ACTA ALIMENTARIA

An International Journal of Food Science

Editor
J. HOLLÓ

Volume 30 **March 2001** **Number 1**

Akadémiai Kiadó
Budapest

ISSN 0139-3006
CODEN ACALDI

ACTA ALIMENTARIA

An International Journal of Food Science

Sponsored by the Joint Complex Committee on Food Science of the Hungarian Academy of Sciences and published by financial support of the Ministry of Agriculture and Regional Development.

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Acta Alimentaria is a quarterly journal in English, publishing original papers on food science. The main subjects covered are: physics, physical chemistry, chemistry, analysis, biology, microbiology, enzymology, engineering, instrumentation, automation and economics of foods and food production.

Distributor:

AKADÉMIAI KIADÓ
H-1519 Budapest, P.O. Box 245, Hungary

Fax: (36-1) 464-8221
E-mail: kiss.s@akkrt.hu
http://www.akkrt.hu

Publication programme, 2001: Volume 30 (4 issues)

Subscription price per volume: US$ 236.00, including normal postage, airmail delivery US$ 20.00.

Acta Alimentaria is published 4 times per annum: March, June, September and December

Acta Alimentaria is surveyed by Chemical Abstracts, Current Contents/Agriculture, Biology and Environmental Science, ASCA, BIOSIS, Nutrion Abstracts and Reviews.

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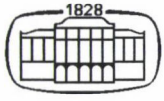
AN INTERNATIONAL JOURNAL OF FOOD SCIENCE

VOLUME 30
2001



AKADÉMIAI KIADÓ
BUDAPEST

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Editorial

NEW TRENDS FOR THE NEW MILLENIUM IN FOOD QUALITY AND SAFETY

Consumers desire better quality food and safe food. Food quality means different things to different people (energetic value, sensory appeal, healthiness). Good quality food contains a high proportion of a valued ingredient (quality refers to richness) and provides the necessary contribution to a nutritionally balanced diet (nutritional value and labelling). Consumers select food to satisfy the senses (appearance, taste, smell, texture and feel) and relate quality to the freshness of food (minimally processed). Functional food is aimed to maintain health, and to avoid diseases by enrichment food with vitamins and minerals (fortified foods, dietary supplements). Consumer education is needed to read the information on prepackaged food label to be familiar with food ingredients and nutrition facts. Food quality means conformity with existing new and harmonized regulation to the European Directives adapted in the international and national Food Books (e.g. Codex Alimentarius Hungaricus). The food control problems in Hungary are mostly in the field of food quality: whereby consumers may be misled regarding composition, labelling, sensory value, errors in contents and mistakes in packaging, whereas food safety regulation is more respected by manufacturers and retailers in the field of microbiology and toxicology.

One important aspect of food safety: measures are taken to control contamination of raw materials in the process of agricultural production and towards the whole food chain. Hygiene problems are now better approached by using HACCP and risk analysis in the production, processing, trade and household or in the gastronomy. Risk assessment is used to determine the probability of a chemical, physical or biological (microbiological) hazard. Safe and wholesome food is that which avoids contamination and prevents infection from the environment (health hazard and food-born diseases). Before the present century, international trading in food took place between buyers and sellers on the basis of contract (agreed prices and quality specification). Dishonest food traders, unregulated markets, unfair trade practices threatened the health of consumers (e.g. toxic colouring and flavouring agents). Consumers pressurised governments to protect them from poor quality food and food-borne health hazards. As a consequence of public pressure countries enacted food laws and established food import and export control agencies to protect consumers. Governments became directly involved in the food trade and this intervention created new problems, the international labyrinth of

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requirements (different national requirements for products of the same kind). There is now a need to standardize food standards regionally and internationally (harmonization). The role of government is to protect consumers adequately from illness or injury, provide assurance that food is suitable for normal human consumption, maintain confidence in internationally traded foods, provide health education programmes which effectively communicate the principles of food hygiene to industry and consumers.

