depolymerization and de- esterification, where both degradation ways can be enzymatically catalysed. Due to the complexity o pectins, many different enzymes are required to achieve their degradation.

Generally, pectolytic enzymes can by divided into two groups[1]:

1. Pectolytic enzymes of microbial nature (exogeneous enzymes).

These participate on degradation of plant cell walls. In this way they help the microorganisms to colonize the plant tissues and assure their alimentation.

These properties are utilized in food and textile industry. Microbial endopolygalacturonases are potential avirulent fac-

tors. They can initiate immune reaction of plants, synthesis and cumulation of e. g. phytoalexins or protein inhibitors.

2. Pectolytic enzymes of plant nature (endogeneous enzymes).

These enzymes play important role by physiological processes of plant materials e. g. by growth and ripening.

Commercial pectinases are produced by cultivation of Aspergillus species. They are utilized in the industrial treatment of fruit and vegetables in following procedures.

Maceration- in maceration process, the fruit tissue is transformed in a cell suspension, resulting in pulpy products that can be used as a base for nectars, baby foods and ingredients for dairy products.

Liquefaction- liquefaction process induces complete degradation of cell wall, leading to the release of protoplasts. This technology is applied to fruits that cannot be easily pressed, especially tropical fruits and stored apples.

Pressing- this step allows a partial degradation of the cell walls, with a subsequent increase in juice yield, as well as a better extraction of pigments and aroma.

Clarification- cloud particles consist mainly of proteins and pectins. Proteins are positively charged and coated by negatively charged pectins. Pectin degradation reveals the positive charges from one particle to the negative charges of another particle, allowing them to aggregate and flocculate.

Results and discussion

In the framework of this paper commercial preparations of pectinases were investigated, where polygalacturonase activity was determined. By the study of viscosity decrease and percentage of split glycosidic bonds of lemon pectin, by all preparations was found out that 50% decrease of viscosity occurs after splitting 0.5–2.0 % of glycosidic bonds. Commercial preparations mutually differed in activity of pectinlyase (Table I) and pectinmethylesterase. By some preparations no cellulolytic activity was evidenced. Also the determined values of pH optimum Fig. 1., heat stability and temperature optimum by individual preparations mutually varied. The experiments with individual preparations contributed to the correlation of conditions for their final application[2].

Pectolytic enzymes, occurring in plant materials (fruits and vegetables) play important role by their ripening and storage. Therefore, the attention was devoted to the study of pectolytic enzymes in carrot and potatoes. By carrot the presence of pectolytic enzymes was investigated in protein precipitates from extracts, obtained in summer and autumn. Besides the expected pectinesterase and exopolygalacturonase activity, also the pectinacetylesterase, ramnogalacturonase and ramnogalacturonanlyase activity was determined. The experiments have also confirmed the absence of endopolygalacturonase. This paper represents the first study which reports on the presence of ramnogalacturonan degradation enzymes in these plant materials[3]. Up to present days, also the occurrence of pectinacetylesterase was not reported.

The presence of polygalacturonase or exopolygalacturonase in potatoes was not described yet. The probably reason is the huge amount of starch in potatoes interferencing with the enzyme assay and the possible presence of some natural regulator of polygalacturonase activity. The presence of

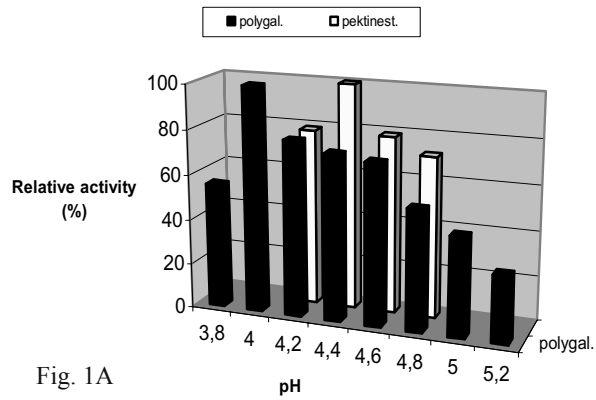

Fig. 1A

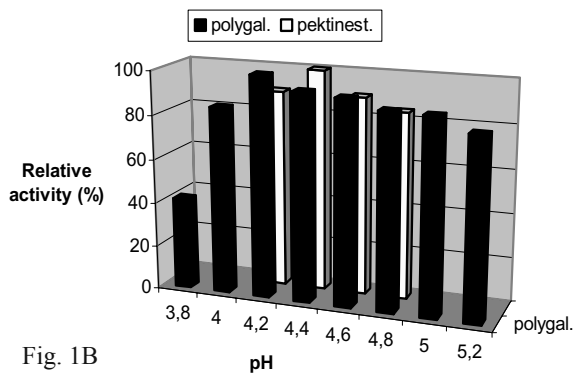

Fig. 1B

Fig. 1. **The pH optimum of polygalacturonase and pectinesterase of preparations VINOZYM G13 (A) and VINOFLOW (B)**

Table I
Activity of pectinlyase in commercial preparations

Commercial preparations	Vinozym G	Vinoflow	Trenoline rouge 5	Ovopres	Gammapect W2L	Gammapect W70L
Specific activity	1.32 10^{-4}	1.25 10^{-4}	0.828	0.760	0.583	0.289
Unite	A_{235} min g^{-1}	A_{235} min g^{-1}	A_{235} min g^{-1}	A_{235} min g^{-1}	A_{235} min g^{-1}	A_{235} min g^{-1}

exopolygalacturonase or oligo-D-galacturonid hydrolase can be at lest supposed as it was in carrot roots[3,4]. The production of polygalacturonase with random action pattern was not expected, because healthy potatoes did not yield to a softening. Activity of polygalacturonase cannot be evaluated directly in protein precipitate of potatoes[5]. It appears only after its partial purification and increases with each purification step. As shown by isoelectric focusing with activity detection, both low- and highmolecular mixtures of proteins with polygalacturonase contained multiple forms of these enzymes. All isoelectric points of them were in slightly acidic region. The values of isoelectric points of majority forms were 6.5 and 6.7. Enzymes in low- and high- molecular mixtures were characterized by their pH optima (Fig. 2.), optima of temperature (Fig. 3.) and thermal stabilities (Fig. 4.).

Till now, only pectinases produced by fungi are utilized within industrial application. Generally, there is a lack on information about the production and properties of pectolytic enzymes from yeasts. Study of pectolytic enzymes from yeasts and yeasts microorganisms was performed with following strains of yeasts:

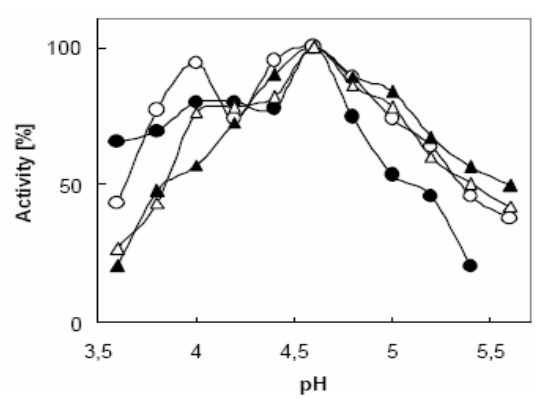

Fig. 2. **pH optimum of polygalacturonases from potatoes. (▲–▲) – juice, highmolecular, (△–△) – juice, lowmolecular, (●–●) – pulp, highmolecular, (○–○) – pulp, lowmolecular**

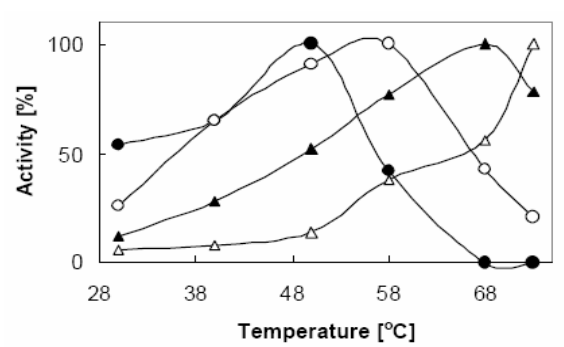

Fig. 3. **The optimum of temperature of polygalacturonases from potatoes. (▲–▲) – juice, highmolecular, (△–△) – juice, lowmolecular, (●–●) – pulp, highmolecular, (○–○) – pulp, lowmolecular**

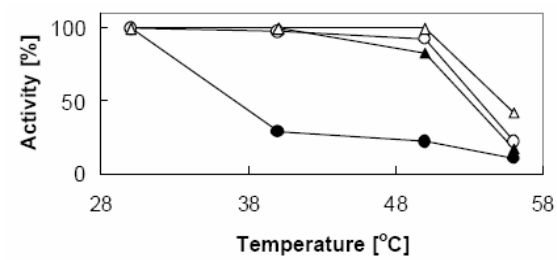

Fig. 4. **Thermal stability of polygalacturonases from potatoes. (▲–▲) – juice, highmolecular, (△–△) – juice, lowmolecular, (●–●) – pulp, highmolecular, (○–○) – pulp, lowmolecular**

Cryptococcus laurenti	(CCY 17-3-16)
Candida boidinii	(CCY 29-37-13)
Aureobasidium pullulans	(CCY 27-1-98)
	(CCY 27-1-111)
	(CCY 27-1-115)

Production of pectolytic enzymes was induced by the presence of pectin substances in cultivation medium, representing the only source of carbon.

C. boidinii was able, as methylotrophic microorganism, to grow on pectan only after adaptation on pectin (primary utilization of methanol).

The strains *A. pullulans* produced during the first steps of growth the polygalacturonases with random and terminal way of action. In later steps of growth only exopolygalacturonases were present (influence on phytopathogenity of microorganism). Active site of endopolygalacturonase was identical with that of enzyme produced by fungi.

The presence of some stress factors during cultivation of *A. pullulans*, such as oxygen limitation, osmosis or the presence of heavy metal in cultivation medium, influenced the first (virulent) phases of the production of polygalacturonases. Generally, the polygalacturonases appeared later and the presence of cadmium supported their production – higher activities were observed and the time of production was prolonged (Fig. 5.). The influence of osmosis (4% NaCl) was strain dependent – 27-1-111 (Fig. 5B) and 27-1-115 (Fig. 5C) showed similar effect as by the presence of heavy metal, while by the 27-1-98 (Fig. 5A) the production was depressed.

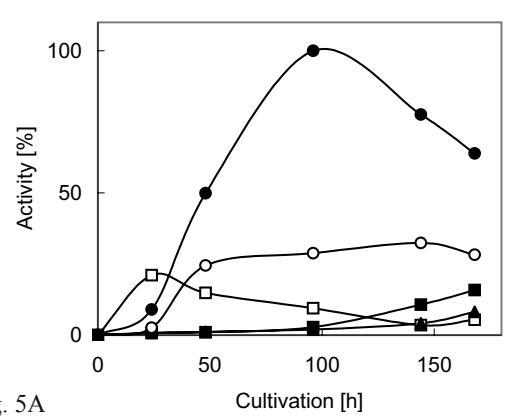

Fig. 5A

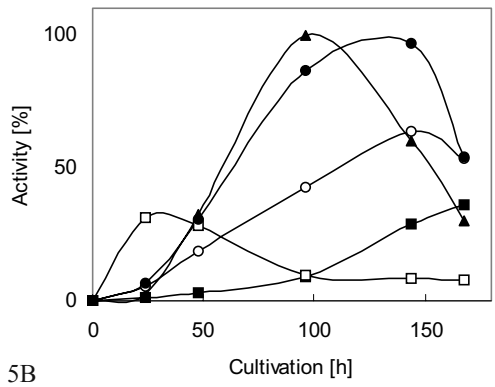

Fig. 5B

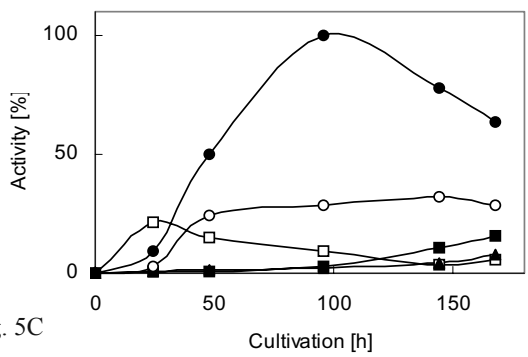

Fig. 5C

Fig. 5. **Production of polygalacturonases by three strains of A. pullulans in physiological conditions and in stress. A – 27-1-98, B – 27-1-111 and C – 27-1-115. (□–□) – physiological conditions, (■–■) – oxygen limitation, (▲–▲)– 4 % of NaCl (osmosis), (○–○) – 5 mM Cd^{2+} and (●–●) – 10 mM Cd^{2+}**

Reduction of polygalacturonase production by all strains can be observed by oxygen limitation (Fig. 5.), but the curves indicated only even more delayed production of these enzymes by all strains. This phenomenon can lead to prolonged and more effective destructive phase of *A. pullulans* on pectin substances and the behaviour of this yeast-like microorganism may correspond to the behaviour of phytopathogenic fungi[6].

REFERENCES

1. Steinhübel A.: Biopolymers *6*, 345 (2002).
2. Omelková J., Škvařilová D., Indruch P.: *Porovnávací studie vlastností vybraných komerčních pektolytických preparátů*. XXXII. Symposium o nových směrech výroby a hodnocení potravin, p. 50, Skalský Dvůr.
3. Stratilová E., Markovič O., Dzúrová M., Malovíková A., Capek P., Omelková J.: Biologia *53*, 731 (1998).
4. Omelková J., Dzúrová M., Stratilová E.: *Degradation of pectate and oligogalacturonides by exo-glycosyl hydrolases produced in carrot roots*. The CD-ROM from the International Carbohydrate Symposium, p. 376, 2002, Cairns, Australia
5. Dzúrová M., Omelková J., Stratilová E.: *Polygalacturonases from potatoes*, 9th Bratislava Symposium on Saccharides, p. *67*, 2000 Smolenice,Slovakia.
6. Omelková J., Dzúrová M., Stratilová E.: *The partial characterization of majority basic form of extracellular polygalacturonase produced by Aureobasidium pullulans*. VII. Pracovní setkání biochemiků a molekulárních biologů, p. *44*, 2003, Brno.

L06 MICROBIAL PREPARATION OF POLYUNSATURATED FATTY ACIDS BY FUNGAL SOLID STATE FERMENTATION

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Introduction

Microorganisms have often been considered for the production of oils and fats as an alternative to agricultural and animal commodities[1]. One of the main targets for microbial oils is construction of healthy and dietary important polyunsaturated fatty acids (PUFAs), such as γ-linolenic acid (GLA), dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA). Particularly active in the synthesis of PUFAs are lower filamentous fungi belonging to *Zygomycetes*[2]. Anticipated success in the microbial PUFAs production has led to a flourishing interest in developing fermentation processes and enabled several processes to attain commercial production levels[3]. With the advent of biotechnological innovations, many new avenues have opened for the application of solid state fermentations (SSF) in food, feed, pharmaceutical, veterinary and environmental fields. Technique of solid state fermentation has shown many promises in the development of several bioprocesses and products. Thus, the association of oleaginous fungi with solid state fermentations has created new perspectives for the economic competitiveness and market of microbial PUFAs. The present study deals with preparation of bioproducts enriched with PUFAs by two selected strains, *Thamnidium elegans* and *Mortierella alpina* that were successfully employed for synthesis of various PUFAs during SSF of some agroindustrial materials.

Materials and Methods

Microorganisms. *Thamnidium elegans* CCF 1456 and *Mortierella alpina* CCF 185 were obtained from the Culture Collection of Fungi (Charles University, Prague, Czech Republic). The cultures were maintained on modified Czapek-Dox agar slants with yeast extract (2.5 g l^{-1}) at 4 °C (water leachate of soil was used instead of water). The spore suspension for inoculation was prepared by washing the mycelium with a sterile distilled water to reach the final concentration of 1–2.10^6 spores per ml.

Substrates and cultivation conditions. Depending on the microorganism, various types of substrates were employed during SSF experiments. Spent malt grains (SMG) were added to some substrates and served as an internal support. Autoclavable microporous polypropylene bags (157×265 mm^2) were filled with 10 g of dry substrate [wheat sprout/SMG (3:1, w/w), wheat bran/SMG (3:1, w/w), rye bran/SMG (3:1, w/w), rice, peeled barley, sesame seeds, peeled barley/linseed oil/spent malt grains (0.5:1:3, w/w), wheat flour /linseed oil/yeast extract/spent malt grains (0.5:2:0.1:3, w/w)], moistened by the addition of 15 ml distilled water, soaked for 2 h at laboratory temperature and sterilized in autoclave (120 kPa, 120 °C, 20 min). The bags filled with oat flakes or noodles were first sterilized and then consequently moistened with 10 ml sterile distilled water. The substrates were inoculated and mixed with 2 ml of spore suspension. Then each bag was closed with sterile cotton plug, inoculated substrate was spread in the bags to obtain substrate layer of about 1 cm and incubated statically at 24 °C for 4–6 days (*T. elegans*) or 10–14 days (*M. alpina*), respectively. Following carbon sources were added to substrate in some bags (%, w/w): whey 5, glucose 5, sunflower oil 50, triloleine 30 and linseed oil 30. Triplicate SSF experiments for each substrate were prepared to assess reproducibility and average results are presented.

Lipid extraction and fatty acid analysis. Bioproduct gained after cultivation was gently dried at 65 °C for 10 hours and weighed. Lipid from homogenized bioproduct was isolated with chloroform/methanol (2:1, v/v) mixture[4] and total lipid was determined gravimetrically. Fatty acids of total lipids were analyzed as their methyl esters[5] by gas chromatography (GC-6890 N, Agilent Technologies) equipped with DB-23 column according to Čertík et al.[6]

Results and discussion

Solid-state fermentations can be carried out on a variety of agricultural materials and residues that have limited nutritional values. To optimize the potential of microorganisms to transform agroindustrial materials and residues into desired metabolites, emphasis is put on the fungal SSF processes to achieve bioproducts with high PUFA content. Moreover, because filamentous fungi simultaneously decrease anti-nutrient compounds and partially hydrolyze substrate biopolymers, fermented bioproduct may be used as an inexpensive food and feed supplement that may fill marketing claims. Two lower filamentous fungi, *T. elegans* and *M. alpina*, with the appropriate capacity to synthesize various PUFAs, were chosen as production microorganisms in these studies. The first step was to screen and to select agroindustrial material as suitable substrates for fungal overproduction of PUFAs. Generally, the surface of substrates was not only covered by the fungal mycelium during cultivation, but the fungal hyphae also penetrated into the substrates. Thus, fungal PUFAs were accumulated in the newly formed bioproduct and their amount depended on the substrates, microorganisms and cultivation conditions used.

G L A p r o d u c t i o n

T. elegans effectively utilized various types of cereals and other materials and enriched them with oil containing GLA. This strain usually covered substrates after 2 days from inoculation and the whole cultivation takes 4–6 days to complete. The ratio of spent malt grains (SMG) to the substrates was optimized during preliminary experiments (data not shown). SMG served as an internal support, which enhanced porosity of the cereal substrate and thereby improved the oxygen transfer within the substrate layer. Addition of SMG to all tested substrates resulted in rise of GLA content in total lipid and GLA yield (Table I). The content of GLA in bioproducts after fermentation of some other substrates is shown in Table II. None of the tested substrates contained GLA befor fermentation. Mixture of spelt flakes/spent malt grains (3:1, w/w) was found the best substrate and its solid state fermentation by *T. elegans* resulted in bioproduct containing 7.2 g GLA/kg bioproduct. Further improvement of GLA formation was achieved by physiological regulation of the SSF process employing following steps:

a) gradual elevation of carbon/nitrogen ratio with addition of glucose or whey[7];
b) optimization of water activity, temperature and oxygen availability[8];
c) transformation of exogenously added oils consisting of linoleic acid as a precursor of GLA[9].

The final fermented bioproduct contained 25 % lipid and the total GLA amount yielded almost 10 g/kg bioproduct (Fig. 1.). T. elegans was also used as a production microorganism by other authors and its growth on pearl barley or apple pomace with addition of peanut oil resulted in 8.0 or 8.7 g GLA/ kg bioproduct, respectively[10,11].