Healthy nutrition is served with many functional foodstuffs, fortified foods and dietary supplements introduced to the food market. Public action is needed to promote and protect the health of the population using dietary guidelines and prevention measures for good nutrition. The Heart Healthy Nutrition project in Hungary supported by the Ministry of Public Health and the World Bank is one of the good examples. Public health action requires an integrated approach encompassing environmental, educational, economic, technical, and legislative measures, together with a health care system oriented to the early detection and management of obesity in children and adolescents, cardiovascular disease, cancer, and osteoporosis.

The role of the food industry is to provide food which is safe and suitable for consumption, to ensure that consumers have clear and easily comprehensible information (regarding hygienic food handling), and to maintain confidence in internationally traded foods. Growth in the international food trade beyond 2000 is based on the growing world population, on changes in the ethnic composition of national populations, on improvements in personal incomes, on advances in agricultural sciences, food technology and related scientific disciplines, supported by the broadening of food tastes, the methods of preservation and the continuing development of transport systems. An increasing range of food from an increasing number of countries will enter into international trade and science should face with new global requests and challenges.

P. A. BIACS

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FACTORS AFFECTING CELLULASE PRODUCTION BY *TRICHODERMA KONINGII*

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(Received: 22 November 1999; revision received: 5 June 2000; accepted: 29 August 2000)

Because of the need for renewable energy resources, cellulose, which can be enzymatically hydrolyzed to glucose, has drawn lot of attention during the past decade. However, the process of cellulose conversion using cellulase is not yet economically feasible because of the high cost of enzymes. Factors influencing the cellulase production of *Trichoderma koningii* using both acid and steam treated sugar cane bagasse and rice straw as carbon sources were investigated. The highest levels of cellulase activities were obtained using a culture medium containing urea and $(NH_4)_2SO_4$ together as nitrogen sources at 0.217% and 0.241% for both carbon sources. When the culture medium was supplemented either with 0.5% Tween 60 or Tween 80, the rate of cellulase production was increased considerably. Maximum levels of both filter paper and CMC-ase activities produced on both media were obtained at 25 °C and 100 r.p.m., while the highest level of β-glucosidase production was obtained at 30 °C and 200 r.p.m.

Keywords: cellulase production, sugar cane bagasse, rice straw, pretreatment

There has been a continuously increasing interest towards the saccharification of cellulosic waste materials by enzymes in recent years (ENAYATI & PARULEKAR, 1995). The enzymatic conversion of native cellulose is catalyzed by the synergistic action of enzymes including endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). These enzymes are synthesized by a large variety of microorganisms including fungi and bacteria as well. However, fungal species have been more intensively studied because they produce extracellular cellulases.

Due to the low specific activity of cellulases, a large quantity of enzyme per unit amount of substrate is required to obtain high conversion yield of cellulose. An important obstacle in the exploitation of lignocellulosics is the rather costly production of cellulases contributing as much as 50% of the overall cost of saccharification process (MANDELS, 1985). Furthermore, microbial synthesis of cellulase enzymes is subjected to catabolite repression, and it has to be induced by cellulose. In the present study, the production of cellulolytic enzyme system of *T. koningii* using two different carbon

sources, i.e. acid and steam treated sugar cane bagasse and rice straw was examined. Various factors, such as quality and concentration of nitrogen source; addition of surfactants; incubation temperature as well as agitation speed, were studied in order to optimize the cellulase production of *T. koningii*.

1. Materials and methods

1.1. Microorganism

Freeze-dried spore preparation of *Trichoderma koningii* 2691 was obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM), Budapest, Hungary. The fungal stock culture was maintained on PDA slants medium at 4 °C.

1.2. Enzyme production

The growth and production medium (TÜRKER & MAVITUNA, 1987) contained 7 g l^{-1} $(NH_4)_2SO_4$, 10 g l^{-1} KH_2PO_4, 1.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1.5 g l^{-1} $CaCl_2 \cdot 2H_2O$, 1.5 g l^{-1} urea, 1 g l^{-1} glucose. The medium was supplemented with trace elements: 25 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 10.3 mg l^{-1} $MnSO_4 \cdot 4H_2O$, 7 mg l^{-1} $ZnSO_4 \cdot 7H_2O$ and 18.3 mg l^{-1} $CoCl_2 \cdot 6H_2O$. Steam pretreatment of both sugar cane bagasse and rice straw was carried out according to CARRASCO (1994) at 120 °C for 100 min in the presence of 1% sulfuric acid. Pretreated sugar cane bagasse and rice straw were used as carbon sources. The production medium contained 1.5% of carbon source. A 3 ml spore suspension obtained from 7-d PDA slant culture was used to initiate growth in a shake flask containing 50 ml of sterile culture medium at pH 5.0. Cultures were incubated on a rotary shaker at 100 r.p.m. and 30 °C. The fermentation broth was harvested then filtered and analyzed for various enzyme activities.