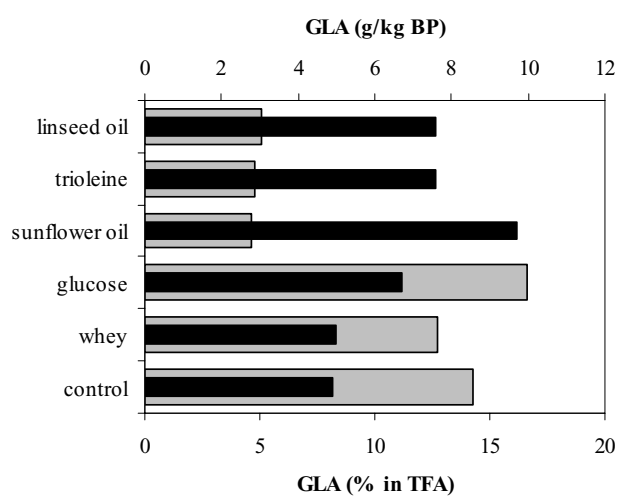

Fig. 1. **Effect of various carbon compounds addition to the substrate on GLA concentration in total fatty acids (TFA) ( ) and GLA yield in bioproduct (BP) ( ) after solid state fermentation. The substrate used for fermentation was the mixture of wheat bran/spent malt grains (3:1, w/w). The amounts of carbon sources addition: whey 5 %, glucose 5 %, sunflower oil 50 %, trioleine 30 %, linseed oil 30 %**

Table I
Bioproduct (BP), total lipid content in bioproduct (TL/BP), fatty acid composition in total fatty acids (FA/TFA) and γ-linolenic acid yield in bioproduct (GLA/BP) after solid state fermentation by *Thamnidium elegans* grown on various agroindustrial substrates. The substrates before fermentation contained no GLA

Substrate	BP [g]	TL/BP [%]	FA/TFA [%] 16 : 0	18 : 0	18 : 1	18 : 2	18 : 3n6	18 : 3n3	GLA [g/kg BP]
amaranth	7.4	7.5	20.3	3.6	33.2	35.9	5.4	0.6	3.1
amaranth/SMG[a] (3 : 1)	7.5	7.8	18.9	4.3	32.9	33.6	7.8	0.5	4.7
spelt flakes	5.8	5.8	18.4	4.4	35.1	28.6	10.8	0.9	4.6
spelt flakes/SMG[a] (3 : 1)	6.6	7.2	16.5	2.6	34.5	28.3	13.1	2.7	7.2
spelt/SMG[a] (3 : 1)	8.4	2.0	16.9	2.6	26.6	42.5	8.6	1.7	1.2
buckwheat	7.2	6.8	16.2	3.5	46.4	22.9	7.6	1.8	3.9
buckwheat/SMG[a] (3 : 1)	7.7	5.7	18.1	3.4	39.3	26.0	10.1	1.4	4.3
control (non-fermented substrate)									
amaranth		4.2	23.7	4.3	24.8	46.5	–	0.5	–
amaranth/SMG[a] (3 : 1)		5.5	24.9	4.4	23.8	46.1	–	0.6	–
spelt flakes		1.0	16.1	0.6	22.3	58.5	–	2.2	–
spelt flakes/SMG[a] (3 : 1)		2.2	25.7	1.5	19.3	48.9	–	3.5	–
spelt/SMG[a] (3 : 1)		1.8	23.2	1.4	20.1	51.5	–	3.4	–
buckwheat		2.5	14.8	1.5	38.4	39.6	–	5.1	–
buckwheat/SMG[a] (3 : 1)		2.6	20.6	1.7	32.8	39.5	–	4.5	–

[a]SMG – spent malt grains

Table II
Total lipid content in bioproduct (TL/BP), γ-linolenic acid (GLA) and arachidonic acid (AA) composition in total fatty acids (TFA) and yield in bioproduct (BP) after solid state fermentation of *Thamnidium elegens* or *Mortierella alpina* grown on various agroindustrial substrates. The substrates contain neither GLA nor AA

Substrate	Thamnidium elegans			Mortierella alpina		
	Lipid [% in BP]	GLA [% in TFA]	[g/kg BP]	Lipid [% in BP]	AA [% in TFA]	[g/kg BP]
rice	4.1	4.2	1.2	8.1	31.1	21.4
wheat sprout/SMG[a] (3 : 1)	6.8	8.6	4.5	10.3	42.7	36.1
rye bran/SMG[a] (3 : 1)	5.4	9.5	3.8	9.2	34.4	21.9
wheat bran/SMG[a] (3 : 1)	4.7	14.3	5.0	11.2	45.3	42.3
noodles	0.6	11.0	0.4	4.2	40.1	14.6
peeled barley	2.4	7.8	1.3	5.4	37.6	16.2
oat flakes	9.3	6.4	4.7	10.7	35.7	31.2

[a]SMG – spent malt grains

AA, DGLA and EPA production

Solid-state fermentations were also employed to improve the market for arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) and eicosapentaenoic acid (EPA). Screening of many fungi has resulted in selection of *Mortierella alpina* that has also been used for preparation of microbial oil with high polyunsaturated fatty acid (PUFA) content by submerged fermentations[3]. The growth of this fungus on agroindustrial materials during SSF was much slower compare with *T. elegans* and fermentation was completed after 10–14 days. Nevertheless, search for optimal substrate revealed that *M. alpina* satisfactory converted mixture of wheat bran/spent malt grains (3 : 1, w/w) leading to bioproduct with 4.2 % AA (Table I). Similar results with *M. alpina* were also published by Streďanská et al.[12]

Basic cultivation of *M. alpina* usually leads to standard fatty acid profile with predominant concentration of AA and only low level of DGLA. Since bioconversion of DGLA to AA is catalyzed by Δ^5-desaturase, inhibition of this metabolic step is accompanied by rapid increase of DGLA/AA ratio. Addition of sesame seeds (they contain sesamin analogous, which are known as an effective inhibitors of Δ^5-desaturase) to peeled barley rapidly enhanced DGLA levels in bioproduct and DGLA/AA ratio as well. However, when *M. alpina* utilized only crushed sesame seeds (they are characterized by sufficient amount of saccharides, proteins and lipids), the DGLA/AA ratio in bioproduct after 13 days of cultivation reached value of 2.9 that corresponded to almost 20 g DGLA/kg bioproduct[13].

SSF process has been developed to prepare EPA-rich bioproducts using *M. alpina* that can rapidly utilize, incorporate and modify exogenously added oils. Linseed oil consists of about 57 % α-linolenic acid that is a precursor of PUFAs belonging to ω-3 fatty acid family. This effort has led to fermentation of the optimized mixture of peeled barley/linseed oil/spent malt grains (0.5:1:3, w/w) by *M. alpina* that simultaneously yielded 23.4 g EPA/kg bioproduct (17.8 % EPA in oil) and 36.3 g AA/kg bioproduct (27.6 % AA in oil). Thus, such strategy allows the preparation of oils with desirable ω-6/ω-3 PUFA ratio finally leading to more beneficial human applications.

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REFERENCES

1. Čertík M., Shimizu S.: Agro Food Industry Hi-Tech *10*, 26 (1999).
2. Ratledge C.: in: *Biotechnology* (Rehm H. J. and Reed G., ed.), p. 133. VCH, Weinheim 1997.
3. Čertík M., Shimizu S.: J. Biosci. Bioeng. *87*, 1 (1999).
4. Čertík M., Andráši P., Šajbidor J.: J. Am. Oil Chem. Soc. *73*, 357 (1996).
5. Christoperson S. W., Glass R. L.: J. Dairy Sci. *52*, 1289 (1969).
6. Čertík M., Breierová E., Juršíková P.: Int. Biodet. Biodegrad. *55*, 195 (2005).
7. Sláviková L., Čertík M., Masrnová S., Škrinárová B., Šajbidor J.: Chem. Listy *96*, 179 (2002).
8. Sláviková L., Čertík M., Masrnová S., Škrinárová B., Valík Ľ., Šajbidor J.: Chem. Listy *96*, 181 (2002).
9. Čertík M., Sláviková L., Masrnová S., Škrinárová B., Angelovičová M., Šajbidor J.: *Proceedings of XIV. International Conference LABORALIM* (Staruch L., Dandár A., ed.), p. 133. STU Press, Bratislava 2003.
10. Conti E., Streďanský M., Streďanská S., Zanetti F.: Biores. Technol. *76*, 283 (2001).
11. Streďanský M., Conti E., Streďanská S., Zanetti F.: Biores. Technol. *73*, 41 (2000).
12. Streďanská S., Slugeň D., Streďanský M., Grego J.: World J. Microbiol. Biotechnol. *9*, 511 (1993).
13. Huong L. M., Slugeň D., Šajbidor J.: Biologia *53*, 801 (1998).

L07 BIOTECHNOLOGICAL PRODUCTION OF ASTAXANTHIN

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Introduction

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is the main carotenoid pigment found in aquatic animals and many of seafood including salmon, trout, red sea bream, shrimp, lobster and fish eggs. It is also present in birds such as flamingoes and quails. Astaxanthin has several essential biological functions in animals[1] and is known as a free radical quencher and powerful antioxidative reagent[2]. Industrially, astaxanthin has been exploited as a pigmentation source and feed supplement for marine fish aquaculture and poultry. It is evident, therefore, that astaxanthin is a molecule with potential to the pharmaceutical, food, cosmetic and medical fields.

Astaxanthin has been produced mainly through the complex of chemical reactions on an industrial scale. However, it is not in the same form as found in nature, and its stability and activity is lower than that of natural product. Since astaxanthin molecule has two identical chiral centers, synthetic astaxanthin exists in three configurational (stereo) isomers: (3*S*,3′*S*) (3*S*,3′*R*), and (3*R*,3′*R*)[1]. The natural astaxanthin is in the 3*S*,3′*S* configuration. Moreover, the use of chemical synthetic compounds as food additives has been strictly regulated in recent years. Therefore attention is paid on the finding of suitable natural methods for its production. One method is based on fermentation techniques employing microorganisms that are able to convert various substrates into this pigment.

Astaxanthin biosynthesis has been observed in a limited number of microorganisms, e. g. in marine bacteria (*Agrobacterium aurantiacum* and *Alcaligenes* sp. strain PC-1), yeast *Phaffia rhodozyma* and in some freshwater green algae, mainly *Haematococcus pluvialis*[3]. Of them, *Phaffia rhodozyma* has some advantageous properties that make it attractive for commercial astaxanthin production: (i) it synthesizes astaxanthin as a principal carotenoid, (ii) it does not require light for its growth and pigmentation, and (iii) it can utilize many

kinds of carbon and nitrogen sources[4]. In order to improve yield of astaxanthin and subsequently decrease the cost of this biotechnological process, diverse works have been published on the using less expensive substrates, on increasing production of this pigment by optimizing the culture conditions including physical (pH, temperature, oxygen availability) and nutritional factors (carbon and nitrogen sources, vitamins)[5]. Other approaches that have been applied to increase astaxanthin yield comprise mutagenesis[6], stimulation by stress factors[7], and genetic and metabolic engineering[8]. The present work was focused on physiological regulation and scale up of astaxanthin production by *Phaffia rhodozyma*.

Material and methods

Microorganisms and flasks experiments: Three strains of *Phaffia rhodozyma* were used in this study: MUCL 31142 (Agroindustrial Fungi & Yeast Collection, Mycotheque de l'Universite Catholique de Louvain), CCY 77-1-1 (Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava) and UCD 77-61 (Yeast Culture Collection, California Institute for Food and Agricultural Research, University of California). The cultures were maintained on both yeast malt agar and wort malt agar at 4 °C. The yeast suspension for inoculation was prepared by washing the cells with sterilized distilled water (10 mL/slant) and diluted with additional 40 mL of sterilized distilled water. Basic cultivation medium for flasks experiments consisted of (g/L): glucose – 20, yeast autolysate – 2.0, KH_2PO_4 – 0.4, $(NH_4)_2SO_4$ – 2.0, $MgSO_4.7H_2O$ – 0.5, $CaCl_2$ – 0.1, NaCl – 1.0. The cultivation medium (100 mL) was inoculated by 1 mL of the prepared spore suspension and cultivation was carried out in 500 mL flasks on a rotary shaker (150 rpm) at 20 °C.

Fermentation experiments: The basic medium contained (g/L): glucose – 3/15/30, yeast autolysate – 7.5, KH_2PO_4 – 2.0, $(NH_4)_2SO_4$ – 2.0, $MgSO_4.7H_2O$ – 2.0, $CaCl_2$ – 0.1, NaCl – 1.0, $FeSO_4$ – 0.08, $ZnSO_4$ – 0.02, $MnSO_4$ – 0.004, $CuSO_4$ – 0.0014, $CoSO_4$ – 0.43 and vitamins (mg/L) as biotin – 0.57, thiamine – 0.28, pantothenate Ca – 36.0 or inositol – 39.0. In batch fermentation, the culture was after inoculation allowed to grow to stationary phase. In fed-batch regime, the operation was started prior to complete glucose exhaustion from the medium (at a residual glucose concentration of approximately 0.25 g l^{-1}). The feed rate varied in order to maintain a constant residual glucose concentration. Nitrogen and phosphate levels in feed medium were not increased with addition of glucose concentration in order to ensure that carbon was the only limiting nutrient. The experiments were conducted in 20 L (SLF-20) and 100 L (Bio-la-fite) fermentors with an agitation rate of 250–450 rpm and a temperature of 20–22 °C. The pH was in individual cases uncontrolled or controlled at pH 5.0 by the addition of NH_4OH. The dissolved oxygen concentration (DO) was maintained by supplying sterile air at a flow rate equivalent to 0.3–0.7 vvm.

Analytical methods: Biomass was measured gravimetrically as dry cell mass. Reducing sugars were assayed colorimetrically in the supernatant by the 3,5-dinitrosalicylic acid method. Astaxanthin was extracted into ethanol after disruption of the yeast cells in desintegrator and was measured by HPLC. Chromatographic separations were performed on a LiChrospher 100 RP-18 (5 μm) with ethanol as eluting solvent (flow rate 1ml/min). The eluent was monitored at 476 nm. Synthetic astaxanthin (Sigma) was used as an external standard.

Results and Discussion

Screening experiments. To find suitable producer of astaxanthin, three species of *P. rhodozyma* were tested in this study. Kinetic analysis revealed that these strains differed each other from the view of both growth rates and astaxanthin formation. After basic optimization of cultivation conditions, maximal yield of 2.1 mg astaxanthin/L was reached by *P. rhodozyma* UCD 77-61 (Fig. 1.). Therefore, this strain was selected for further investigations.

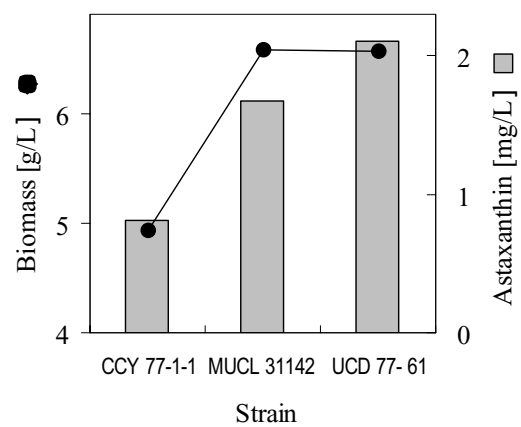

Fig. 1. **Growth and astaxanthin production by three strains of *P. rhodozyma***

Flasks experiments. To improve biosynthesis of this pigment, various sources and concentrations of carbon (glucose, saccharose, fructose, galactose, maltose, lactose, xylose, starch, glycerol) and nitrogen sources (inorganic sources, yeast extract, a range of commercially available proteine hydrolysates) were studied (data not shown). Maltose enhanced astaxanthin yield about 1.5 times compared to glucose. The pigment formation was also improved by increasing initial glucose concentration up to 30 g L^{-1}. Addition of glucose to medium at exponential growth phase resulted in further improvement of astaxanthin biosynthesis by *P. rhodozyma* UCD 77–61. Experiments with nitrogen sources showed that combination of $(NH_4)_2SO_4$ with yeast extract or some soy protein hydrolysates favoured astaxanthin production. It should be noted that cultivation in flasks is more limited to oxygen compare to cultivation in well-regulated fermentation tanks. In order to check effect of oxygen availability on astaxanthin formation, the strain was cultivated on various amounts of cultivation medium in flasks (dissolved oxygen is reduced

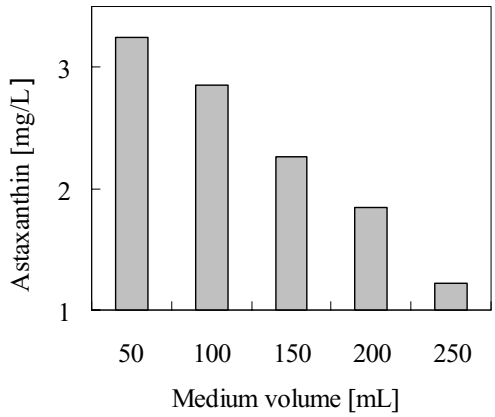

Fig. 2. **Effect of medium volume on astaxanthin production by *P. rhodozyma***

with increased medium volume). Our results confirmed that cultivation of the strain must be carried out in flasks containing small amount of cultivation medium, where formed astaxanthin reached maximal levels (Fig. 2.). Other approach how to enhanced astaxanthin production is to employ stress factors to medium. Many yeasts have been described with an increased ability to produce carotenoids when they grown under unfavorable environment[9]. Schroeder and Johnson[10] have reported that carotenoid biosynthesis is regulated by singlet oxygen and peroxyl radicals in the *P. rhodozyma* cell. Fig. 3. demonstrates that addition of H_2O_2 and ethanol stimulates astaxanthin biosynthesis. However, such stimulation was more effective if stress factors were employed to the medium in exponential growth phase than from the beginning of cultivation. Interestingly, addition of selene not only improved astaxanthin production but also other forms of astaxanthin were detected under selene presence (data not shown). Effect of other heavy metals on astaxanthin formation is still under investigation. Nevertheless, activated biosynthesis

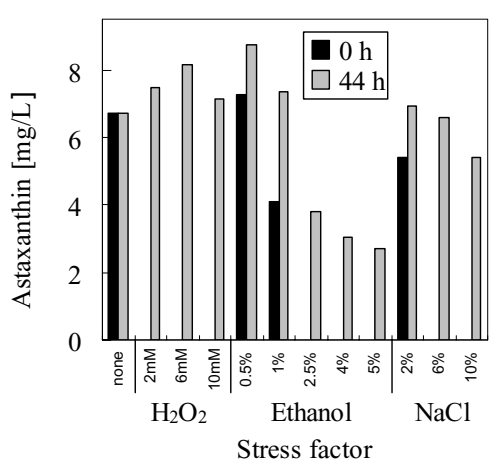

Fig. 3. **Effect of stress factors on astaxanthin production by *P. rhodozyma*. Stress factors were added to the medium either at the beginning of cultivation (0 h) or at exponential phase of cultivation (44 h)**

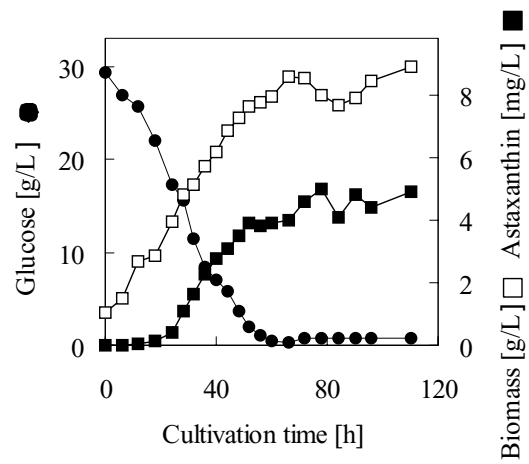

Fig. 4. Growth, glucose consumption and astaxanthin production by P. rhodozyma **during batch fermentation in 100 L fermentor**

of astaxanthin by *P. rhodozyma* exposed to heavy metal presence could be in a part explained by its scavenger characters as a protection against the harmful effect of cultivation conditions.

Fermentations in bioreactors. Information about physiological regulation of astaxanthin in flasks cultivations was verified in bioreactors. Basic kinetic of growth and astaxanthin production by *P. rhodozyma* in batch fermentation is displayed in Fig. 4. It is obvious that glucose utilization is accompanied by increased astaxanthin biosynthesis. When glucose was exhausted from the medium, astaxanthin formation was also reduced. Therefore glucose was added several times to the medium during fed-batch fermentations (data not shown) what resulted in enhanced production of the pigment. Yamane et al.[4] also reported that high carbon/nitrogen ratio induced amount of astaxanthin and C/N-regulated fed-batch fermentation of *P. rhodozyma* led to 16 mg astaxanthin/L. Our experiments with optimized fermentation condition finally yielded up to 20 mg astaxanthin/L (0.6 mg astaxanthin/g cell). Thus, this strain can be considered as a potential producer of astaxanthin.

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REFERENCES

1. Guerin M., Huntley M. E., Olaizola M.: Trends Biotechnol. *21*, 210 (2003).
2. Naguib Y.: J. Agr. Chem. *48*, 1150 (2000).
3. Johnson E. A., Schroeder W. A.: Adv. Biochem. Eng. Biotechnol. *53*, 119 (1995).
4. Yamane Y-I., Higashida K., Nakashimada Y., Kakizono T., Nishio N.: Appl. Environ, Microbiol. *63*, 4471 (1997).

5. Ramirez J., Gutierrez H., Gschaedler A.: J. Biotechnol. *88*, 259 (2001).
6. An G. H., Schuman D. B., Johnson, E. A.: Appl. Environ. Microbiol. *55*, 116 (1989).
7. Gu W. L., An, G. H., Johnson, E. A.: J. Ind. Microbiol. Biotechnol. *19*, 114 (1997).
8. Lodato P., Alcaino J., Barahona S., Retamales P., Jimenez A., Cifuentes V.: Biol. Res. *37*, 83 (2004).
9. Certik M., Breierova E.: Chem. Listy *96*, 147 (2002).
10. Schroeder A. W., Johnson E. A.: J. Biol. Chem. *270*, 18374 (1995).