1.3. Assays

Filter paper activity (FPA) was measured according to MANDELS and co-workers (1976) procedure. The reaction mixture containing 0.5 ml of 0.05M acetate buffer (pH 4.8) and 0.5 ml of culture filtrate was incubated together with a 1×3 cm (25 mg) strip of Whatman No. 1 filter paper at 50 °C for 60 min. The enzymatic reaction was terminated by addition of 1 ml DNS reagent (MILLER, 1959). After 5 min of boiling and addition of 10 ml distilled water the absorbance was measured at 540 nm.

Carboxyl-methyl-cellulose degrading capacity (CMC-ase) was determined by incubating 0.5 ml of enzyme sample together with 0.5 ml of 1% carboxy-methyl-cellulose in 0.05 mol l^{-1} acetate buffer (pH 4.8) at 50 °C for 30 min. The hydrolysis was stopped by addition of 1 ml DNS reagent. After boiling for 5 min and dilution with 10 ml of distilled water the absorbance was read at 540 nm.

For β-glucosidase activity determination 10 µl of culture filtrate was incubated together with 1 ml of 0.67 mmol l^{-1} (0.02%) p-nitrophenyl-β-D-glucopyranoside in 0.05 mol l^{-1} acetate buffer (pH 4.8) ad 50 °C for 10 min. The enzymatic reaction was terminated by addition of 3 ml 0.1 mol l^{-1} NaOH solution. The absorbance was measured at 400 nm (RÉCZEY et. al., 1990).

2. Results and discussion

2.1. The effect of nitrogen source

The effect of alternative nitrogen sources on the production of cellulolytic enzymes using two different media was investigated. Ammonium sulfate and urea of basal medium were replaced with urea or ammonium sulfate separately or with other organic and inorganic nitrogen sources is such amounts that the final nitrogen concentration in the media remained unchanged. The results are summarized in Fig. 1. It can be seen that the highest enzyme activities were obtained when the original culture medium was used in which both urea and $(NH_4)_2SO_4$ were added. With all other nitrogen sources considerably lower activities were obtained.

These findings were in good agreement with the results obtained by MANDELS and WEBER (1969) and TÜRKER and MAVITUNA (1987). It was also observed that using urea or $(NH_4)_2SO_4$ separately resulted in much lower cellulase production, which could be due to pH changes during the cultivation. The final pH of the culture broth was more acidic when ammonium containing compounds were added to the medium compared to urea or other organic nitrogen sources. This observation was in good agreement with HAAPALA and co-workers (1994, 1996). They showed that the deamination of urea resulted in increasing pH in the culture medium.

2.2. The effect of nitrogen source concentration

The effect of various concentrations of urea and ammonium sulfate as nitrogen sources on the cellulase production was also investigated. The results are plotted in Fig. 2.

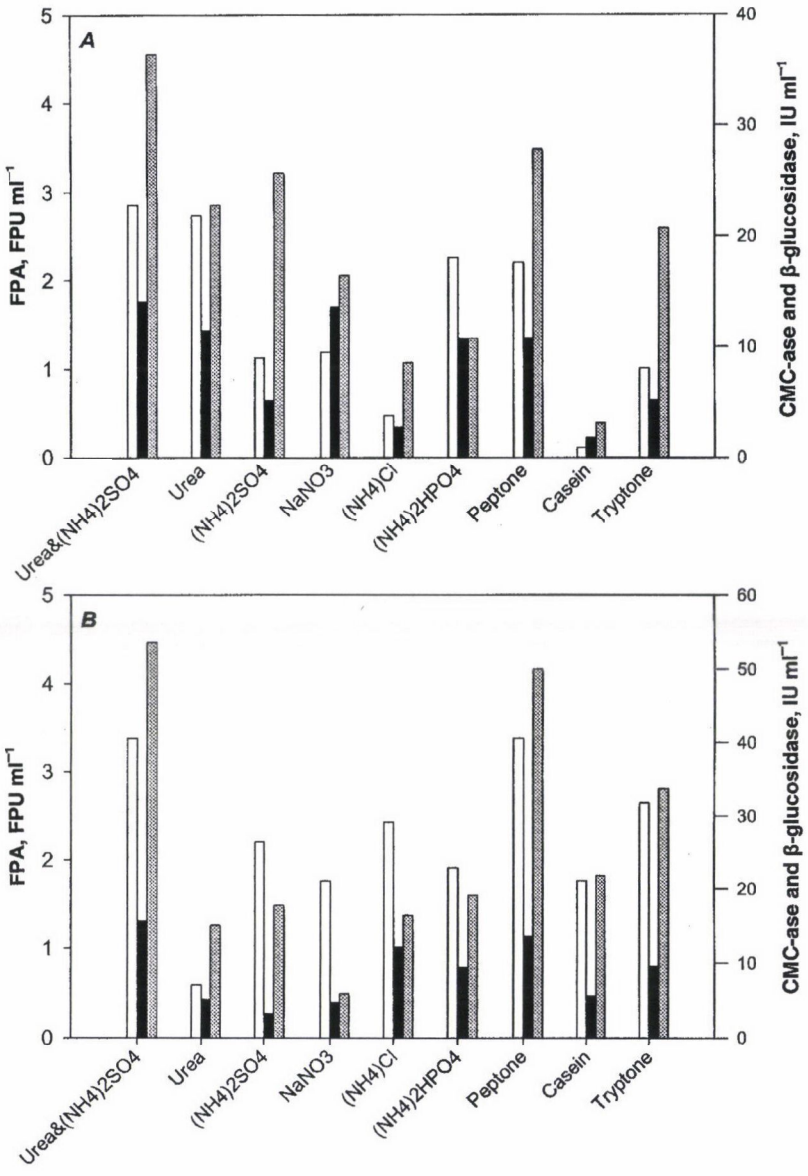

Fig. 1. The effect of nitrogen source on the cellulase production of *T. koningii* using various carbon sources. A: pretreated sugar cane bagasse, B: pretreated rice straw. Filter paper activity (FPA): white bars, CMC-ase activity:black bars, β-glucosidase activity: grey bars