L08 THE YEAST CONTAMINANTS ISOLATED FROM SPOILED SOFT DRINKS

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Introduction

The yeasts are important components of fermentation processes in wines, mainly when the basic substrates are saccharides. Generally, the knowledge about characteristic features of the yeast species in wines and their precise and rapid identification can serve as safety system for control of individual fermentation processes. The presence of some yeast species can highlight the error arising in the course of the fermentation.

The yeasts are everywhere, if there are good conditions for their reproduction and life. They can be found on trees and plants from early spring to winter. They can be a part of many fruits, because fruits give them the nutritive. In general terms, the available information about the presence of microbial communities in vineyards and on grape surfaces may be summarized as follows[1]:

1. Mature sound grapes harbor microbial populations at levels of 10^3–10^5 CFU/g, consisting mostly of yeasts and various species of lactic and acetic bacteria[2] and filamentous moulds,
2. the sources of yeasts and yeast-like microorganisms include all the vine parts, as well as the soil, air, other plants, and animal vectors in the vineyard,
3. insects are the principal vectors for the transportation of yeasts,
4. yeast colonization on grape is influenced by the degree of ripeness of the bunch,
5. the occurrence and growth of microorganisms on the skin of the grapes is affected by the rainfall, temperature, grape variety, and application of agrochemicals,
6. yeasts are mainly localized in areas of grape surface where some juice might escape and are embedded in fruit secrete; outer surface of the berries is covered by a waxy layer, which affects the adherence of microbial cells and their ability to colonize the surface,
7. oxidative basidiomycetous yeasts, without any ecological interest (*Sporobolomyces, Cryptococcus, Rhodotorula, Filobasidium* spp.) are mostly prevalent in the vineyard environment (soil, bark, leaves, grapes), as well as *Aureobasidium pullulans*, which seems to be a normal inhabitant of grape skin,
8. apiculate yeasts (*Hanseniaspora* and *Kloeckera* spp.) and oxidative yeasts (mostly *Candida, Pichia* and *Kluyveromyces* spp.) are predominant on ripe sound grapes,
9. the main wine yeast – *S. cerevisiae*- contrary to many early reports, is virtually absent from sound grapes, being present in one berry among 2016 tested [3] or about 1 in 1000 berries[4].

Table I
Representation of individual species of yeasts in grape syrup during the wine fermentation[5]

Yeast species	Before the fermentation [%]	During the fermentation [%]	After fermentation [%]
S. cerevisiae	23.4	94.9	98
S. bailii	0.3	–	–
H. anomala	1.4	1.5	1.7
P. membranaefaciens	–	–	0.3
Kl. apiculata	42.7	3.7	–
Met. pulcherrima	31.9	–	–

Methods

All methods used for the identification of the isolated yeast strains are described in Kurtzman and Fell[6] .

Results and discussion

In many cases, microbial spoilage is not easily defined, particularly in fermented foods and beverages, where the metabolites produced contribute to the flavour, aroma, and taste of the final products. In fact, for cultural or ethnic reasons, there is little difference between what is perceived as spoilage or beneficial activity. An example of this can be found in the wine industry, where the production of 4-ethylphenol by *Brettanomyces/Dekkera* spp. in red wines is only regarded as spoilage when this secondary metabolite is present at levels higher than about 620 μg l^{-1}. At less than 400 μg l^{-1}, it contributes favorably to the complexity of wine aroma by imparting aromatic notes of spices, leather, smoke, or game, appreciated by most consumers. About 620 μg l^{-1}, the wines are clearly substandard for some consumers, but remain pleasant for others.

One of the most recent handbooks of yeast taxonomy describes the characteristics of 761 species[7]. Of these, about

a quarter may be isolated from foods, but only a handful plays a significant role in food spoilage.

The environment of possible contamination can be divided into two ways: i) the cellar line (grape syrup processing, storage, preparing the finally product; ii) the bottling line (bottling in glass/PET bottle).

Environment of assembly line could be separated into three sections with regard to possibility of the contamination: environment of pasteurization, bottle washing and the bottle filter alone. After 10 min of exposition 20–30 types of microorganisms were found. Most of them were moulds, yeasts and also bacteria. Based on pilot identification of isolated yeasts anaerobic yeast species (*Saccharomyces* sp.) were found. These strains probably come to grape syrup from cellar. It seems, that the critical time for contamination is during preparation of wine soft.

Among another identified yeast strains, the red yeast *Rhodotorula* come to grape syrup probably from Italy. These yeasts are mostly osmotolerant, thus, they are able to grow in the presence of high concentration of saccharides.

REFERENCES

1. Loureiro V., Malfeiro-Ferreira M., Int. J. Food Microbiol. *86*, 23 (2003).
2. Fleet G. H.,: Int. J. Food Microbiol. *50*, 101, (1999).
3. Vaughan-Martini A., Martini A.: J. Ind. Microbiol. *14*, 514 (1995).
4. Mortimer R., Polsinelli M,: Res. Microbiol. *150*, 199 (1999).
5. Romano P., Suzzi G., Comi G., Zironi R.: J. Appl. Bacteriol. *73*, 126 (1992).
6. Kurtzman C. P., Fell J. W., The Yeasts, A Taxonomic Study, Elsevier (1998).
7. Boekhout T., Robert V., Smith M., Stalpers J., Yarrow D., Boer P., Gijswijt G., Kurtzman C., Fell J., Guého E., Guillot J., Roberts I.: *Yeasts of thew World-Morphology, Physiology, Sequences and Identification*. CD-ROM from ETI Information Service, Wokingham UK, 2002

L09 EVALUATION OF HONEY AUTHENTICITY

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Introduction

Honey is a valued sweet and viscous substance produced by bees from flower nectar or from honeydew. Floral honey is composed mainly of the carbohydrates, fructose and glucose, but these sugars can be artificially added to falsify honey. Because of its nutritional value and unique flavour, the price of natural bee honey is much higher than of other sweeteners, such as refined cane sugar, beet sugar and corn syrup. The authenticity of honey is of great importance for commercial and health reasons[1].

The aim of this study was to detection of adulteration of commercial samples of honey. In the samples of honey selected analytical parameters were determined.

Results were compared with composition criteria for honey present in Decree of Slovak Republic No. 1188/2004 – 100 (ref.[2]) which was transformed by Council Directive 2001/110/EC relating to honey.

Experimantal part

Honey samples. The present study made use of 11 commercial honey samples obtained from Slovak market in 2004. Botanical origin of commercial samples were certified as multifloral (samples A, B and C), acacia (samples E and I), linden (samples F and H), forest (samples D and G), honeydew (sample J) and sunflower (sample K).

Physico-chemical parameters. *Water content* (moisture) was determined with refractometer (Carl Zeiss, Jena, Germany), reading at 20 °C, using the Wedmore table[3].

pH was measured in pH-meter (inoLab pH Level 2, WTW, Weilheim, Germany).

Total acidity was determined by the titrimetric method[3].

Electrical conductivity of 20 % honey solution was measured at 20 °C in a conductimeter (type OK-102/1, Radelkis, Budapest, Hungary) [3].

Insoluble material was determined by gravimetric method[3].

Reducing sugars were determined by Lan and Eynon method modified by Soxhlet[3].

Glucose was determined by Auerbach-Bodlär method[3].

Fructose was determined by biosensor constructed by the immobilisation of the enzyme D-fructose dehydrogenase.

Sucrose was determined by Lan and Eynon method[3].

Proline was measured according to the original method of Ough[4].

Hydroxymethylfurfural was determined by spectrophotometric method according to Winkler using Zeiss Specol 11 VEB (Carl Zeiss, Jena, Germany)[4].

Adulteration of honey by starch hydrolyzates was demonstrated according to Fie reaction II (ref.[3]).

HPLC method was optimised for determination of HMF in honey. Five grams of honey were diluted up to 50 ml with demineralized water, clarified with Carrez solutions to stabilisation of HMF, filtered on 0.45 μm filter and injected in a HPLC (HPP 5001, Laboratorní přístroje, Praha, Czech Republic) equipped with UV detector (LCD 2040, Laboratorní přístroje, Praha, Czech Republic). The HPLC column was a Nucleosil C18-RP (250 × 4 mm, 5 μm) (Watrex, Germany). The HPLC conditions were the following: isocratic mobil phase, 90 % demineralised water and 10 % acetonitrile; flow rate 1 ml min^{-1}; injection volume 20 μl. All the solvents were

Table I
Physico-chemical parameters determined in commercial samples of honey

Sample	pH	Water [%]	Acidity [mekv kg^{-1}]	Proline [mg kg^{-1}]	Conductivity [mS cm^{-1}]	Water insoluble solids [%]	HMF [mg kg^{-1}] by HPLC	HMF [mg kg^{-1}] by Winkler	Glucose [%]	Fructose [%]	Sucrose [%]	Reducing sugars [%]
A multifloral	4.1	18.96	7.10	169	0.1318	0.015	55.3	68.7	35.38	40.59	2.80	71.67
B multifloral	3.6	19.12	19.46	339	0.2439	0.003	29.8	32.9	35.25	41.50	2.95	67.56
C multifloral	3.9	15.92	16.71	520	0.3877	0.009	13.3	13.6	35.12	43.50	4.05	69.81
D forest	4.3	15.92	20.84	591	0.6556	0.027	13.4	11.7	32.16	41.33	2.98	72.05
E acacia	3.9	16.16	9.39	246	0.1727	0.006	15.0	11.5	32.65	42.71	4.22	71.17
F linden	4.5	17.32	6.18	79	0.3208	0.003	45.9	46.8	31.71	37.82	5.46	69.44
G forest	3.9	15.64	12.82	356	0.2517	0.020	49.2	59.3	32.30	42.05	6.29	70.28
H linden	4.1	16.93	18.86	461	0.4406	0.021	12.7	9.9	33.07	37.67	4.98	69.92
I acacia	3.8	16.32	10.96	218	0.1459	0.013	16.0	14.0	30.63	40.00	6,10	71.67
J honeydew	4.3	15.50	34.74	821	0.9870	0.015	7.6	4.4	32.70	35.71	3.29	66.23
K sonefower	3.7	18.84	14.80	320	0.2806	0.017	51.0	55.8	38.04	39.38	3.39	68.36

HPLC grade (Merck, Milan). HMF was identified by splitting the peak in honey with a standard HMF (Sigma-Aldrich, Milan). The amount of HMF was determined using an external calibration curve, measuring the signal at 280 nm.

Results and discussion

Table I shows the means of the data obtained from the analysis of the different physico-chemical parameters.

Water content, a parameter that is related to the climatic conditions, the season of the year and the degree of maturity, showed values between 15.50 % to 19.20 % and none of honeys exceeded the permitted limit established by the Directive[2].

pH values, which are of great importance during the extraction and storage of honey, range between 3.6 to 4.5 which are typical pH values for honey[5].

Honey regulation depend on total acidity which can indicate the history of honey. A high total acidity may mean that the honey had undesirably fermented at some time and that the resulting alcohol was converted into organic acids. Despite the variation in total acidity among tested honeys summarised in Table 1 all total acidity values fell within limits below 40 mekv kg^{-1} (ref.[2]).

Reducing sugars, mainly fructose and glucose, represented the largest portion of honey composition. The reducing sugars were above 65 % in all samples such level complied with requirements of Slovak Republic[2].

The electrical conductivity of the honey is closely related to the concentration of mineral salts, organic acids and proteins. This parameter shows great variability according to the floral origin and is considered one of the best parameters for differentiating between honeys with different floral origins especially for differentiating of honeydew honey[5]. The results obtained for the electrical conductivity show that the sample G was mislabelled as forest honey because of the value of its electrical conductivity 0.3208 mS cm^{-1} (ref.[2]).

Proline is unique because this amino acid come mainly from the honeybee during the conversion of nectar into honey. The amount of proline in honey has been proposed as an indicator of honey ripeness or to the detection of syrup addition to honey[3,6]. The International Honey Commission recommends taht the proline in honey should account for more than 180 mg kg^{-1} (ref.[3]).

HMF content is widely recognised parameters in evaluating the freshness and quality of honey. Legal regulation in Slovak Republic set a maximum HMF content of 40 mg kg^{-1} and 80 mg kg^{-1} respectively for honey from tropical climate[2].

HPLC method is more appropriate for HMF determination in honey, because the presence of substances which interfere with the UV method did not reveal and also because of carcinogenic of *p*-toluidine used in Winkler method[3,7].

Results of physico-chemical parameters were compared with composition criteria for honey present in Decree of Slovak Republic No. 1188/2004 – 100 (ref.[2]). Some of commercial samples of honey do not fulfil this standards. The content of sucrose in two samples of honey (G and F) exceeded the limit by 1.3 % and 0.5 % respectively. The content of proline was lower than 180 mg kg^{-1} in the sample F (79 mg kg^{-1}) and in the sample A (169 mg kg^{-1}) that suggests adulteration with sugars. Addition of starch hydrolysates was detected in the samples A, G and J. The value of electrical conductivity in the sample G (0.3208 mS cm^{-1}) also suggested the substitution of the honey type. The content of HMF in the sample A, F, G and K was up the limit of Decree of Slovak Republic.

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REFERENCES

1. Sivakesava S., Irudayaraj J.: J. Food Sci. *66*, 787 (2001).
2. Decree of Slovak Republic No. 1188/2004-100 relating to honey (2004).
3. Slovak Technical Norm 57 0190. Methods of examination of honey-bee honey (1973).
4. Bogdanov S.: Apidologie, extra issue, *1* (1997).
5. Terrab A., Recamales, A. F., Hernanz D., Heredia F.: Food Chem. *88*, 537 (2004).
6. Hermosín I., Chicón R. M., Cabezudo M. D.: Food Chem. *82*, 263 (2003).
7. Zappalá M., Fallico B., Arena E., Verzera A.: Food Control *16*, 273 (2005).

L10 IDENTIFICATION OF SOME SLOVAKIAN WINES ORIGIN BY THE USE OF FACTOR ANALYSIS OF ELEMENTAL DATA

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Introduction

The recent outbreak of food origin diseases, and scares in different parts of the world have heightened consumer awareness in food quality and safety concerning with aspects of geographical origin, agricultural practices and accurate labelling of food products as well. Wine adulteration, mainly in terms of varieties and regions of geographical origin, has been very widespread. Commission Regulation (EC) No 753/2002 (ref.[1]) lays down certain rules for applying Council Regulation (EC) No 1493/1999 as regards the description, designation, presentation and protection of certain wine sector products. Regulation EC 753/2002 governing the designation, naming and protection of wines has been amended by regulations[2,3].

The development of reliable methods for traceability is strongly required in grapevines, as market needs for knowledge of the product source, its protection and verification of declared origin. For identification of wine origin some new rapid, robust, accurate and cost effective sophisticated techniques are searched, from which namely analysis of trace elements and ratios of stable isotopes have been shown to be a valuable tool to discriminate wines according to their geographical and technological processes applied during manufacture. Elements can be considered as good indicators of wine origin since they are not metabolized or modified during the vinification process[4]. The ability for discriminating wines by regions through their micro- and macro element patterns suggests that the elements are regulated mainly by their movement from soils to grapes. Wine multielemental composition is strongly influenced by the solubility of inorganic compounds of the soil. In principle, the pattern of a wine will reflect the geochemistry of the provenance soil. However, several factors, such as environmental contamination, agricultural practices, climatic changes, and vinification processes, may change markedly the multielement composition of the wine and may endanger the relationship between wine and soil composition. All of these factors may imperil the usefulness of wine multielement composition as a fingerprint of the wine region of origin[5].

Trace elements have well assisted in typing the provenance of wines. The most frequently quantified and cited elements used for wines authentication[6,7] are: K, Na, Fe, Yn, Rb, Ca, Cu, Cr, Co, Sb, Cs, Br, As, Ag, Li, Ba, Sr, Mg, Al, Mn. For differentiation of 18 German wines according to nine minerals (Na, K, Rb, Cs, Cr, Fe, Co, Zn, and Ag) cluster analysis was used. The significant correlation between the content of the wine and soil was found namely in the alkaline metals content Na, K, Rb, and Cs (ref.[8]). For correct classification of 14 French (Bourgogne) wine only Ca and Ba was sufficient. For differentiation of these wines from 26 American wines, results of 17 elements analysis were used[9]. Using of Rb and Li determination 85 % of French wines from two regions were correctly classified[10]. Similarly using of only Rb and Li was successful for differentiation of Spain wines at the regional level. A correct classification of a reduced set of wines from three French regions was obtained using Li, K, Ca, Cu, Ni and linear discriminant analysis. Mn, Li, K and Fe were the most important elements for 93 % successful classification of the three regions of Spanish wines[6]. Eleven elements, K, Na, Ca, Mg, Fe, Cu, Yn, Mn, Sr, Li and Rb, were determined in dry and sweet wines bearing the denominations of origin of Canary Islands, Spain[11]. Multi-element analysis using of 48 elements determined by inductively coupled plasma mass spectrometry (ICP-MS) was successful for differentiation of 55 Spanish and 67 English wines[12]. Trace elements composition and used statistical methods were able to distinguish not only the geographical origin of tested wines, but also their type red, white and rosé. Differentiation of some Hungarian red wines using of 16 elements (Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, P, Sr and Zn) was successful and similar results were obtained according to principal component analysis using of determinations of Zn, Na, B, Cu and strontium[13]. For some vineyard regions differentiation following discriminating elements were used: Ba, Ca, Mg (France and USA), Ba, Ni, B, Sr, Ca, Al, K (USA), Rb, Na, Fe (Germany), Rb, Li, Mg (France), Mn, Sr (Romania), and Mn, P, Ag, Ni, and Cr (Hungary)[14]. Correct differentiation of the German Mosel and Rhein-Pfalz wines according to their production district was achieved by using the concentration of only two elements Rb and Fe[15]. Extensive research on ultra/trace elements, the rare earth elements – lanthanides, has lead to efficient identification of wine authenticity[16].

An interpretation of wine differences, related to variety and geographical origin, is based on statistical analysis.

Multivariate statistical methods can be very useful to explore of measured data. Principal component analysis (PCA) is very often used to establish the relationships and differentiation among the variables. Cluster linear analysis (CLA) is employed to discover natural groupings of samples. Stepwise discriminant analysis (SDA) is applied to select the most important and significant variables. Very useful for differentiation of region of origin is generalized canonical analysis (GCA)[4]. According to this method using of seven elements were satisfactorily classified some Spanish wines[10]. The canonical discriminant analysis (CDA) is a method applied to identify similarities between wines from the same or different region of origin[4]. Thus 22 French red wines were well classified according to their geographical origin[17]. Thirty-four French red wines from three regions were well classified according to variety and geographic origin from the results of statistical analyses: F statistic, PCA and SDA[18]. Stepwise discriminant analysis was satisfactory used for discrimination of the wine-growing regions of France according to stable isotopes and trace elements determination[19]. Characterization of the geographic origin of Bordeaux wines from the results of isotopic and trace element measurements was realised by analysis of variance (ANOVA) and PCA methods[20]. Cluster and principal component analysis classified correctly samples of wines from seven clones of Monastrell grapes[21]. For the classification of some German wines according to type and region PCA and discriminant space with the K Nearest Neighbour (KNN) methods were used[15]. Linear discriminant analysis (LDA) allowed a reasonable classification of red wines from the Canary Islands according to the island of origin. When artificial neural networks were applied on the matrix of data constituted by the analyzed metals (K, Na, Ca, Mg, Fe, Cu, Zn, Mn, Sr, Li, and Rb), the results improved in relation to those obtained by other multivariate methods observing a differentiation of wines according to island of production[22].

Experimental

For the database creation of model for differentiation of white dry wines according to Slovak vineyard regions were analysed 8 wines from Central Slovakian vineyard region (VR), 16 wines from Nitra VR, 17 from Little Carpathian VR, 10 from South Slovakia VR, 10 from Eastern Slovakia VR, and 8 wines from Tokaj vineyard region. Samples of wines were given from commercial sources. The most analysed were wines made from Müller Thurgau, Veltliner Green, and Riesling Wälsch grape varieties with the vintage from 2001 to 2003. All of the samples were analysed for the content of elemental markers As, Ba, Ca, Co, Cr, Li, Mg, Rb, Sr, Sn, and V, which were selected on the base of geochemical characterization of Slovakian vineyard regions using of the distribution data in Slovakian geochemical atlas of soils[23].

AAS conditions: The selected specific markers of wine origin were determined by AAS method. Samples of a wine were digested by microwave digestion system – Milestone MLS 1200 MEGA (Sorisole, Italy). A Perkin Elmer 4100 atomic absorption spectrometer (Norwalk, CT, USA) equipped with a deuterium lamp background-correction system and HGA 700 graphite tube atomizer with pyrolytically coated graphite tubes and flame was used for metal determination involving atomic absorption spectrometry. Metal Ca, Mg were determined from atomic spectrometry using an air/acetylene flame, and Sr was characterized by using an acetylene/nitrous oxide flame. Metals As, Ba, Co, Cr, Li, Sn, Rb and V were measured on graphite tube atomizer.

Wine sample preparation: 2.5 ml of wine sample was introduced into a tephlon microwave digestion vessel, and 2 ml of 65% HNO_3 (Suprapur, Merck) was added. Vessel was closed and fastened into the rotor. The rotor with six loaded vessels was placed into the microwave oven. The microwave digestion program was applied: 250 W (1 min), 0 W (1 min), 250 W (5 min), 400 W (5 min), and 650 W (5 min). The digested sample was adjusted to the volume of 10 ml with ultra pure water. Spiked sample for recovery determination was prepared by adding of two concentrations of stock solution to 2.5 ml wine sample before microwave digestion. The peak height of the absorbance was used for calculation of metals content, only for Cr was area of the absorbance. All results were expressed as the average of triplicate measurements.

Statistics: For the visualization of segregated vineyard regions the principal component analysis (PCA) and Principal Component Factoring (PCF) were applied using of statistical programme Unistat® (Unistat Ltd., 4 Shirland Mews, London W9 3DY, England). At the PCA and PCF statistics standardized criteria with maximal number of iteration of 50 were set.

Results and discussion

Selection of the traceability markers started from the study of distribution of about 36 different elements in vineyard regions using of Slovakian geochemical atlas of soils[23]. On wine regional differentiation the As, Ba, Ca, Co, Cr, Li, Mg Rb, Sr, Sn, and V were selected according their variability as the most useful markers for geographical

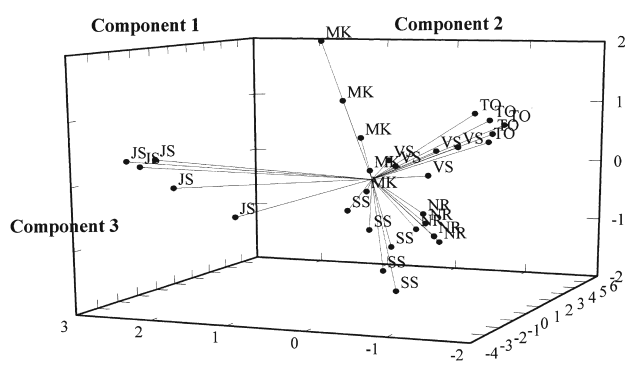

Fig. 1. **Segregation of Slovak vineyard regions using of principal component visualization (Soil composition markers: As, Ba, Ca, Co, Cr, Li, Sn and V; Vineyard regions: MK – Little Carpathians, JS – Southern Slovakia, NR – Nitra, SS – Central Slovakia, VS – Eastern Slovakia and TO – Tokaj region)**

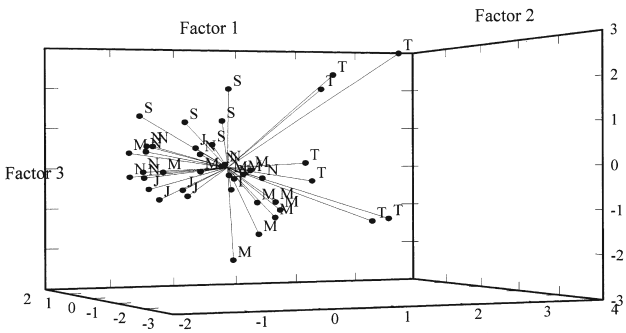

Fig. 2. **Segregation of Slovak vineyard regions by principal component factoring (Plot of factor scores – unrotated; Markers: Ca, Mg, Rb, Sr, Ba and V; Vineyard regions: M – Little Carpathians, J – Southern Slovakia, S – Central Slovakia, N – Nitra, and T – Tokaj)**

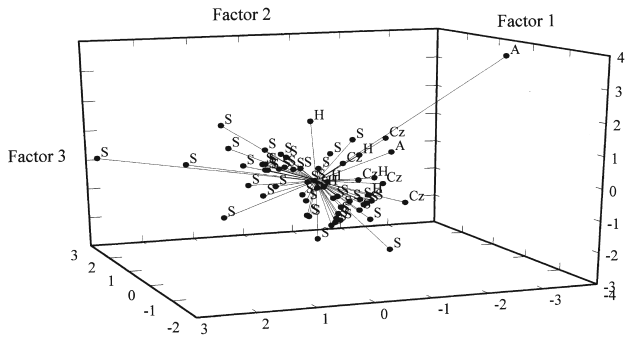

Fig. 3. **Principal component factoring – segregation of Slovakian wines and some neighbouring central European wines (Markers of origin: Ba, Ca, Co, Cr, Li, Rb, Sr, V and Mg; Country of wine origin: S – Slovakia, H – Hungary, Cz – Czech, A – Austria)**

authentication. On the Fig.1. we can see an example of an ideal differentiation of all Slovak vineyard regions using of PCA visualization of some selected soil composition markers obtained from geochemical atlas of Slovakian soils.

On the Table I are summarized results from the distribution of selected elemental markers in some white dry wines originated from Slovakian vineyard regions. A large proportion of total variance of this data can be expressed mainly with the content of elements Ca, Mg, Rb, Sr, and Ba. The best segregation of all Slovakian vineyard regions was achieved by using of these elements in combination with vanadium and PCF statistics, Fig. 2. There a very valuable segregation of all main Slovakian vineyard regions was reached with the exception of Eastern Slovakia, which is not, by the way, very significant vineyard district. Data from this region decreased the distinguish results, before of its geochemical composition similarities to the other national vineyard regions. Due to the same reason we can observe a small regional intersection of neighbouring vineyard regions in the case Small Carpathian and Nitra districts. Extra strong segregation from other regions shows Tokaj district.

Tested model for traceability of Slovak wines was tried for differentiation of Slovakian wines from foreign ones. It was found, that the best results gives a model which exploit Ba, Ca, Co, Cr, Li, Mg Rb, Sr, and V. In the Fig. 3. there is shown the situation of differentiation of Slovakian wines from wines of neighbouring countries. Good separation was achieved with Czech and Austrian wines, not with Hungarian ones. This is caused by Tokaj region, which was disconnected and divided between Slovakia and Hungary, when the Austro-Hungarian Empire broke up in 1920. The distribution of all elements is very similar for both of compared vineyard regions because of their common and identical vineyard soil composition.

For the differentiation we tried to apply the commonly used PCA statistics, but this method did not achieve so good differentiation and discrimination than PCF method. It may be because the basic idea behind PCA is only to redraw the axis system for multi dimensional data and simply to transform the original variables into a new set of variables. Factor analysis attempts to construct a mathematical model explaining the correlation between a large set of variables.

Conclusions

Some of the specified elemental markers used in our national model for white dry wines geographical authentication were successfully applied in many studies identifying the European wines origin on the regional level, such as As, Ba, Ca, Cr, Co, Li, Mg and Rb. It seems many some of them may be regarded as some common regional or to some extent sub-regional markers of wine origin. For full differentiation of European wines more research in this field is advisable.

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REFERENCES

1. Commission Regulation (EC) No 753/2002. Official Journal of the European Communities *L 118*, 1(2002).
2. Commission Regulation (EC) No 316/2004. Official Journal of the European Communities *L 055*, 16 (2004).
3. Commission Regulation (EC) No 1429/2004. Official Journal of the European Communities *L 263*, 11 (2004).
4. Arvanitozannis I. S., Katsota M. N., Psarra E. P., Soufleros E. H., Almeida C. M. R., Vasconcelos M. T. S. D.: J. Anal. Atom. Spectrometry. *16*, 607 (2001).
5. Almeida C. M. R., Vasconcelos M. T. S. D.: J. Agric. Food Chem. *51*, 4788 (2003).
6. Ashurst P. R., Dennis M. J.: *Food Authentication*, Blackie Academic & Professional, London, Madras, 399 p. (1996).
7. Arvanitozannis I. S., Katsota M. N., Psarra E. P., Soufleros E. H.: Trends in Food Sci. and Tech. *10*, 321 (1999).
8. Siegmund H.., Bächmann K.: Z. Lebensm. Unters-Forsch. *166*, 1 (1977).

Table I
Distribution of some elemental markers of white dry wines according to affiliation to vineyard regions

Elements	Values* [mg l^{-1}]	Slovak vineyard region					
		Middle Slovakia	Nitra region	Small Carpathian	South Slovakian	East Slovakian	Tokaj Region
As	x_{mean}	0.005	0.008	0.008	ND	0.013	0.007
	s_x	0.005	0.011	0.009	ND	0.012	0.006
	x_{min}	ND**	ND	ND	ND	ND	ND
	x_{max}	0.015	0.037	0.034	0.009	0.035	0.019
Ba	x_{mean}	0.156	0.159	0.208	0.150	0.235	0.245
	s_x	0.040	0.032	0.098	0.044	0.112	0.083
	x_{min}	0.101	0.110	0.107	0.114	0.121	0.154
	x_{max}	0.213	0.210	0.408	0.265	0.485	0.374
Ca	x_{mean}	110.9	145.7	97.2	100.8	108.8	171.1
	s_x	25.3	24.8	26.1	26.1	22.0	48.4
	x_{min}	82.2	112.7	62.0	72.7	74.0	93.0
	x_{max}	166.4	182.8	152.4	134.0	154.3	236.4
Co	x_{mean}	0.007	0.004	0.004	0.003	0.006	0.005
	s_x	0.005	0.001	0.003	0.001	0.003	0.003
	x_{min}	ND	0.003	ND	ND	0.003	ND
	x_{max}	0.017	0.008	0.013	0.006	0.011	0.009
Cr	x_{mean}	0.016	0.015	0.019	0.016	0.022	0.033
	s_x	0.004	0.005	0.009	0.008	0.006	0.014
	x_{min}	0.011	0.008	0.007	0.006	0.013	0.014
	x_{max}	0.023	0.030	0.040	0.028	0.032	0.055
Li	x_{mean}	0.015	0.019	0.028	0.016	0.018	0.035
	s_x	0.006	0.022	0.019	0.010	0.008	0.011
	x_{min}	0.008	0.005	0.007	0.010	0.008	0.022
	x_{max}	0.025	0.098	0.063	0.044	0.036	0.049
Rb	x_{mean}	1.019	1.019	0.621	0.845	0.429	0.333
	s_x	0.320	0.179	0.486	0.295	0.229	0.210
	x_{min}	0.649	0.710	0.119	0.564	0.080	0.030
	x_{max}	1.410	1.370	1.570	1.340	0.783	0.793
Sr	x_{mean}	0.452	0.581	0.616	0.795	0.699	0.961
	s_x	0.177	0.332	0.229	0.875	0.212	0.224
	x_{min}	0.318	0.128	0.353	0.323	0.312	0.555
	x_{max}	0.844	1.257	1.113	3.222	1.104	1.293
Sn	x_{mean}	0.007	0.006	0.007	0.005	0.008	0.008
	s_x	0.003	0.002	0.004	0.002	0.004	0.003
	x_{min}	ND	ND	ND	ND	ND	ND
	x_{max}	0.013	0.009	0.016	0.010	0.016	0.015
V	x_{mean}	0.038	0.014	0.014	0.009	0.020	0.088
	s_x	0.021	0.006	0.012	0.010	0.010	0.122
	x_{min}	0.017	ND	ND	ND	ND	0.013
	x_{max}	0.079	0.025	0.035	0.034	0.033	0.399
Mg	x_{mean}	104.1	106.3	87.3	100.9	82.1	100.8
	s_x	23.0	11.0	13.5	15.2	20.9	21.7
	x_{min}	69.7	87.2	57.2	82.8	58.8	82.2
	x_{max}	145.0	129.4	103.6	121.3	116.2	140.9

* Values: x_{mean} – average value, s_x – standard deviation; ** ND: not detected

9. Kwan W. O., Kowalski B. R., Skogerboe R. K.: J. Agric. Food Chem. *27*, 1321 (1979).
10. Lacasta F.: Rapport de stage BTAO, DGCCRF 33405 Talence, France (1982).
11. Frías S., Conde J. E., Rodríguez M. A., Dohnal V., Trujillo J. P. P.: Nahrung/Food. *46*, 370 (2002).
12. Baxter J. M., Crews M. E., Dennis J., Goodall I., Anderson D.: Food Chem. *60*, 443 (1997).
13. Szentmihály K., Kiss G. A. C., Keszler Á., Kótai L.: Acta Alimentaria. *29*, 105 (2000).
14. Day M. P., Zhang B. L., Martin B.: J. Am. J. Enol. Vitic. *45*, 79 (1994).
15. Maarse H., Slump P., Tas A. C., Schaefer J.: Z. Lebensm. Unters. Forsch. *184*, 198 (1987).
16. Klimmek A.: Dissertation. Prozesswissenschaften der Technischen Universität, Berlin. 259 p. (2003).
17. Sivertsen H. K., Holen B., Nicolaysen F., Risvk E.: J. Sci. Food Agric. *79*, 107 (1999).
18. Etiévant P., Schlich P.: J. Sci. Food Agric. *45*, 25 (1988).
19. Day M. P., Zhang B., Martin B. J.: J. Sci. Food Agric. *67*, 113 (1995).
20. Martin G. J., Mazure M., Jouitteau C., Martin Y. L., Aguile L., Allain P.: Am. J. Enol. Vitic. *50*, 409 (1999).
21. Plaza E. G., Muňoz R. G., Cutillas A. M.: J. Sci. Food Agric. *80*, 497 (2000).
22. Díaz C., Conde J. E., Estévez D., Olivero S. J. P., Trujillo J. P. P.: Agric. Food Chem. *51*, 4303 (2003).
23. Čurlík J., Šefčík P.: Geochemical Atlas of the Slovak Republic, Part V: Soils. Bratislava, 182 p. (1999).

L11 SPECIATION OF CONSIDERED ELEMENTS BY ATOMIC SPECTROMETRY METHODS

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Introduction

Analytical chemists have increasingly realized that determining total contents of the elements cannot provide the required information about mobility, bioavailability, and finally the impact of elements on ecological systems or biological organisms. Only knowledge about the chemical species of the elements can lead to understanding of chemical and biochemical reactions involving these species, thus providing information about toxicity or essentiality.

Many laboratories deal with speciation analysis of various elements, especially antimony, arsenic, bismuth, selenium, tin, chromium and mercury. We study in details determination of species of selenium and tin compounds.

Concerning ion exchangers used for ICP-AES detection, organically bonded selenium and tin weren't sorbed and eluted when batching already. Using off-line coupling of liquid chromatography and ETA-AAS, all forms were separated on the appropriate column but from practical point of view the method is more difficult and time-consuming than e. g. HPLC-(UV)-HG-AFS which is being thoroughly tested in our laboratory. The use of suitable sample introduction system is necessary for on-line hyphenation of CZE and ICP-AES. At first we used micro concentric nebulizer MCN-100, model M-2 and now we plan to use the direct injection high efficiency nebulizer (DIHEN). We intend to apply the developed methods to real samples of food supplements.

Although selenium ranks among essential elements, the range between concentrations with favourable and toxic effects on the human organism is relatively narrow. Toxic doses are only hundred times higher than those required for physiological functions[1,2,3]. This dual effect has been recognized for many naturally occurring chemicals. In Europe there is a zone from Finland to Turkey that is poor for selenium so most of local citizens have an insufficient selenium uptake in food, which influences their health condition. In food, selenium is contained mainly in meat whereas its content depends on the amount of selenium in the livestock feed. Satisfactory quantity of selenium is also in vegetables grown on a soil with plentitude of selenium. Today supplementation is already carried out by various food supplements. The Czech Republic belongs to the zone with deficiency of selenium and that is why this study was aimed on monitoring the amount and forms of selenium just in food supplements.

Speciation analysis of tin is of special importance. There is no evidence that tin is an essential element for humans but the toxicities of various tin species are significantly different[4]. Inorganic tin compounds leave body rapidly, having been breathed in or eaten, thus they do not usually cause harmful effects; swallowing large amounts can however cause stomach aches, anemia, and liver and kidney problems. Exposure to organotin compounds can cause skin and eye irritation, respiratory irritation, gastrointestinal effects and neurological problems. It is not known whether or not it causes cancer in humans.

Speciation analysis can be achieved by various species-selective techniques (Fig. 1., adapted from ref.[5]).

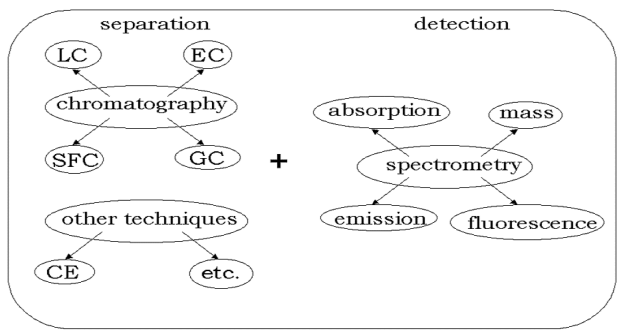

Fig. 1. **Species-selective techniques for speciation analysis**

Experimental

Reagents and Apparatus

Selenium(IV) stock solution containing 1.000 g dm^{-3}, 37% hydrochloric acid, 48% hydrobromic acid and 65% nitric acid, 1 g dm^{-3} Ni solution in 2% HNO_3 as a matrix modifier, Sn(IV) as stannic nitrate by Analytica Co, CZ. Selenate stock solution 1.000 g dm^{-3} prepared from $Na_2SeO_4.10\ H_2O$, L-(+)-selenomethionine and Se-(methyl)-selenocysteine, solutions 0.7% $NaBH_4$ in 0.1% NaOH and citric acid prepared from solid chemicals by Aldrich, Germany. Inorganic tin species (Sn(II) as stannous chloride dihydrate purchased from Lachema Brno Czech Republic). The commercial pills containing selenium were Antioxidant and Selenium (Walmark, CZ), Bioactive selenium and zinc (Whithall, Denmark).

The technique used for organic tin species (dibutyltin dichloride, triphenyltin chloride and tributyltin chloride, acetonitrile (for HPLC) and triethylamine, all purchased from Sigma-Aldrich s. r. o. CZ and acetic acid, p. a., Onex Rožnov pod Radhoštěm as chemicals for mobile phase) separation was HPLC (HPLC pump P 580, from Gynkotech) with ACE C-18 column (ACE C-18, 3 μm, 15 cm × 1.0 mm, from ACT); detection was accomplished by atomic absorption spectrometry with graphite furnace ETA-AAS (Thermo Elemental, model Solaar M6, UK). All chemicals were diluted with deionised water if necessary.

The chromatographic system used for the separation of selenite and selenate consisted of low-pressure pump Ismatec and anion exchanger Separon SGX-AX, 60 μm, type of column 421-310063, Tessek, CZ. This chromatographic system was coupled on-line with atomic emission spectrometer with ICP (Thermo Jarrell Ash, model IRIS/AP, USA). Selenium amino acids were separated by HPLC (Pye Unicam, PU4011 Pump, Philips, UK, with a 100 μl loop), column used in this system was Supelcosil LC-18-DB, 58355-U, Col. 18557-06, 15 × 4.6 mm, 5 μm, Sulpeco, USA and PRP-X100, 250 × 4.1 mm, 10 μm, polystyrene divinylbenzene copolymer, Hamiton, USA; detection was off-line by ETA-AAS and on–line atomic fluorescence spectrometer with hydride generation HG-AFS, Millennium Excalibur, PSA, UK. Inorganic tin species were separated by ion-exchange chromatography and detected by optical emission spectrometry with inductively coupled plasma ICP-AES.

Selenium speciation procedures

Extraction was used for dissolving selenium[6,7]. 0.5 g of pulverized sample was extracted in 20 cm^3 of water. As an ancillary technique ultrasound was used[8].

First of all it was necessary to optimise work conditions for final selenium determination (selection of suitable absorption and emission lines, setting gases flow and the rate of sample suction and plasma conditions for ICP-AES, time – temperature program for ETA-AAS and parameters for HG-AFS) and in addition calibration curves were adjusted for each method.

Selenium determination by ICP-AES

Different types of ion exchangers in low-pressure system coupled with ICP-AES were tested for the separation of inorganic forms of selenium. Anionic chromatographic columns were used to separate Se(IV) and Se(VI)[9]; Separon SGX-AX was found to be a sufficient ion exchanger, hydrochloric acid to be a suitable mobile phase for the elution of selenite and selenate – 0.1 M hydrochloric acid eluated selenite and 1 M hydrochloric acid eluated selenate. In Fig. 2. the first peak corresponds to selenite and the second one to selenate.