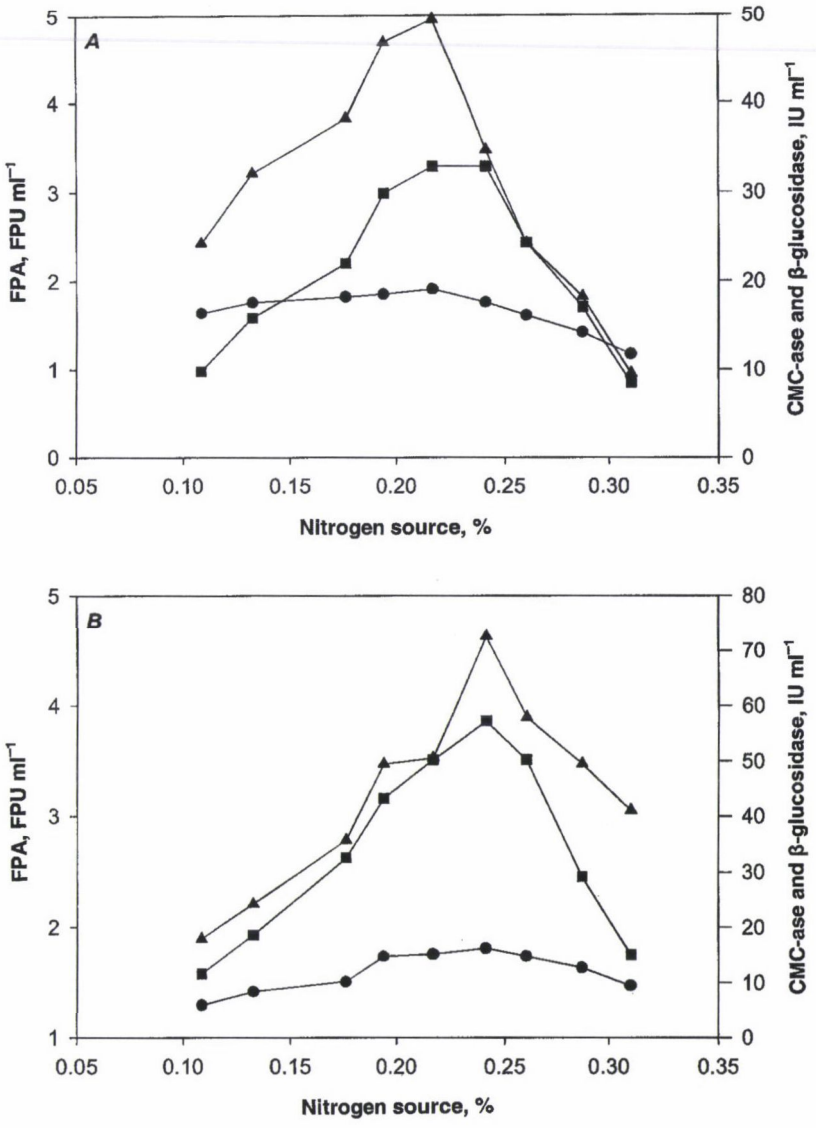

Fig. 2. The effect of nitrogen source concentration on the cellulase production of *T. koningii* using various carbon sources. A: pretreated sugar cane bagasse, B: pretreated rice straw. Filter paper activity (FPA): ■, β-glucosidase activity: ▲, CMC-ase activity: ●

It can be seen that 0.217% nitrogen from urea and $(NH_4)_2SO_4$ containing medium was optimal for cellulase production by *T. koningii*. However, maximum CMC-ase and β-glucosidase activities were obtained at a lower nitrogen source concentration (see Fig. 2). At 0.194% nitrogen concentration, the CMC-ase and β-glucosidase activities reached 96% of the activities obtained on the control medium (0.217% nitrogen source). It is clear from Fig. 2/A that applying the original amount of ammonium sulfate (7 g l^{-1}), while increasing the urea from 1.5 g l^{-1} to 2.0 g l^{-1} (which increase the total nitrogen source concentration from 0.217% to 0.241%) caused the increase in the enzyme production of *T. koningii* by about 11.18% in the case of FPA. The increase in the CMC-ase due to increasing the amount of urea was about 7.2%, while significantly higher, 42.10%, β-glucosidase activity was obtained.

These results were in line with WEBB and co-workers (1986) and KHALAF ALLAH and co-workers (1993).

2.3. The effect of surfactants

The effect of Tween 40, 60, 80 on the cellulase production of *T. koningii* was studied at two different concentrations i.e. 0.1 and 0.5% surfactant in the culture medium. The results are shown in Fig. 3. The highest enzyme production rates were obtained when 0.5% Tween 60 was applied in the medium.

The rate of enzyme production of *T. koningii* using acid-steam treated sugar cane bagasse was increased about 1.3 times compared to the control medium, in which there was no surfactant added. However, using acid-steam treated rice straw as the carbon source 0.5% Tween 80 added to the medium was optimal for cellulase production (see Fig. 3/B).

Cellulase production was 3.43%, 2.49% and 13.24% higher for FPA, CMC-ase and β-glucosidase compared to the control medium. The final pH of the medium was slightly affected by Tween 80. The effect of Tween 80 on the cellulase production by various fungi was observed by HUNG and co-workers (1988). LONG and KNAPP (1991), and STUTZENBERGER (1987) reported the mechanism of enhancement in the enzyme secretion by Tween. Surfactants can increase the permeability of the cell membrane, facilitating more rapid secretion of extracellular enzymes, which in turn leads to greater enzyme synthesis.