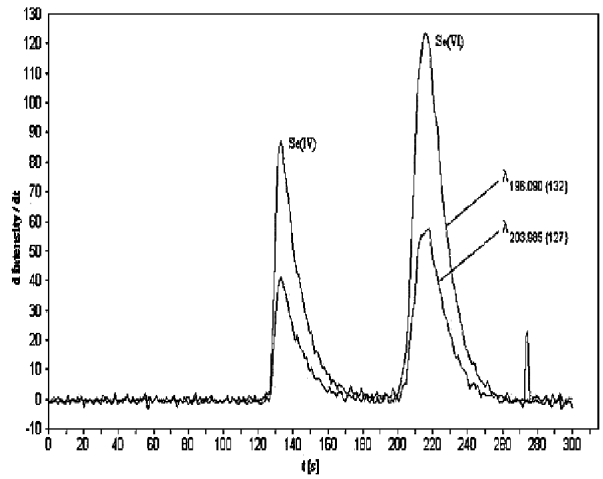

Fig. 2. **Derivation of signal intensity–time characteristic for Se(IV) and Se(VI) with concentration of 5 mg dm^{-3} on a Separon SGX-AX column. 2 cm^3 of sample batched, after gradient elution by 2 cm^3 of 0.1 M HCl followed by 2 cm^3 of 1 M HCl. (λ_1 = 196.090 nm; λ_2 = 203.985 nm)**

The process of batching consisted of pumping 2 cm^3 of selenium solution and then 2 cm^3 of water to the column. A gradient elution was then optimised for the separation. At first selenite was eluted by pumping 2 cm^3 0.1 M HCl. Afterwards the concentration of acid was increased to 1 M HCl for elution of selenate. Separation of a mixture of selenite, selenate, L-(+)-selenomethionine and Se-(methyl)--selenocysteine was tested. However, this column did not retain selenium amino acids.

Selenium determination by ETA-AAS

Separation of organically bonded forms of selenium was performed by HPLC on a Supelcosil LC-18-DB column[10] (reversed-phase columns were used for selenium amino acids separation[7]) with off-line detection by ETA-AAS (Fig. 3.). The elution reagent was deionised water. For injecting an analytical batching stopcock (100 μl) was used. Fractions of the eluate were collected into beakers after certain time and they were analysed off-line by ETA-AAS. Selenocysteine

was eluted sooner than selenomethionine. At lower concentrations the retention time is shifted a bit to lower levels.

The real samples were concentrated by evaporating 3 cm^3 of sample to 0.5 cm^3 from which 50 µl was taken for analysis. In certain time the fractions were collected and they were analysed by ETA-AAS.

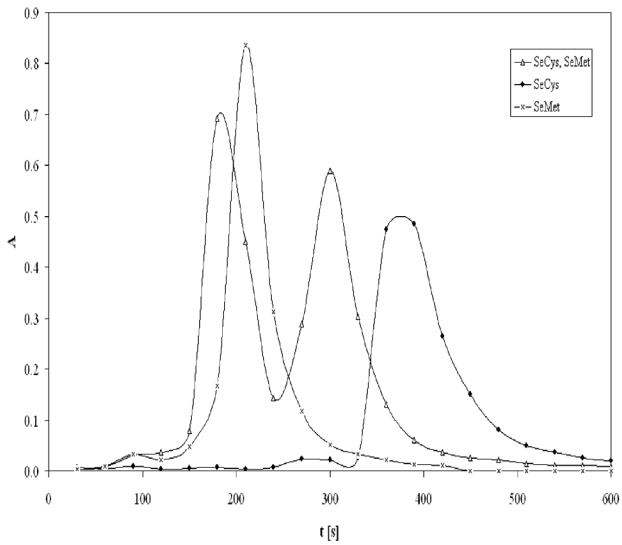

Fig. 3. **Absorbance–time characteristic for selenocysteine and selenomethionine separated by HPLC with fractions detection by ETA-AAS**

ETA-AAS has sufficient low detection limits for selenium determination but then it is time and operation skills demanding in off-line arrangement. Therefore it would be convenient for on-line connection of ICP-AES with hydride generation. Another possibility for selenium speciation is the use of high-performance liquid chromatography connected on-line with direct hydride generation atomic fluorescence spectrometry[1].

Selenium determination by HG-AFS

Separation of organically bonded and inorganic forms of selenium was performed by HPLC on the Hamilton column PRP-X100, 250×4.1 mm, 10 µm, based on polystyrene divinylbenzene copolymer with quaternary ammonium groups suitable for the separation of anions[9] with on-line detection by HPLC-(UV)-HG-AFS (Fig. 4.). Tested elution reagents were buffered solutions with different pH values. As the best the solution of 10 mM citric acid with adjusted pH 5 by solution of NH_4OH was selected. For injecting an analytical batching stopcock (100 µl) was used. Output from column was connected into atomic fluorescence system with hydride generation in speciation mode supported by UV-radiation and heating. This arrangement supposes addition of concentrated hydrochloric acid to main stream and conversion of all forms of selenium compounds on Se(IV) suitable for hydride generation with supporting UV radiation (throughput through UV-cell) and heating (throughput through temperature controlled-cell). (See Fig. 4.)

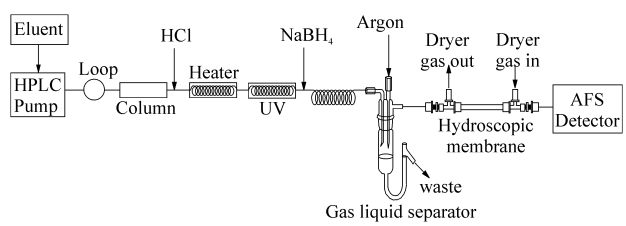

Fig. 4. **HPLC-Heating-(UV)-HG-AFS scheme**

Tin compounds speciation

For inorganic tin separation various cation exchangers were tested (Amberlite IRC 50, Cellulose CM 23, Servacel CM 32, Dowex 50WX, Trisacryl M CM, Sephadex CM 50).

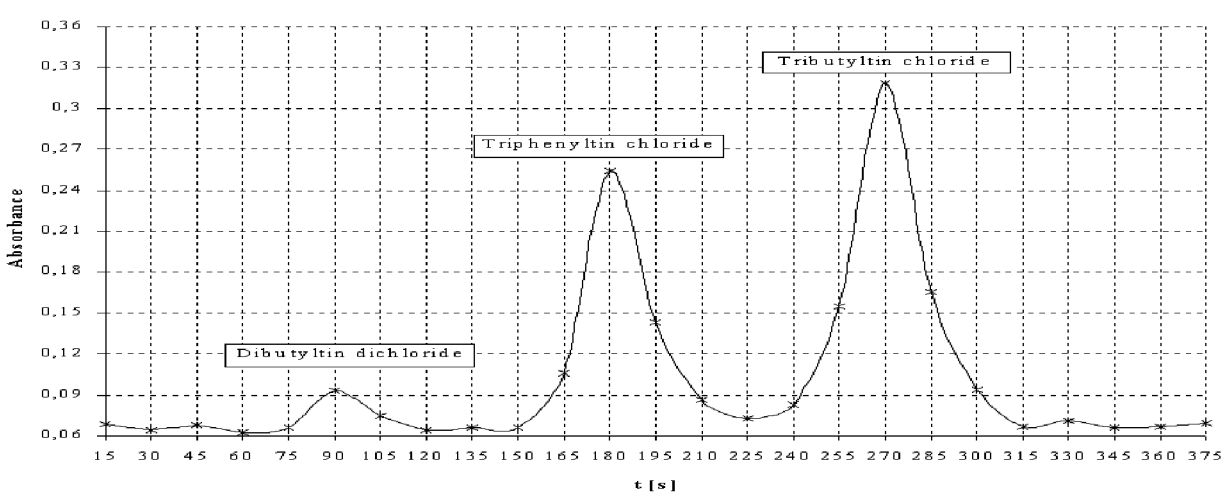

Fig. 5. **Separation of organic tin species on the ACE C-18 column; eluted by the mixture of acetonitrile–water–acetic acid with 0.05% triethylamine (65 : 23 : 12)**

Amberlite IRC 50 was evaluated to be the best one; Sn(II) was not retained and was eluted immediately after injection; for Sn(IV), tall and narrow peaks were obtained. The elution reagent was 1 mol dm^{-3} HCl. Detection was performed by ICP-AES at $\lambda = 189.989$ nm.The optimised factors were the used ICP-AES method (direct or hydride generation, while hydride generation was better having detection limits by one order lower), the concentration of sodium borohydride for hydride generation (the tested concentrations were 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4; 5% $NaBH_4$, the best one was 3% $NaBH_4$ giving the highest signal) and concentration of hydrochloric acid for hydride generation (the tested concentrations were 0.1; 0.25; 0.5; 0.75; 1 mol dm^{-3} HCl, the best one was 0.25 mol dm^{-3} HCl with the highest signal).

The optimised factors for organotin speciation analysis were the used column and mobile phase (both of these were optimised according to literature source11; the column chosen was ACE 3 μm C-18 15 cm × 1.0 mm; the mobile phase was the mixture of acetonitrile–water–acetic acid with 0.05% triethylamine (65 : 23 :12), pH 5). The separation can be seen in Fig 5.

Results

In the analysed real samples selenium was included in the forms of sodium selenite and yeasts cultivated on a selenium enriched substrate. The analysis of samples containing selenite confirmed the presence of this species. In the samples with organic selenium species we vouched that in the preparation there are not only organic forms but also inorganic selenium that is present in the largest amount (Fig. 6.). That proves that the transformation of the inorganic form to the organic form by yeasts cultivated on selenium enriched substrate was not perfect.

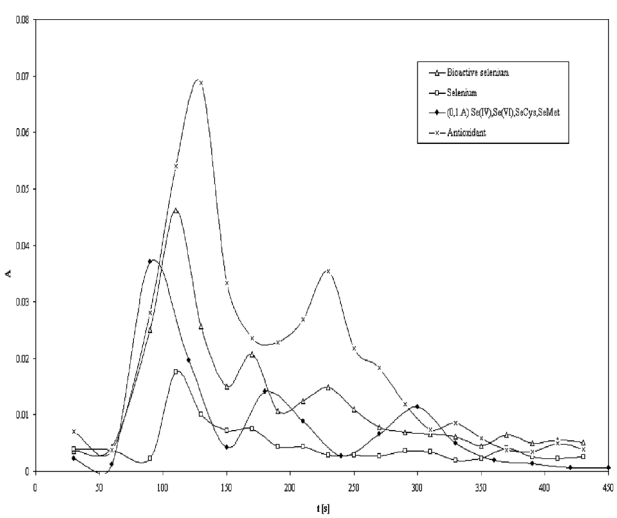

Fig. 6. **Absorbance–time characteristic for samples containing organically bonded vselenium and the mixture of standards separated by HPLC with detection by ETA-AAS (the absorbance of standards was scaled down 10times to be more apparent)**

Conclusion

The analysis was aimed on optimisation of conditions for standards separation above all and it's necessary to keep on working on samples extraction (e. g. try other extraction solutions – buffers solutions based on citric acid and ammonium dihydrogen phosphate with different adjusted pH).

For separation of all selenium species together it would be possible to connect the separation on ion exchangers with separation on a column with high performance liquid chromatography. After batching the sample the inorganic forms of selenium would be retained on an ion exchanger in the first column and the organic forms would continue to a HPLC column. As an elution reagent it would be good to use water that would elute selenium amino acids at first and then HCl to wash inorganic forms of selenium compounds or buffers solutions based on citric acid and ammonium dihydrogen phosphate with different adjusted pH and concentrations from 10 to 50 mM for all forms. It would be possible to detect single fractions or to realize on-line connection with hydride generation and ICP-AES, CZE-ICP-AES or HPLC-(UV)-HG-AFS. The latter is indicated as the best from point of view of sensitivity and detection of wide range of various selenium compounds.

Optimisation of methods for speciation analysis of two inorganic and three organic tin species were achieved. For inorganic tin species separation and detection, on-line coupling of ion exchange chromatography and ICP-AES were used, organotin speciation analysis was performed by off-line coupling of HPLC and graphite furnace AAS.

The subsequent activities will include testing of other separation and detection techniques (e. g. on-line coupling of capillary zone electrophoresis with ICP-AES), speciation analysis of other elements (arsenic, chromium and selenium) and real sample application.

REFERENCES

1. Ipolyi I., Stefánka Z., Fodor P.: Anal. Chim Acta *435*, 367 (2001).
2. He Y., Moreda J., Cervera M., Guardia M.: JAAS *13*, 289 (1998).
3. Cava-Montesinos P., Cervera M., Pastor A., Guardia M.: Anal. Chim. Acta *481*, 291 (2003).
4. Wade T. L., lecture online available on http:// 165.95.49.52/OCNG647/Presentation.htm page 19.
5. Szpunar J., Lobinski R.: Journal of Analytical Chemistry *363*, 550 (1999)
6. Gómez-Ariza J. L., Caro de la Torre M. A., Giráldez I., Sáchez-Rodas D., Velasco A., Morales E.: Applied Organometallic chemistry *16,* 265 (2002)
7. Ochsenkühn-Petropoulo M., Michalke B., Kavouras D., Schramel P.: Anal. Chim. Acta *478*, 219 (2003).
8. Alsing Pedersen G., Larsen E. H.: Fresenius J. Anal. Chem. *358*, 591 (1997)
9. Gómez M. M., Gasparic T., Palacios M. A., Camara C.: Anal. Chim. *Acta 374*, 241 (1998).

10. Gómez-Ariza J. L., Sánchez-Rodas D., Caro de la Tore M.A., Giráldez I., Morales E.: J. of Chromatography A *889*, 33 (2000).
11. Wahlen R., Catterick T.: Journal of Chromatography B 783, 221 (2003).

L12 DETERMINATION OF LYCOPENE IN FOOD BY ON-LINE SFE-LC ELIMINATING ITS DEGRADATION DURING THE ANALYTICAL PROCEDURE

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The sample was pre-treated by supercritical fluid extraction (SFE) and afterwards on-line analyzed by liquid chromatography (LC) with UV detection. One single monolithic column was employed for trapping of analytes and their subsequent separation. In SFE, dependence of temperature, addition of modifier, and duration of static and dynamic period on extraction recovery were studied. Presence of methanol as a modifier (100 µl) and sufficient static period (10 min) was observed to be essential for quantitative extraction. Optimum extraction temperature and pressure was 90 °C and 400 atm respectively. Flow rate of CO_2 was set to 1.5 ml min^{-1}. Analysis of lycopene, including sample preparation, extraction, and analysis was performed in 35 minutes. The repeatability of the method was 3.9 %. Limit of detection (LOD) of LC was 0.5 ng. However, the LOD of the whole method depends on the sample amount used for extraction and can be even smaller. The analytical procedure is performed in the closed system and therefore no loses of lycopene can occur due to the no presence of atmospheric oxygen, moisture, and UV light. Further, the method benefits from the on-line set up by increased sensitivity. Lycopene was determined in tomato, tomato paste, tomato ketchup, pasta sauce, ruby grapefruit, red grapefruit, rosehip paste, water melon, papaya, date, and guava. The results have been published[1].

REFERENCES

1. Pól J., Hyötyläinen T., Ranta-Aho O., Riekkola M.-L.: J. Chromatogr. A *1052,* 25 (2004).

L13 HOW ANTIOXIDANT INTAKE INFLUENCES LIPID METABOLISM AND ANTIOXIDANT STATUS IN PATIENS WITH TYPE 2 DIABETES AND/OR HYPELIPIDAEMIA

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Introduction

For the majority of organisms on Earth, life without oxygen is impossible. Aerobic organisms rely on oxygen for an efficient production of energy equivalents in the form of ATP. Despite its many advantages, O_2 is a poisonous molecule; aerobes only survive in its presence because they have evolved antioxidant defence. Oxidative stress was defined as a disturbance in the cell or organism pro-oxidant–antioxidant balance in favour of the former[1]. Oxidative stress differs from many other stresses in that its primary effectors, the reactive oxygen species (ROS), can arise largely in the course of normal cell metabolism. The effects of oxidative stress are manifold and may differ from cell type to type. In humans, free radicals have been implicated in over 100 diseases[2].

Each aerobic organism possesses a number of mechanisms both to control the production of ROS and to limit or repair the damage to tissues. The integrated antioxidant system comprises preventative antioxidants, scavenging antioxidants (remove ROS once formed, thus preventing radical chain reactions) and repair enzymes (repair or remove ROS-damaged biomolecules). Scavenging antioxidants are divided into two large groups: enzymes (superoxide dismutase, glutathion peroxidase, catalase) and small molecules, both hydrophilic (e. g. glutathione, ascorbate, bilirubin, uric acid, flavonoids) and lipophilic (e. g. tocopherols, carotenoids). Many of low molecular weight antioxidants are introduced into human body by the ingestion of natural food sources only, because humans are unable to produce some of the components of this antioxidant system, so the efficiency of a complex antioxidant system depends on the intake of antioxidants and/or their precursors in nutrition[1].

Oxidative stress is involved in many pathological processes, especially in chronic degenerative diseases as diabetes, atherosclerosis, cancer, Alzheimer disease etc. Thus, some ways to reduce negative effects of oxidative stress are studied. In recent years our understanding of the various diets and food supplements intake has been increased substantially. Numerous studies indicate the beneficial effects of certain elements of some specific diets (e. g. Mediteranean diet) and of these diets as whole. Very little is known about the current role of local food products (e. g. regional vegetables and varieties of fruit).

This work is focused on specific situation in Czech population. Several intervention studies were realized, in which relation between certain complex antioxidant micronutriens intake and some parameters characterizing antioxidant and metabolic status in non-insulin dependent diabetics, patents with hyperlipidaemia and healthy subjects. The summary of results of these studies will be demonstrated and some diet recommendation will be fomulated.

Methods

S u b j e c t s

Four intervention studies were conducted in co-operation with the Department of Clinical Biochemistry and the Department of Internal Diseases, Kyjov Regional Hospital, Czech Republic according to Table I.

Table I
Clinical studies

Study/year	Nr of subjects	Diagnosis	Type of preparative
2001/2002	80	Type 2 diabetes	antioxidant trio (A, E, β-carotene)
2002/2003	60	Type 2 diabetes	antioxidant trio + bioflavonoids
2003/2004	45	hyperlipidaemia	Chlorella pyrenoidosa
2004/2005	40	hyperlipidaemia	PUFA + tocopherol

B i o c h e m i c a l e x a m i n a t i o n s

Blood samples were taken 4 times during the whole period of each experiment. The plasma concentrations of glucose, glycated haemoglobin (HbA1c), total protein, urea, creatinin, uric acid, bilirubin, ALT, AST, total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerols, apolipoproteins A1 and B were examined in each sample using a Hitachi apparatus and standard diagnostic kits.

The plasma levels of retinol, γ- and α-tocopherols as well as the plasma levels of carotenoids - lutein, lycopene, α-carotene and β-carotene, were determined by RP- HPLC with spectrophotometrical detection.

Serum antioxidant activity was measured using a "Total antioxidant status" kit (TAS; Randox). As a biomarker of oxidative damage, serum advanced glycation end-products (AGEs) were measured fluorimetrically and the total sum of oxidized substances in serum was measured spectrophotometrically. Analysis of serum fatty acids was done by using gas chromatography.

Results and discussion

All studies were realized during 8 months (October – May). During the 1st period of experiment (3 months) took all groups normal diet. During consecutive 90 days (2nd period) both groups A and B were treated by individual supplements. During the 3rd period (2 months) rate of antioxidants utilization in tested organisms was followed.

Levels of antioxidants and TAS in the control groups in all studies exhibited typical seasonal decrease in the 2nd period. After 90-day of antioxidant treatment statistically significant changes in some parameters of saccharid (HbA1c) as well as lipid metabolism (LDL cholesterol and TAG decrease) were observed in group of patients with diabetes and hyperlipidaemia.

Complex preparative containing several types of antioxidants (e. g. carotenoid group and flavonoid group) exhibited better effect than simple antioxidant trio intake. Effect of Chlorella pyrenoidosa was substantially lower than effect of preparatives containing mixture of antioxidant vitamins and provitamins. It seems, that the most active component that positively influences lipid and sacahride metabolism in Czech population is probably α-tocopherol. But, 90-day intake of natural sources (500 g of fruit and vegetables daily in addition to normal diet) led to higher and, moreover, long-term effect, when compared with any food supplements.

The main problem with any epidemiological study is that correlation does not imply causation. It is commonly agreed that diets rich in vegetables and fruit are associated with a lower incidence of certain types of degenerative diseases, e. g. cancer, coronary diseases. For example, β-carotene is a common constituent of plants. The higher the blood plasma β-carotene level in humans, the lower the risk (on a population basis) of developing some forms of cancer. It is not clear whether β-carotene itself gives protection against cancer or whether it is simply a diet marker. Thus eating more vegetables might raise β-carotene levels, but the real protective agent could be anything (or any combination of things) in vegetables, because plants contain a huge range of potentially protective agents.

Recently it was shown that the levels of antioxidants in a random cohort of the Czech population (n = 450) exhibited typical seasonal differences, with the maximum in the summer and autumn and the minimum in the winter and spring seasons, when a number of individuals including healthy subjects were deficient in many of the parameters tested. According to the National Institute for Public Health study focused on monitoring the total daily intake of fruits and vegetables including processed foods, the average total daily intake of these sources in 1997 was about 280 g (based on 7-day recall). This finding particularly agrees with the data obtained in the present study. Based on the average intake of fruits and vegetables, it is possible to relate the Czech population to the north European countries, such as Ireland, Scotland and Norway. With regard to the generally higher incidence of cardiovascular and other degenerative diseases observed in these countries, enhanced consumption of antioxidants, predominantly in a natural form, could be recommended to the Czech population. Further it should be noted that it is unnecessary to enrich a diet with some exotic and/or imported sorts of fruits and vegetables. Regular consumption of a sufficient amount of common natural sources (about 500 g daily, on average) would be an optimum method of solution.

It is an old concept that food and health are intimately linked; in 400 B. C. the physician Hippocrates wrote, "Let food be your medicine and medicine be your food". It is clear that there is no universal "best" antioxidant; individual antioxidants can protect well against one type of damage (e. g. lipid peroxidation) but not against the other types of modification (e. g. protein damage). This study confirmed that the intake of complex antioxidant micronutrients can positively influence total antioxidant status in healthy subjects. According to our findings, an optimum supplement should contain a complex mixture of naturally occurring antioxidants to ensure an adequate ratio for the synergistic biological effect of the whole group of antioxidants. Additionally, local specialities in the accessibility of natural sources could be taken into account when general public diet recommendations are formulated.

REFERENCES

1. Sies H.: Oxidative stress II. *Oxidants and Antioxidants*. Academic Press, London (1991).
2. Halliwell B., Gutteridge J. M. C.: *In*: *Free Radicals in Biology and Medicine*. 3rd edition, Oxford University Press (1999).
3. Kankova K., Marova I., Zahejsky J., Muzik J., Stejskalova A., Znojil V., Vacha: Metabolism *50*, 1152 (2001).
4. Buring J. E., Hennekens C.H. Nutr. Rev. *55*, S53, Part 2 (1997).
5. Burri B. J. Nutr. Res. *17,* 547 (1997).
6. Halliwell B.: Free Rad. Res. *25*, 57 (1996).
7. Willett W. C.: Science *264*, 532 (1994).
8. Gey K. F.: J. Nutr. Biochem. *6*, 206 (1995).

L14 INTERACTION OF POLYCYCLIC AROMATIC HYDROCARBONS WITH PLASTIC PACKAGE MATERIALS – EFFECTIVE TOOL FOR ELIMINATION OF CARCINOGENIC COMPOUNDS FROM FOODS

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent the largest class of known environmental carcinogens. Some of them, even though not carcinogenic, may act as synergists. In a number of papers, remarkable high concentrations of PAHs in fats and oils have been reported. Moret et al[1] analysed 51 samples of olive oils and determined the total PAHs concentrations from 2.94 to 143.12 μg kg^{-1}. Pupin & Toledo[2] determined benzo(a)pyrene (BaP) in 40 olive oil samples available on Brasilian market. As found, BaP was present almost in all samples when the highest concentration reached the level of 164 μg kg^{-1}. Stijve & Hischenhuber[3] tested 12 samples of vegetable oils. They determined the highest concentration of BaP in coconut oil (up to 581.7 μg kg^{-1}). Hopia et al[4] determined the concentrations of numerous PAHs in Finnish butters, margarines and vegetable oils, and some raw vegetable oil materials. Total PAH concentrations in 25 samples varied from 0.17 (corn oil) to 4600 (crude coconut oil) μg kg^{-1}. It was concluded that enormous PAHs concentrations in coconut oil could be brought about by direct drying of copra with smoke. In general, smoke is an important source of PAHs because they are being formed during a thermal combustion of wood at a limited access of oxygen[5]. From there reasons, EU has adopted the regulation 208/2005 limiting BaP content at the level of level of 2 μg kg^{-1} in oils and fats intended for direct human consumption or use as an ingredient in foods. Also, International Olive Oil Council has recommended maximum tolerable concentration of BaP in olive–pomace oil the same level of 2 μg kg^{-1}. When thinking about the interactions between foodstuff and packaging, the contamination of food by substances migrating from the polymer packaging is mostly intuitively considered. However, several papers have announced that the polymers could be used for removal of contaminants from foodstuff with regard to their ability to adsorb some organic contaminants from liquid media. In this way, PAH concentrations were reduced in a liquid smoke flavour by two orders during 14 days[6]. The possibility of PAH removal from liquid media as well as roasted meats by sorption into polyethylene was mentioned as the effective procedure for elimination of contaminants from foods[7]. As found[8] the rate-limiting step of the PAH sorption from liquid into polyethylene is the diffusion in liquid media. PAHs are primarily adsorbed on polyethylene surface, with subsequent migration into bulk polymer[9]. At present time, the dominant plastic material used for vegetable oil and non-alcoholic drinks packaging is polyethylene terephtalate (PET). For packaging purposes, food industry uses-up 20 % of total world production with expected increasing by 15 % yearly in near future. The ability of PET to decrease PAHs concentrations in polar and non-polar liquid media has already been unambiguously proven[10]. Concurrence effects of other compounds on extend of adsorption processes of BaP from rapeseed oil onto PET surface has been confirmed[11]. Since there are no any data available in the literature regarding the interaction of PET with BaP contained in the sunflower oil, the aim of this work was to study the possibility of removal of PAHs by sorption onto PET.

Experimental

A sunflower oil was purchased in local marked, produced by Palma-Tumys Ltd., Bratislava, Slovak Republic. In the experiment, pre-bubbled PET receptacles of cylindrical shape were used with i.d. of 21.4 mm. The receptacles were also provided by Palma-Tumys Ltd. The company uses them for oil and fruit syrup packaging after blowing to volume of 2L. BaP of analytical grade was purchased from Promochem, Germany. Acetonitrile was of gradient grade (Merck,

Germany), methanol of UV purity (Lachema, CR), chloroform for analysis (Lachema, CR), cyclohexane for analysis (ITES, Slovak republic). All solvents were rectified just before use.

Experiment

First of all, the oil was analysed for the presence of BaP. Subsequently, 100 g of the oil was spiked with PAHs solution in 2 dm^3 volume glass flask and left to evaporate the solvent spontaneously. To accelerate the evaporation, the oil was mixed up occasionally. Then, roughly 900 g of the oil was added and the content of the flask was mixed thoroughly. At this stage, the sample of spiked oil was taken to determine the initial BaP concentration. Than, the receptacles were filled by the spiked oil and placed into a polystyrene box to protect them from the light and to maintain the constant temperature of 18.3 °C. Samples for analysis were taken after 1, 3, 5, 7, 11, 24 and 49 hrs by a glass pipette from a couple of receptacles. To maintain equal conditions during the experiment, new couples of receptacles were taken for each analysis. After sampling, the contents of receptacles were than discharged. The preparation of samples and HPLC conditions are described in[11]. All measurements were done in duplicate.

Results and discussion

The hanges in BaP concentrations in the sunflower oil are shown in Fig. 1. As follows from Fig. 1., BaP concentration started to decrease immediately after filling the PET receptacles by sunflower oil until an equilibrium state between liquid and solid phases was reached. As the spiked samples were not stirred during the experiment, it is reasonable to assume that the factor limiting the rate of BaP concentration decrease is the diffusion in liquid media. Because the PET receptacles were of cylindrical shape, we therefore used for quantitative description the relationship derived for the diffusion in a cylinder:

$$\frac{n_t}{n_\infty} = 1 - \sum_{n=1}^{\infty} \frac{4}{a^2 \alpha_n^2} \exp\left[-D\alpha_n^2 t\right] \qquad (1)$$

where n_t is the amount of diffused BaP, which has left the sample as a consequence of the diffusion into PET at time t, and n_∞ is the amount of BaP corresponding to infinite time, D is the diffusion coefficient, a is the radius of the cylinder, and α_n are the roots of the equation:

$$J_0(a \cdot \alpha_n) = 0 \qquad (2)$$

where J_0 is the zero-order first-class Bessel function. In the experiments, not the amount of BaP adsorbed on PET was measured, but their residual concentration in the liquid media. After recalculation of the amount of BaP to their concentration, Eq. 1 can be modified into the form

$$c_t = c_\infty + (c_0 - c_\infty) \sum_{n=1}^{\infty} \frac{4}{a^2 \alpha_n^2} \exp\left[-D\alpha_n^2 t\right] \qquad (3)$$

where c_0, c_t and c_∞ are the initial concentration, concentration at time t and equilibrium concentration, respectively. The diffusion coefficient of BaP was calculated by the non-linear least squares method by minimizing the sum of squares of differences between the concentrations of BaP measured experimentally and those calculated by Eq. 3, and his reached the value of 3.92 $cm^2\ h^{-1}$. The minimization was carried out by the simplex method. Minimized parameters were c_∞, $c_0 - c_\infty$ and D. In calculations, the first 20 terms of Eq. 3 were taken into account. The values of α_n were taken from tables. Eq. 3 makes it also possible to calculate the time necessary for a decrease of the BaP concentration from the initial value to a value requested, or the equilibrium value, respectively. Of course, the value of diffusion coefficient is also transferable to another geometry of plastic containers. As known, there are three main routes of human exposure to BaP – food 80 %, air 19.7 % and drinking water 0.2 %). As the experiments have demonstrated, the use of PET for food packaging could be also useful in preventive human health protection. This fact was especially underlined two years ago when serious problems appeared in Czech Republic and Slovak republic with over-limit concentrations of BaP in olive oils packed in glass bottles. If producers had used PET packaging materials, they could have avoided serious troubles associated with banning the sale and returning the oils back to the country of origin. The results of this study lead to the implication that the used PET bottles could act as a conceal source of BaP, and for this reason their reuse, or recyclation should be being carried out only at the control for presence of these compounds in PET bulk.

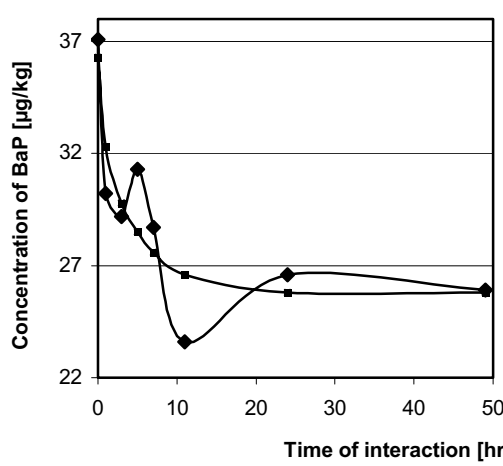

Fig. 1. **Changes in BaP concentration in rapeseed oil stored in PETreceptacles. ■ – dependance calculated by modified kinetic equation, ♦ – experimentally obtained data**

Conclusion

Summarising the results and findings obtained in this work, the following conclusions could be postulated:

1. The BaP concentration in liquid media can be reduced on the basis of interaction between BaP contained in the sunflower oil and PET as a solid phase.

2. The rate of decrease in BaP concentration describes the modified kinetic equation using calculated diffusion coefficients.
3. The equation makes it possible to calculate the BaP concentration at any time during the interaction as well as to predict the final equilibrium concentration
4. The equation is of general use, transferable also to another geometry of package. However, the oscillations in BaP concentrations at the beginning of the experiment are out of known theory of adsorption.
5. This way of BaP elimination could be useful especially in cases, where the usage of organic compounds is impossible, improper, or banned by legislation, respectively.
6. The interactions between PET package and foods, in general, are suitable from the point of view of a consumer health protection to decrease the real exposition of human organism to carcinogenic compounds, what is also important from point of correct approach to risk analysis associated with food consumption.

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REFERENCES

1. Moret S., Piani B., Bortolomeazzi R., Conte L. S.: Z. Lebensm. Unters. Forsch. *205*, 116 (1997).
2. Pupin A. M., Toledo M. C. F.: Food Chem. *55*, 185 (1996).
3. Stijve T., Hischenhuber C.: Deutsch. Lebensm. Rundsch. *83*, 276 (1987).
4. Hopia A., Pyysalo H., Wickström K.: J. Am. Oil. Chem. Soc. *63*, 889 (1986).
5. Šimko P.: J. Chromatogr B, *770*, 3 (2002).
6. Šimko P., Bruncková B: Food Addit. Contam. *10*, 257 (1993).
7. Chen J., Chen S.: Food Chem. *90*, 461 (2005).
8. Šimko P., Šimon P., Khunová V., Bruncková B., Drdák M. Food Chem. *50*, 65 (1994).
9. Šimko P., Šimon P., Khunová V.: Food Chem. *64*, 157 (1999).
10. Šimko P., Šimon P. Belajová E.: Eur. Food. Res. Technol. *219*, 273 (2004).
11. Šimko P., Skláršová B., Šimon P., Belajová E.: Eur. J. Lipid Sci. Technol. *107*, 187 (2005).

L15 DEGRADATION OF PLANT DNA BY FOOD PROCESSING

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Introduction

Genetically modified foods belong to the most controversial issues in the last decade. The risk assessment of foods and feeds obtained from genetically modified plants requires a holistic approach, taking into account all aspects that may affect the health of the consumer[1]. To perform the risk assessment, sound data on hazard identification, hazard characterization, exposure assessment and risk characterization are needed for each aspect[2].

DNA has never been considered to constitute a health risk, except its potential to couse gout, but it is an agent capable of transforming bacteria[3]. It has been considered that the recombinant DNA should be transfered by bacteria present in food, feed and gastrointestinal tract of animals by the mechanism called horizontal gene transfer. Trasnsformation, transduction and conjugation by bacteria are three main mechanisms of the horizontal gene transfer[4,5,6].

Structural genes range typically between 150 and 6000 bp and regulatory sequences between 100 and 150 bp, but detection of DNA sequences of GMO in foods aim at short DNA sequences (100 bp)[7,8]. Thus, for the risk assessment, not only the presence of recombinant DNA in food but also the maximal detectable fragments length of the recombinant DNA have to be known[2].

Estimation of the likelyhood of such transfer in food requires data on the persistence of DNA as well as the competence development of bacteria in this enviroment. It has already been demonstrated that food-associated bacteria can develop competence during growth in foods and thus become transformed with free DNA in the food matrix[9,10,11]. In contrast to rich data on the presence of DNA in processed food[12,13,14] or its stability in rumen models[15,16], the knowledge of the kinetics of DNA degradation under the conditions of food processing is restricted.

In addition, the degradation of the plant DNA during production of bread was monitored indicating that temperature and pH were the major effective factors[17,18].

Important source of proteins in food and feed are legumes. Proteins alternative to soya, which are grown in Slovak Republic, are bean and pea. Recently, maize and soya are the most frequently used genetically modified plants[19]. In our study we studied the kinetics of DNA degradation in legumes and maize affected by heat processing, by methods of molecular biology, PCR in particular.

Material and methods

Processing of DNA degradation

Table I
Processing of samples by temperature and time

Temperature/Time [min]	0	7	15	30	45	60	75	90	120	150	225	360
Pea, bean, maize												
80 °C, 100 °C	+	+	+	+	+	+	–	+	+	+	+	+
Pea, maize 200 °C	+	+	+	+	+	+	+	+	+	–	–	–
bean 200 °C	+	+	+	+	+	+	+	–	–	–	–	
bean 200 °C	+	+	+	+	+	+	+	–	–	–	–	–

The seeds of pea (Gloriosa), bean (Bobovica biela maslová) and maize (NK 603) were subjected to heat processing at 80 °C, 100 °C and 200 °C. Samples were taken in various time – 0 to 360 min at 80 °C and 100 °C, 0 to 120 min at 200 °C (pea, maize) and 0 to 75 min at 200 °C (bean) (Table I).

DNA isolation

DNA was extracted by the commercial kit GeneSpin for food (GeneScan, Teltow, Germany) after the homogenization of the processed seeds in the mixer AY47R1 (Moulinex, Barcelona, Spain) and/or in mortars. The final volume of eluted DNA solution was 100 µl. The quality of isolated DNA was detemined by agarose gel (1%) electrophoresis[20].

Monitoring the kinetics of DNA degradation

PCR was performed in 25 µl volumes using GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California, USA) or using Personal Cycler (Biometra, Göttingen, Germany). The amplification protocols for each reaction are in Table II and Table III. Each reaction consisted of: 10 mmol l^{-1} Tris HCl, pH 8,8; 50 mmol l^{-1} KCl; 1,5 mmol l^{-1} MgCl2; 0,05% Tween 20; 200 µmol l^{-1} dNTP (Invitrogen, Carlsbad, CA, USA); 50 pmol l^{-1} primers (Table IV, Table V, Table VI) 1U HotStar Taq polymerase (Qiagen, Hieden, Germany), and 1 µl of DNA. Pea lectin gene (X66368), bean lectin gene (AJ439563) and bean phaseolin gene (J01263) were compared with sequences in GeneBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) and the primers were designed by the program Primer3 (Whitehead Institute Nine Cambridge Center, Cambridge, MA, USA). PCR products were analysed by electrophoresis in 1,5 % agarose gel and photographed by using a digital camera.

Table II
PCR cycle for amplification of lectin and phaseolin gene

Step	Temperature	Time
Initial denaturation	95 °C	15 : 00 min
30 cycles	94 °C	0 : 30 min
	58 °C	0 : 30 min
	72 °C	1 : 00 min
Final extension	72 °C	5 : 00 min
cooling	4 °C	∞

Table III
PCR cycle for amplification of invertase gene

Step	Temperature	Time
Initial denaturation	95 °C	15 : 00 min
40 cycles	95 °C	0 : 30 min
	64 °C	0 : 30 min
	72 °C	0 : 40 min
Final extension	72 °C	3 : 00 min
cooling	4 °C	∞

Table IV
Pea lectin gene primers

Primer	Sequence (5′→3′)[2]	Size of PC products
HRACH F3	ttgtcataaatgcacccaacagttacaacg	122 bp
HRACH R1	catactctgcgctattgaaaactccgag	
HRACH F2	atggcttctcttcaaacccaaatgatctcg	417 bp
HRACH R1	catactctgcgctattgaaaactccgag	
HRACH F2	atggcttctcttcaaacccaaatgatctcg	748 bp
HRACH R2	gcatattctgctcctgtggtagctgag	
HRACH F3	ttgtcataaatgcacccaacagttacaacg	874 bp
HRACH R3	ccaaaatgttgagaggtgcacatgaacc	

Table V
Bean lectin and phaseolin gene primers

Primer	Sequence (5′→3′)[2]	Size of PCR products
FAZ F2	cagtagacctgaagagcgttcttcc	116 bp
FAZ R2	cggagagcttggaagcaaaagacc	
FAZ F1	cctcttccttgtgcttctcaccc	469 bp
FAZ R1	tgatggagttcacgtcgatgcc	
FAZ F1	cctcttccttgtgcttctcaccc	724 bp
FAZ R2	cggagagcttggaagcaaaagacc	
Psn-F	tcgtcttggtgaaacctgat	1371 bp
Psn-R	ttttgctgttcctgttggtg	

Table VI
Maize invertase gene primers

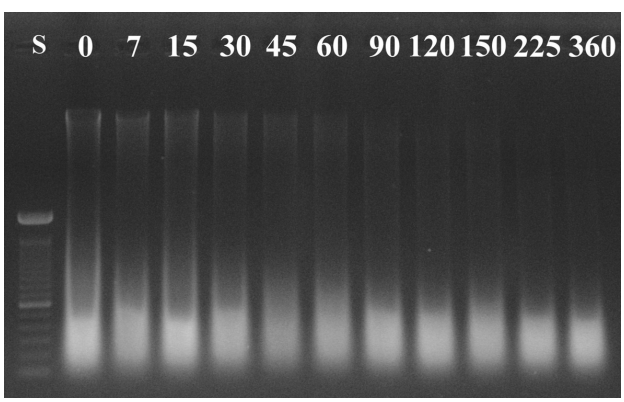
Primer	Sequence (5′→3′)[2]	Size of PCR products
IVR1F-A	ccgctgtatcacaagggctggtacc	224 bp
IVR1R-B	ggagcccgtgtagagcatgacgatc	
IVR1F-I	tcctccactggctgcacctaccg	124 bp
IVR1R-B	ggagcccgtgtagagcatgacgatc	
IVR1F-A	ccgctgtatcacaagggctggtacc	696 bp
IVR1R-C	cgtaggtgccgatcgcgtagtagtc	
IVR1F-E	agtgggtcaagtcggacgccaacc	401 bp
IVR1R-C	cgtaggtgccgatcgcgtagtagtc	
IVR1F-A	ccgctgtatcacaagggctggtacc	1339 bp
IVR1R-D	aggatcggggcctctctgcttgaac	

Results and discussion

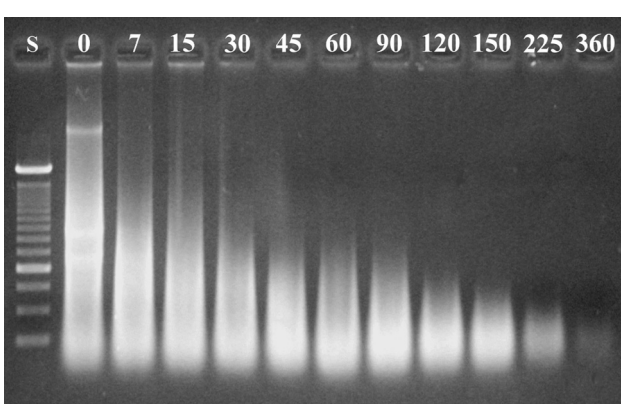
The kinetics of degradation of plant DNA by the process parameter temperature was monitored using PCR. Plant DNA isolated from maize, bean and pea was incubated in distilled water at 80 °C, 100 °C and 200 °C (Fig. 1.). Five PCR systems were established to amplify maize DNA fragments of different sizes (Table VI) and four PCR systems were established to amplify bean and pea DNA fragments of different sizes (Table IV, Table V).