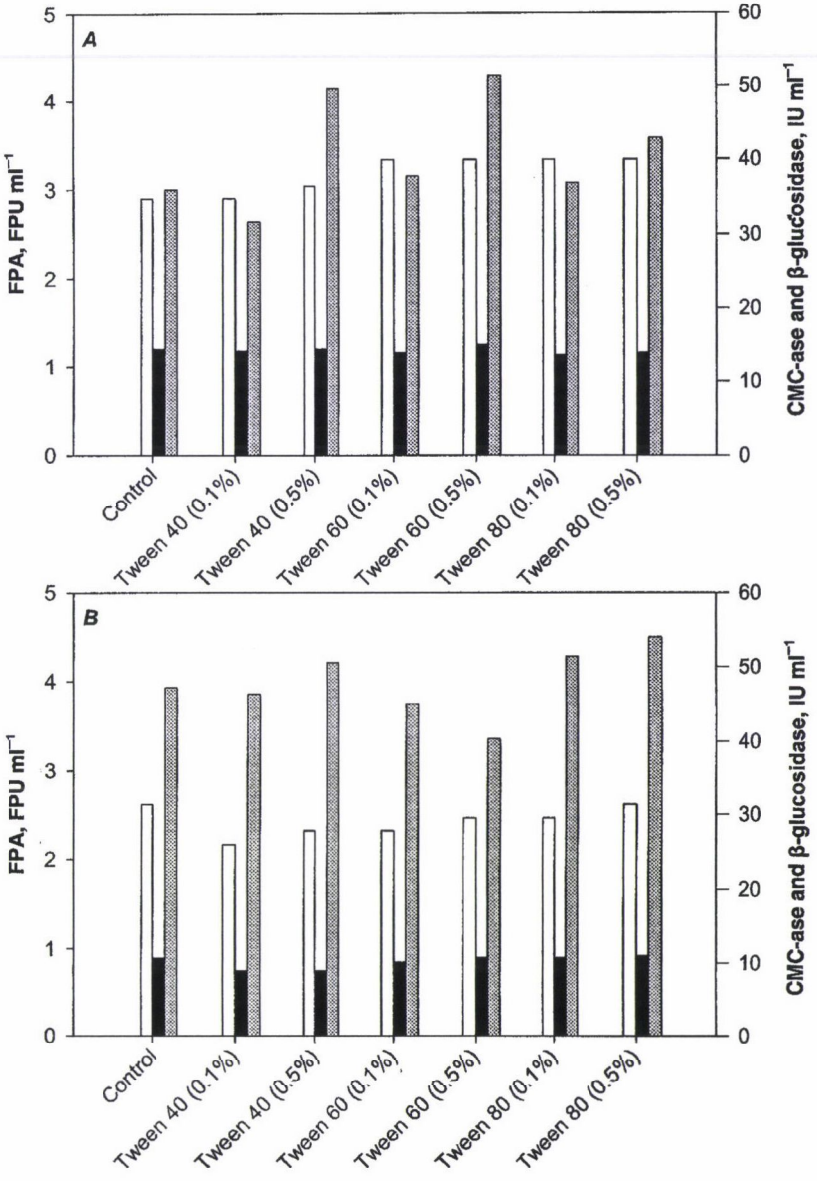

Fig. 3. The effect of surfactants on the cellulase production of *T. koningii* using various carbon sources. A: pretreated sugar cane bagasse, B: pretreated rice straw. Filter paper activity (FPA): white bars, CMC-ase activity: black bars, β-glucosidase activity: grey bars