Higher temperature (100 °C and 200 °C) dramatically reduced the size of extracted DNA. Treatment of plant tissues at 100 °C and 200 °C for more than 15 to 60 min was sufficient for the degradation of DNA to take place to the extent that it should be incapable of transmitting genetic information. These conclusions are based on PCR amplification of about 1000 bp DNA fragments and the survival of these specific gene sequences. Because the average size of structural genes range about 1000 bp primers for the amplification of DNA fragments were designed in this size and in smaller size.

Under the heat processing at 80 °C, 100 °C and 200 °C the amplification of the 1379 bp fragment (phaseolin gene) failed after 45 min of incubation and 1339 bp fragment (invertase gene) failed after 15 min, respectively. Bauer and co-workers[2] revealed that 1225 bp DNA fragment of maize Bt-176 was no longer detectable after the 30 min incubation at pH 4.0 and 65 °C. All fragments exclusively could be



a) 80 °C



a) 100 °C

Fig. 1. **Comparison of the level of DNA degradation in samples of the pea after isolation of DNA and heat processing, S – standard n . 250 bp a) at 80 °C and b) at 100 °C, 0–360 min**

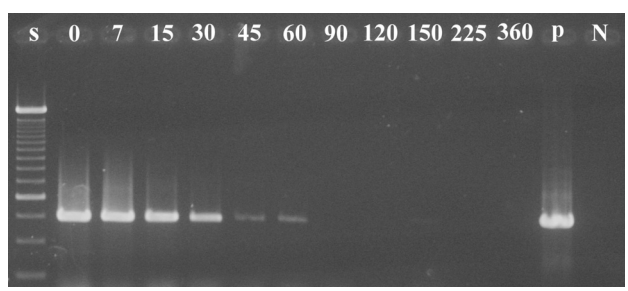


Fig. 2. **Amplification of 748 bp fragment of pea DNA after isolation of DNA and heat processing at 100 °C. S – standard n . 50 bp, P – positive control, N – negative control, 0–360 min**

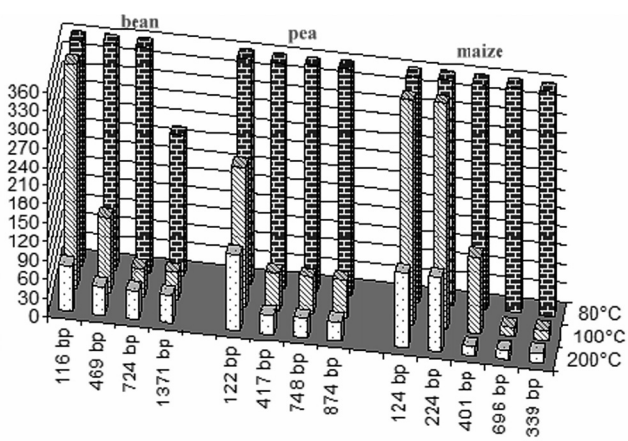


Fig. 3. **Comparison of the kinetics of DNA degradation in samples of bean, pea and maize based on the temperature, the time of heat processing and the maximum size of detectable PCR product**

detected after 360 min incubation at 80 °C. 400 to 1000 bp fragments were not amplificable after 30 to 120 min of the incubation at 100 °C and after 15 to 45 min incubation at 200 °C (Fig. 2., Fig. 3.).

Maximum size of PCR products, that were detectable, reflected the level of DNA degradation depending on temperature of heat processing.

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REFERENCES

1. Recommendation 97/618/EC of the Commission, Official Journal L253.
2. Bauer T., Weller P., Hammes W. P., Hertel C.: European Food Research and Technology *217*, 338 (2003).
3. de Vries J., Wackernagel W.: National Academy of Sciences of the United States of America *99*, 2094 (2002).
4. Doolittle W. F.: Trends in Genetics *14*, 307 (1998).
5. Dutta C., Pan A.: J. Biosci. 27, Suppl. *1*, 27 (2002).
6. de Sousa C. P.: The Brazilian Journal of Infectious Diseases *7*, 23 (2003).
7. Berdal K. G., Holst Jensen A.: European Food Research and Technology *213*, 432 (2001).
8. Hubner P., Waiblinger H. U., Pietsch K., Brodmann P.: Journal of AOAC International *84*, 1855 (2001).
9. Bauer F., Hertel C., Hammes W. P.: Systematic and Applied Microbiology *22*, 161 (1999).
10. Bräutigam M., Hertel C., Hammes W. P.: FEMS Microbiology letters *155*, 93 (1997).
11. Zenz K. I., Neve H., Geis A. Heller K. J.: Systematic and Applied Microbiology *21*, 28 (1998).
12. Allmann M., Candrian U., Luthy J.: Lancet *309* (1992).
13. Ebbehoj K. F., Thomsen P. D.: Meat Science *30*, 221 (1991).
14. Meyer R., Chardonnens F., Hubner P., Luthy J.: Zeitschrift für Lebensmitteluntersuchung und Forschung A *203*, 339 (1996).
15. Duggan P. S., Chambers P. A., Heritage J., Forbes J. M.: FEMS Microbiology letters *191*, 71 (2000).
16. Martín-Orúe S., O´Donnell A. G., AriDů J., Netherwood T., Gilbert H., Mathers, J.: British Journal of Nutrition *87*, 533 (2002).
17. Moser M. A., Kniel B., Schmitz-Winnenthal J., Hupfer C., Engel K. H.: Getreide Mehl und Brot *53*, 334 (1999).
18. Straub J. A., Hertel C., Hammes W. P.: Zeittschrift für Lebensmittel Untersuchung und Forschung *208*, 77 (1999).
19. James C.: *Preview: Global Status of Commercialised Transgenic Crops.* ISAAA Briefs No. 30. ISAAA, Ithaca, N. Y (2003).
20. Maniatis T., Fritsch E. F., Sambrook J.: *Molecular cloning. A laboratory manual.* Cold Spring Harbor Laboratory (1982).

L16 DETECTION AND QUANTIFICATION OF PATHOGENIC BACTERIA IN FOODS BY REAL-TIME POLYMERASE CHAIN REACTION (PCR)

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Introduction

Foodborne diseases are a widespread and growing public health problem, both in developed and developing countries. Bacteria are the causative agents of foodborne illness in 60 % of cases requiring hospitalization[1]. Detection of pathogenic bacteria in food and the determination of the level of contamination are important from the point of food safety. In addition to conventional microbiological methods for the detection of pathogens in food, rapid methods based upon polymerase chain reaction (PCR) have become available[2,3]. (PCR) is a method of a great potential to speed up the detection of pathogenic bacteria. Recently, relatively laborious as well as time-consuming amplicon detection by gel electrophoresis is getting changed for a new closed-tube detection method, 5' nuclease PCR with fluorescent TaqMan probes. It detects the template specifically, sensitively and reproducibly on the basis of the fluorescence emitted from the hydrolysed probe and proportional to the formation of the amplicon.

The method is based on the 5'-3' exonuclease activity of the Taq DNA polymerase, which results in the cleavage of the fluorescent dye-labelled probes during PCR and the intensity of fluorescence is then measured. The TaqMan probe is located between the two PCR primers and has a melting temperature 10 °C higher than that of the primers: binding of the TaqMan probe prior to the primers is crucial because without it, PCR products would be formed without the generation of fluorescence and thus without being detected. The TaqMan probe probe is an oligonucleotide with a reporter dye attached to the 5' end and a quencher dye attached to the 3' end. Till the time the probe is hydrolysed, the quencher and the fluorophore remain in proximity to each other, separated only by the length of the probe. Degradation of the TaqMan probe by the Taq DNA polymerase, frees the reporter dye from the quenching activity of the quencher and thus the fluorescent activity increases with an increase in cleavage of the probe, which is proportional to the amount of PCR product formed[4,5,6].

We developed a 5'-nuclease PCR system for the detection and quantification of pathogenic bacteria from Enterobacteriaceae family: *Salmonella enterica., Escherichia coli and Citrobacter freundii/braakii.*

Materials and methods

Bacterial strains

Salmonella enterica serovar Enteritidis CCM 4420, *Escherichia coli* CCM 4787 and *Citrobacter freundii* CCM

4475 were used as model microorganisms for developing the detection systems.

R e a g e n t s

Primers and probe specific for *Salmonella enterica*. oriented to *fimC* gene
Srt2F: ATA AAT CCG GCG GCC TGA TG
Srt2R: TGG TAT CGA CGC CTT TAT CTG AGA
Srt2P: 6-FAM-TTA CAC CGG AGT GGA TTA AAC GGC TGG G-TAMRA

Primers and probe specific for *Escherichia coli* oriented to *sfmD* gene
Ert2F: ACT GGA ATA CTT CGG ATT CAG ATA CGT
Ert2R: ATC CCT ACA GAT TCA TTC CAC GAA A
Ert2P: 6-FAM-ATA CCA GCA GCT GGG TTG GCA TCA GTT AT-TAMRA

Primers and probe specific for *Citrobacter freundii/ braakii.* oriented to *cfa* gene
Crt4F: TTGGCGTCCAGCGCATTCA
Crt4R: AATTCCAGCCTTCGGCAAACG
Crt4P: 6-FAM-TCCAGATCGGAAAGGGTTGCGGTGAC--TAMRA

R e a c t i o n m i x t u r e

Conventional PCR: 1×PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM of each primer, 1.5 U Taq DNA polymerase, PCR water to volume 22.5 µl, 2.5 µl of sample 5'-nuclease PCR: 1×PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM of each primer, 1.5 U Taq DNA polymerase, 0.2 mM TaqMan probe, PCR water to volume to 22.5 µl, 2.5 µl of sample.

D N A p r e p a r a t i o n

Cells from 1 ml of decimally diluted overnight culture (37 °C, 120 rpm, 16 h) were subjected to simple lysis by boiling (95 °C, 25 min) in 1×PCR buffer. Lysate (volume 2.5 µl) was added to the PCR reaction mixture.

R e a c t i o n c o n d i t i o n s

Conventional PCR and detection was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems, USA) and used the temeperature programme: 15 min at 95 °C, 30 cycles (15 s at 95 °C, 60 s at 60 °C), 5 min at 72 °C. Real time 5'-nuclease PCR was performed in Opticon 2 DNA Engine (MJ Research, USA) and used the temperature programme: 15 min at 95 °C, 40 cycles (15 s at 95 °C, 60 s at 60 °C).

A m p l i c o n d e t e c t i o n

Conventional PCR

Amplification product was detected by electrophoresis in 1.5% agarose gel, stained by ethidiumbromide and visualized in UV-light. Presence of 102 bp, 106 bp and 100 bp DNA fragments indicated positivity for *Salmonella enterica, E.coli* and *Citrobacter freundii/braakii*, respectively.

Real-time PCR

Kinetics of the fluorescence signal in the FAM channel were recorded and the threshold cycle (C_T) values were calculated from log scale curves using the internal instrument software (Chromo4, MJ Research, Waltham, MA, USA.). For calibration line, averaged C_T values were plotted against decadic logarithm of cell concentration.

Results and discussion

To obtain data on the applicability of the developed real--time PCR system to quantification, decimally-diluted cultures of all three pathogenic bacteria were analysed in three independent experiments. For decreasing concentrations of the culture, amplification curves with proportionally increasing threshold cycle values were recorded (Fig. 1.).

Threshold cycle values were plotted against bacterial concentrations and individual calibration lines were constructed. These were linear over the range of concentrations used (10^2 to 10^8 cfu ml^{-1}) with correlation coefficients $r^2 \geq 0.998$ (Fig. 2.), analytical parameters are given in Table I.

To investigate whether related *Enterobacteriaceae* may interfere with the developed real-time PCR system, a decimally diluted culture of *Salmonella* Enteritidis CCM 4420, *E.coli* CCM 4787 and *Citrobacter freundii* CCM 4475, respectively was analysed on the background of other enterobacteria (*E.coli* CCM 4787 (10^6 cfu ml^{-1}), *Citr. freundii* CCM 4475 (10^6 cfu ml^{-1}). *S. Enteritidis* (10^6 cfu ml^{-1})). Presence of these considerably high amounts of related *Enterobacteriaceae* had practically no effect on the calibration lines obtained.

Table I
Detection limit of the conventional and real-time PCR and parameters of the calibration lines of the threshold cycle vs. log c (cfu ml^{-1}) calculated from 5'-nuclease PCR with decimal dilutions of *S. Enteritidis*, *E.coli* and *Citr. freundii* in pure cultures

Strain	Detection limit [cfu ml^{-1}]		Quantification range [cfu ml^{-1}]	Parameter (average ± SD, n = 3)	
	Gel electrophoresis	Real-time PCR		slope	x intercept
S Enteritidis CCM 4420	10^4	10^2	10^8–10^2	–3.39 ± 0.09	13.68 ± 0.14
E.coli CCM 4787	10^4	10^2	10^8–10^2	–3.46 ± 0.10	13.40 ± 0.12
Citr. freundii CCM 4475	10^4	10^2	10^8–10^2	–3.42 ± 0.13	13.45 ± 0.10

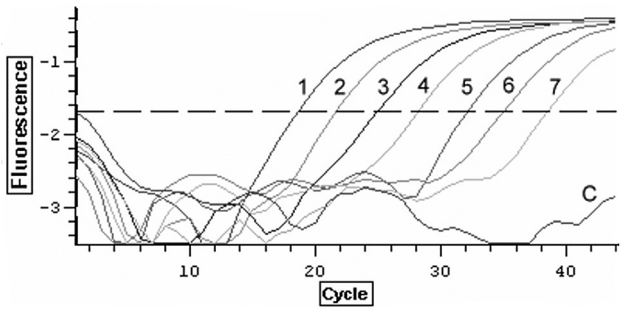

Fig. 1. **Record of a representative real-time 5'-nuclease PCR experiment with decimal dilutions of Salmonella Enteritidis CCM 4420 showing curves for $2.2 . 10^8$ cfu ml^{-1} (1), $2.2 . 10^7$ cfu ml^{-1} (2), $2.2 . 10^6$ cfu ml^{-1} (3), $2.2 . 10^5$ cfu ml^{-1} (4), $2.2 . 10^4$ cfu ml^{-1} (5), $2.2 . 10^3$ cfu ml^{-1} (6), $2.2 . 10^2$ cfu ml^{-1} (7), and no template control (C)**

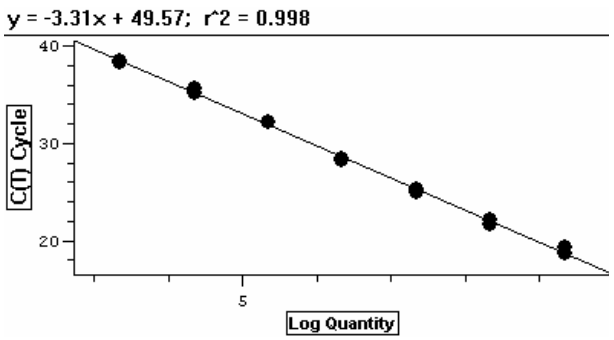

Fig. 2. **A calibration line of the threshold cycle vs. log c (cfu ml^{-1}) calculated from 5'-nuclease real-time PCR with decimal dilutions of S. Enteritidis CCM 4420**

The detection limit of the qualitative method 10^4 cfu ml^{-1} when the amplification was followed by gel electrophoresis (Fig. 2.), 5´-nuclease real-time PCR with 40 amplification cycles showed the detection limit of 10^2 cfu ml^{-1}.

The developed 5'-nuclease PCR system proved to be highly sensitive and specific for *S. enterica*, *E.coli* and *Citr. freundii.* Presented highly specific real-time PCR systems represent a good tool for quantification of these pathogenic bacteria in clinical, food and enviromental samples.

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REFERENCES

1. Mead P. S., Slutsker L., Dietz V., McCaig L. F., Bresee J. S., Shapiro C., Griffin P. M., Tauxe R. V.: Emerg. Infect. Dis. *5*, 607 (1999).
2. De Boer E., Beumer R. R.: Int. J. Food Microbiol. *50*, 119 (1999).
3. Rompré A., Servais P., Baudart J., de-Roubin M. R., Laurent P.: J. Microbiol. Meth. *49*, 31 (2002).

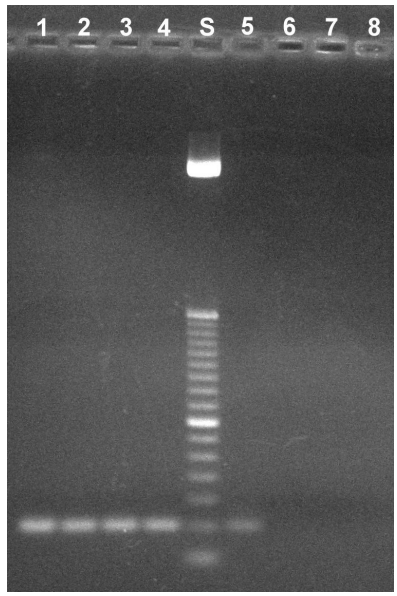

Fig. 3. **Detection limit of the conventional PCR with the amplification followed by gel electrophoresis. Decimal dilutions of S. Enteritidis CCM 4420 were analysed: 10^8 cfu ml^{-1} (1), 10^7 cfu ml^{-1} (2), 10^6 cfu ml^{-1} (3), 10^5 cfu ml^{-1} (4), 10^4 cfu ml^{-1} (5), 10^3 cfu ml^{-1} (6), 10^2 cfu ml^{-1} (7), and no template control (8), standard 50 bp (S)**

4. Annonymous: Qiagen News *3*, 21 (2001).
5. Lie Y. S., Petropoulos C. J.: Curr. Opin. Biotechnol. *9*, 43 (1998).
6. Heid C. A. Stevens J., Livak K. J., Williams P. M.: Genome Research *6*, 986 (2002).

L17 MECHANISM OF ACRYLAMIDE FORMATION IN MODEL FOOD SYSTEMS

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Introduction

In April 2002, a research group at Stockholm University and the Swedish Food Administration announced that significant amounts of acrylamide might be formed during common heat processing of foods[1]. Since acrylamide is classified as "probably carcinogenic to humans", these findings were considered as alarming. Although a final assessment of the toxicological risk is not finished yet, experts all over the world declare, that acrylamide levels in foods should be minimised as far as possible. To eliminate effectively the formation of acrylamide in heated foodstuff, it is necessary to understand

the mechanism of its formation. Among factors influencing acrylamide formation, necessary to investigate, are the precursor molecules of the reaction. Because the highest amounts of acrylamide were frequently found in French fries and potato chips, the attention was focused on heat-processed potato products[2]. Early investigations of the reaction pathways associated with the formation of acrylamide reached a consensus that the process is initiated with the reaction between reducing sugars and asparagine, thus indicating the Maillard reaction as a source of acrylamide[3]. However, subsequent studies have shown that the Maillard reaction may represent only one of a number of pathways to acrylamide formation, while it is still accepted that the presence of asparagine is a crucial factor in the formation of acrylamide in food[4,5]. Potato products have been associated with some of the highest levels of acrylamide, partly due to relatively high levels of suspected acrylamide precursors. Although acrylamide is not present in raw potatoes, or formed during boiling, high levels of acrylamide is be formed at the higher temperatures associated with frying and oven-baking[6]. For that reason, potatoes and potato-like model systems have been the subject of numerous studies of acrylamide formation[7]. The aim of this work was to study the formation of acrylamide from reducing saccharides and asparagines at various thermal regimes of heating reaction mixtures.

Experimental

Materials

Asparagine (Asn),and sugars (fructose, glucose), and ethyl acetate were purchased from the Sigma-Aldrich Co. Ltd. (Poole, U. K.). Acryamide (AA), p. a. purity were purchased from Fisher Scientific Ltd. (Loughborough, U. K.).

Experiment

Reaction mixtures consisted of equivalent mass of reducting sugars (fructose, glucose), and Asn were heated in temperature interval 120–200 °C at various thermal gradients and AA content was isolated and determined by GC–MS analysis, as mentioned in[4].

Analysis

Analyses were carried out using a Agilenta Technologies 6890 gas chromatograph equipped with a Agilenta Technologies 5890 mass selective spectrometer. An injection volume was made using a splitless injector heated to 250 °C. The GC column was a Agilent 122-3232 60 m × 0.25 mm fused silica capillary with a DB-FFAP phase. The column was held at 50 °C for 2 min, before being heated to 250 °C at a rate of 10 °C min^{-1}. Detection was was carried out in selected ion monitoring mode, monitoring ions obtained by negative chemical ionization, the mass of monitored fragments 70.2 and 73.2 m/z, respectively.