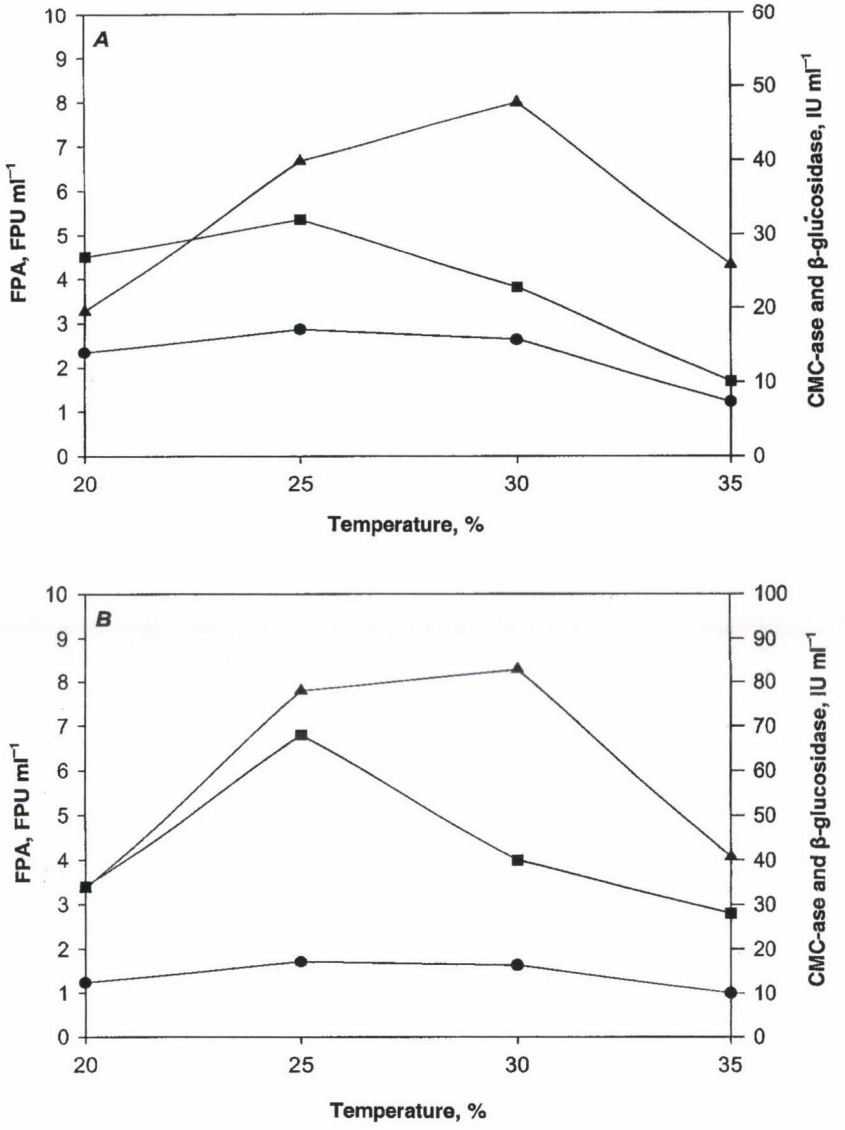

Fig. 4. The effect of incubation temperature on the cellulase production of *T. koningii* using various carbon sources. A: pretreated sugar cane bagasse, B: pretreated rice straw. Filter paper activity (FPA): ■, β-glucosidase activity: ▲, CMC-ase activity: ●