Results and discussion

As results shoved, a velocity of thermal gradient has the decisive effect on AA formation during heating the mixtures.

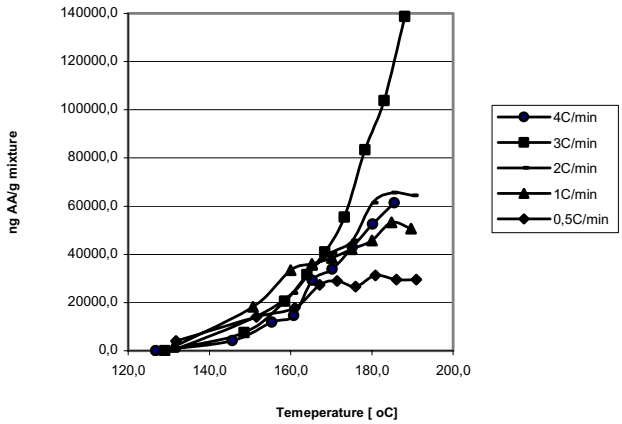

Fig. 1. **Dependence of acrylamide formation on thermal gradient in a model mixture consisted of asparagines and reducing sugars**

As follows from Fig. 1., the highest content of AA was observed at the gradient 3 °C min^{-1}, what is quit surprising at comparison to other gradients. However, AA can be either formed or petered out with regard its high reactivity, or ability to polymerise, respectively. Due to these facts, it will be necessary to carry out other experiment employing diferential scaning calorimetry to determine more exactly the character of all chemical reactions.

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REFERENCES

1. Tareke E., Rydberg P., Karlsson P., Eriksson S., Törnqvist M.: J. Agric. Food. Chem. *50*, 4998 (2002).
2. Weisshaar R.: Eur. J. Lipid Sci. Technol. *106*, 786 (2004).
3. Yaylayan V., Wnorowski A., Perez Locas C.: J. Agric. Food. Chem. *51*, 1753 (2003).
4. Zyzak D. V., Sanders R. A., Stojanovic M., Tallmadge D. H., Eberhart B. L., Ewald D. K. et al.: J. Agric. Food. Chem. *51*, 4782 (2003).
5. Becalski A., Lau B. P. Y., Lewis D., Seaman S. W.: J. Agric. Food. Chem. *51*, 802 (2003).
6. Ahn J. S., Castle L., Clarke D. B., Lloyd A. S., Philo M. R., Speck D. R.: Food Addit. Contam. *19*, 1116 (2002).
7. Friedman M.: J. Agric. Food. Chem. *51*, 4504 (2003).

L18 PESTICIDES RESIDUES ANALYSIS IN BABY FOOD: APPLICATION OF NOVEL APPROACH ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC)

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Introduction

Increasing number of pesticide/food commodity combinations to be controlled for compliance with maximum residues limits (MRL) requires introduction of novel approaches in residues analysis. While most earlier used pesticides were non polar or moderately polar compounds (hence amenable to gas chromatography, GC), recently registered pesticides preparation contain more polar active ingredients. On this account high performance liquid chromatography (HPLC) coupled with mass spectrometry (LC-MS) only method of choice for separation of samples components and identification/quantification of target analytes.

In addition to achieving good performance characteristics (low detection limits, good accuracy etc.) of analytical method high sample throughput is important control criterium when selecting analytical method. Recent introduction of ultra performance liquid chromatography (UPLC) system Acquity by Waters brings a revolutionary solution in increasing the speed of analyses compared to classic HPLC instruments. Separations columns (BEH C18) with very small particles (1.7 μm) can be operated at high mobile flow rates thanks to possibility to set head pressure as high as 15 000 psi. Printed study was conducted thanks to kind loan provided by Waters G.m.b.H. Prague to Department of Food Chemistry and Analysis ICT Prague that had in this way a possibility as the first in the Czech Republic to test the Acquity (demo) instrument. Pesticides residue analysis was selected as a challenging example of application of this system.

Experimental

Pesticide standards (see Table I) were obtained from Dr. Ehrenstorfer (Germany). Methanol was supplied by Meck (USA) and deionized water was produced by Milli-Q RG (Milipore, USA) apparatus.

All UPLC separations were carried by the ACQUITY UPLC™ system (Waters, UK) using a reversed phase column Acquity UPLC BEH C18 (100×2.1 mm, 1.7 μm) (Waters, UK). Mobile phase was methanol-water. LC conditions are shown in Table II.

Total analysis time was 10 min and additional 2 min post run time was required to set the column to starting conditions. Others conditions in all experiments ware as follows. Flow rate of mobile phase 0.3 ml min^{-1}, column and sample temperature 25 °C, injection volume 2 μl.

Table I
Comparison of HPLC/UPLC retention times under conditions

No.	Compound	Retention Time [min] UPLC	HPLC
1	Carbendazim	2.9	6.1
2	Thiabendazole	3.4	7.1
3	Carbaryl	3.8	8.1
4	Carbofuran	4.1	7.8
5	Linuron	5.0	9.6
6	Methiocarb	5.0	9.7
7	Epoxiconazole	5.5	10.2
8	Flusilazole	5.7	10.3
9	Diflubenzuron	5.7	10.4
10	Tebuconazole	6.0	10.7
11	Imazalil	6.0	10.6
12	Propiconazole	6.0	10.8
13	Triflumuron	6.2	10.7
14	Bitertanol	6.2	10.9
15	Prochloraz	6.2	10.9
16	Teflubenzuron	7.2	11.6
17	Flufenoxuron	7.5	11.6

Table II
Optimized UPLC mobile phase gradient

Time [min]	Water [%]	Methanol [%]
0	70.0	30.0
0.50	70.0	30.0
4.00	28.0	72.0
6.30	15.0	85.0
8.00	0.0	100.0
12.00	70.0	30.0

Conventional HPLC separations were carried out using 2695 Alliance module (Waters, UK) quipped a reversed phase column Discovery C18 (150×3 mm, 5 μm) (Supelco, USA), methanol water mobile phase gradient was used (see Table III)

Mobile phase also was methanol-water with LC conditions:

Table III
Optimised HPLC gradient program

Time [min]	Water [%]	Methanol [%]
0	50.0	50.0
6.00	0.0	100.0
15.00	0.0	100.0
25.00	50.0	50.0

Table IV
Optimised MS/MS transitions parameters

Compound	Selected transition [m/z]	Cone Voltage [V]	Colision Energy [V]
Carbendazim	192>160	35	22
Carbaryl	202>145	16	9
Thiabendazole	202>175.	40	25
Carbofuran	222>165	23	12
Methiocarb	226>169	26	9
Linuron	249>159	33	17
Imazalil	297>255	35	20
Tebuconazole	308>70	34	20
Diflubenzuron	311>158	25	13
Flusilazole	316>247	25	19
Epoxiconazole	330>121	30	20
Bitertanol	338>99	20	11
Propiconazole	342>158	43	25
Triflumuron	359>156	29	16
Prochloraz	376>308	24	11
teflubenzuron	381>158	23	13
Flufenoxuron	489>158	25	19

Total analysis time was 15 min and additional 10 min post run time was required to condition the column to starting conditions. Flow rate 0.3 ml min^{-1}, column temperature 25 °C and the injection volume 20 µl were used in all experiments.

Tandem mass spectrometric detector Quattro Premier (Waters, UK) was employed in both series of experiments (UPLC × HPLC). Positive electron ionization (ESI+) was applied for all analyzed compounds. In tuning setup was set for each analyte selective MS/MS transition and all measurement was made in MS/MS mode with this setup. Selective MS/MS conditions are shown in Table IV and common setup was as follows: capillary voltage 3.5 kV, extractor voltage 4 V, source temperature 120 °C, desolvation temperature 250 °C, cone gas flow 100 l h^{-1} and desolvation gas Flow 700 l h^{-1} (both gasses nitrogen). Argon was used as a collision gas in all experiments.

Results and discussion

Significant improvement of the speed of LC-MS analysis of 17 polar pesticides was obtained by replacement of conventional HPLC separation by UPLC see Fig. 1. On the other hand, it should be noted that although narrower elution band was obtained in experiments employing separation no significant increase of detection limits increased (i. e. signal to noise ratio) of target pesticides was achieved as compared to conventional HPLC (Table V). To benefit on improved peaks shapes and increased peak capacity coupling of

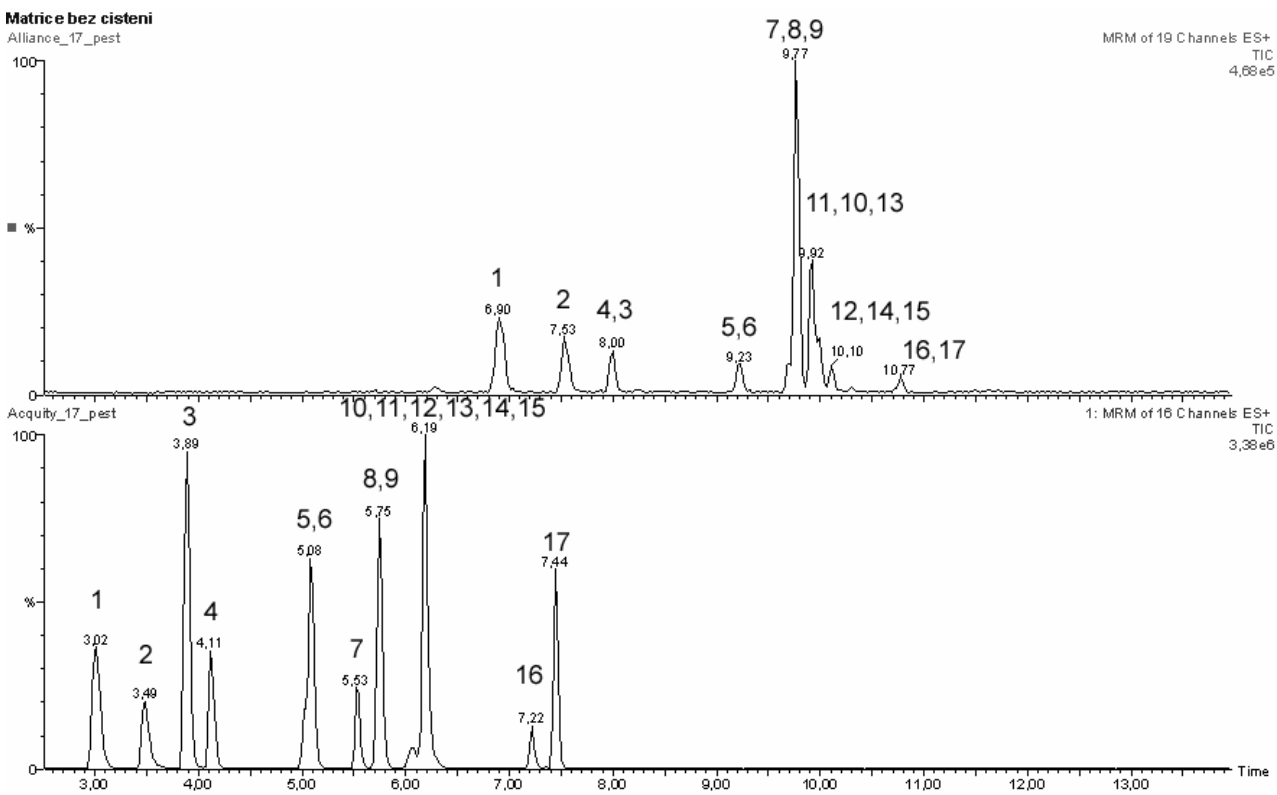

Fig. 1. **Optimized separation of 17 polar pesticides obtained by (A) conventional HPLC and (B) UPLC (TIC chromatogram of MS/MS transitions), for codes of analytes see Table I**

UPLC with time of flight (TOF) mass spectrometric detector or other detector with high scan rate (eg. DAD) might be considered. The total analyses time was reduced to 40 % in comparison to HPLC.

Table V
LOD obtained in LC-MS system employing UPLC and conventional HPLC

Pesticide	LOD [ng ml^{-1}] UPLC	HPLC
Carbendazim	0.1	0.1
Thiabendazole	0.1	0.1
Carbofuran	0.2	2.0
Carbaryl	0.2	1.0
Methiocarb	0.2	1.0
Linuron	1.0	1.0
Imazalil	1.0	1.0
Tebuconazole	1.0	1.0
Diflubenzuron	1.0	1.0
Flusilazole	1.0	1.0
Epoxiconazole	1.0	1.0
Bitertanol	2.0	2.0
Propiconazole	2.0	1.0
Triflumuron	1.0	1.0
Prochloraz	0.5	1.0
Teflubenzuron	5.0	5.0
Flufenoxuron	1.0	1.0

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REFERENCES

1. Mičkova B., Zrostlíková J., Hajšlová J., Rauch P., Moreno M. J., Abad A., Montoya A.: J. Chromatogr. A *495*, 123 (2003).
2. Wren S. A. C.: Journal of Pharmaceutical and Biomedical Analysis, *In Press*.
3. Snyder L. R., Dolan J. W., Gant J. R.: J. Chromatogr. *165*, 3 (1979).
4. Mellors J. S., Jorgenson J. W.: Anal. Chem. *76*, 5441 (2004).
5. Dolan J. W., Snyder L. R., Djordjevic N. M., Waeghe T. J., Hill D. W., J. Chromatogr A *857*, 1 (2003).
6. McCalley D. V.: J. Chromatogr A *1038*, 77 (2005).
7. Snyder L. R., Kirkland J. J., Glajch J. L.: *Practical HPLC Method Development*, 2nd ed., Wiley-Interscience, New York, 1997.
8. Meyer V. R.: *Practical High-Performance Liquid Chromtography*, 2nd ed., John Wiley and Sons, New York, 1996.
9. Zrostlíková J., Hajšlová J., Poustka J., Begany P.: J. Chromatogr A *973*, 13 (2002).
10. Zrostlíková J., Hajšlová J., Kovalczuk T., Štěpán R., Poustka J.: J. AOACr *86*, 612 (2003).
11. European Commission (August 2003) Quality Control Procedures for Pesticide residue Analysis: Guidelines for Residue Monitoring in the European Union, European Commision Document SANCO 10476/2003, Brussels, Belgium.
12. Hajšlová J., Zrostlíková J.: J. Chromatogr A *1000*, 181 (2003).

L19 DETERMINATION OF MYCOTOXINS BY LATERAL FLOW DEVICES (LFD) IN CEREALS

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Introduction

Throughout the world there is increasing consumer demand for high-quality food and drink products with the lowest possible level of contaminants. Many cereals and other crop are susceptible to fungal attack either in the field or during storage. These fungi may produce as secondary metabolites a diverse group of chemical substances known as mycotoxins. Many mycotoxins were initially identified after they caused a variety of health problems in livestock as well as humans, with many target organs and systems affected. There can be wide year to year fluctuations in the levels of mycotoxins in foods, depending on many factors, such as adverse conditions favouring fungal invasion and growth. It is necessary to control mycotoxins levels in whole chain food every time.

Analytical methods for determination of mycotoxins can be devided in two groups. The first one includes classical analytical methods, such as thin-layer chromatography (TLC), high-performance liquid (HPLC), gas chromatography (GC) and mass chromatography (MS). In recent years, most of these techniques have been coupled with immunoaffinity techniques to simplify extraction and improve mycotoxins recovery and measurement from foodstuffs[2]. The second group contains commercial immunological techniques, for example enzyme-linked immunosorbent assay (ELISA).

The aim of this report is to introduce rapid, easy, cheap, semi-quantitative analytical method for determination of mycotoxins – a lateral flow device (LFD).

Experimental

In collaboration between Central Science Laboratory (CSL) in York and Institute of Chemical Technology (ICT) in Prague there was optimized the method for Ochratoxin A

detection by the lateral flow device. The work started at CSL in August 2004 and will be completed in July 2005 with validation of the new method.

Ochratoxin A (OTA) is a mycotoxin naturally produced by several species of *Aspergillus* and *Penicillium*. Cereals and cereal products are the main contributors of OTA intake. EU legislation relating to OTA has recently been introduced and for cereals limits of 3 ppb for processed and 5 ppb for raw cereals have been set.

Lateral flow devices (LFD's) or immunochromatographic assays is one step rapid systems that takes a few minutes to determinate a wide variety of analytes such as mycotoxins. The competitive LFD relies upon the competition for binding sites on sensitised latex particles. An OTA specific monoclonal antibody was bound by passive absorption to dyed latex particles. An additional rabbit serum sensitised latex particle was produced as an internal control bead. Both sensitised latex particle were then applied, using an immersion procedure, onto a release pad, to produce a stable particle reservoir for release onto a nitrocellulose-based membrane. Two lines of reagents were immobilised onto the membrane using a sophisticated reagent dispenser. The target reference or test line was comprised of the OTA-BSA conjugate. The control line was a line of goat anti-rabbit antibody. The release pad and membrane were assembled, together with an absorbent pad, into a plastic housing[2], see Fig. 1.

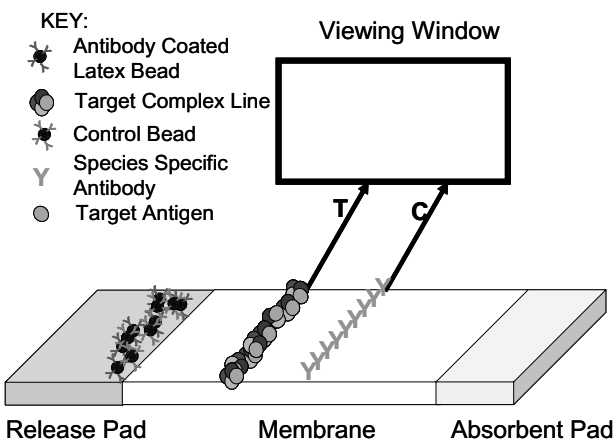

Fig. 1. **Schematic diagram of the LFD**

When a sample is added to the device the latex particles are released from the release pad, which then begin to flow across the membrane. If OTA is present in the sample extract, antibody binding will occur to produce a latex-antigen complex. Any sensitised latex particles that fail to bind to an antigen will attach to the immobilised OTA-BSA conjugate test line as they traverse the membrane, thus producing a visible line of deposited latex. The goat antirabbit antibody control line captures the internal control latex, by detection of the bound rabbit serum, to produce an internal control line. This is independent of OTA binding, and provides a visible confirmation of latex flow[2]. The visible target line indicates a negative result. The intensity of target line was measured with using portable reader, see Fig. 2., and then was found out OTA concentration in sample.

Fig. 2. **Reader for measure of color intensity**

Results and discussion

Statistical sampling methods are being investigated at CSL so that the LFD could be used for the identification of "hot spots" in grains sores. By using a simple extraction procedure and portable reader, multiple samples of 50–100 g can be analysed to determinate relatively low levels of OTA, and identify the occurrence of hot spots. The reader is used to quantify the risk associated with each sample, see Fig. 3. and Table I. An overall assessment is made based on the number of samples tested and their individual levels of risk.

Table I
Hot spot risk of OTA contamination in wheat grains

Hot spot risk	Approx. OTA	Reader value
Low	<12.5 ppb	>60 %
Medium	12.5–50 ppb	59–31 %
High	>100 ppb	<30 %

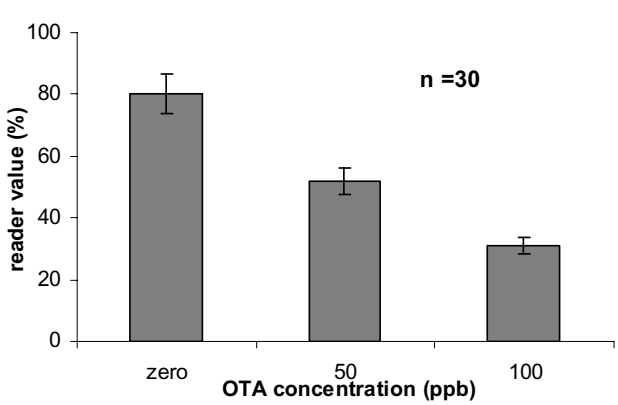

Fig. 3. **The individual levels of OTA contamination in wheat**

Conclusions

A rapid and very reliable method for identification and semi-quantification of OTA has been developed. The LFD takes only a few minutes to obtain a result. The components of the test are relatively inexpensive and the method requires little or no previous experience to successfully perform. The use of the LFD would reduce the number of tests required and possibly reduce the culturing times required. The cost and ease of the device allow testing to occur in simple laboratory environments, replacing the need from sophisticated analytical equipment. The LFD format could be used as part of OTA and other mycotoxins preventative measures, particularly in areas of the world which do not have, or can not afford, the infrastructure of analytical laboratories services.

REFERENCES

1. Magan M, Olsen M.: *Mycotoxins in food*, Woodhead, Cambridge 2004.
2. Danks C., Ostoja-Starzewska S., Flint J., Banks J. N.: Aspect of Applied Biology *68* (2003).