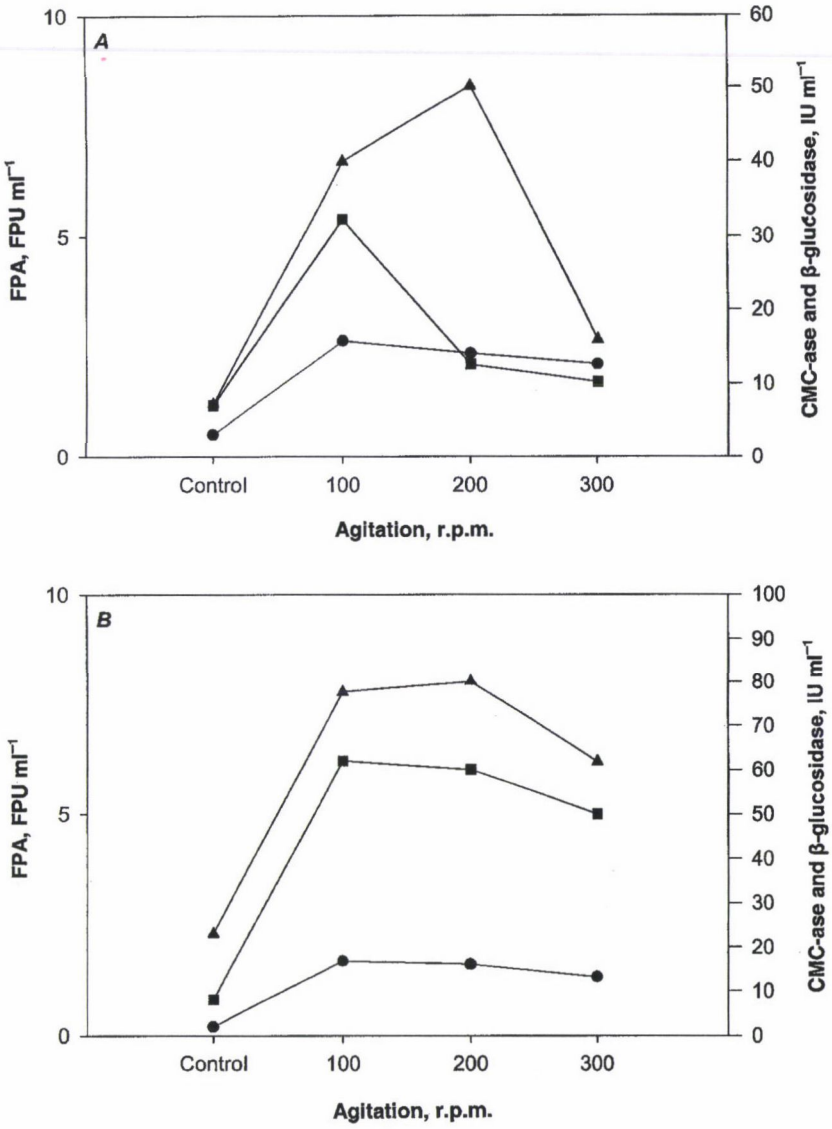

Fig. 5. The effect of agitation on the cellulase production of *T. koningii* using various carbon sources. A: pretreated sugar cane bagasse, B: pretreated rice straw. Filter paper activity (FPA): ■, β-glucosidase activity: ▲, CMC-ase activity: ●

2.4. The effect of incubation temperature

To account for the effect of incubation temperature on the cellulase production of *T. koningii* using two different carbon sources, cultivation was run at 20, 25, 30 and 35 °C. The results are shown in Fig. 4. It can be seen that cultivation temperature of 25 °C was optimal for cellulase production (FPA, and CMC-ase), while 30 °C was favorable for β-glucosidase production for both acid-steam treated bagasse and rice straw.

Higher temperature resulted in progressive decline in cellulase production. The enzyme activities obtained at 35 °C were about 50–60% lower than that of obtained at 25 °C using both acid-steam treated bagasse and rice straw as carbon source. These results were in agreement with those obtained by KNAPP and LEGG (1986), DOPPELBAUER and co-workers (1987), BASTAWDE (1992) and CHAUDHURI and SAHAI (1993).

2.5. The effect of agitation

The effect of agitation on the cellulase production using two different carbon sources was investigated. The results showed that the enzyme production could be increased with increasing agitation (Fig. 5), however increasing the agitation speed above 100 r.p.m. resulted in a decreased cellulase enzyme production. Although, the FPA and CMC-ase activities were decreased when the agitation speed was increased from 100 to 200 r.p.m, the β-glucosidase production was increased considerably.

The high concentration of carbon source resulted in increased viscosity of the medium that may affect the availability of oxygen needed for β-glucosidase production. These results were in agreement with those reported by MAGNELLI and co-workers (1996), BUSWELL and CHANG (1994), SILVA and co-workers (1995) and RÉCZEY and co-workers (1996).

3. Conclusions

Acid-steam pretreatment of both sugar cane bagasse and rice straw proved to be an effective method to increase accessibility for cellulase production with *T. koningii* 2691. Urea and other organic nitrogen compounds were better N-sources than inorganic ammonium containing salts. Supplementing the culture medium with surfactants, such as Tween, increased the cellulase production considerably. The effect of incubation temperature and agitation was also investigated. The results obtained were similar to those of reported earlier using other *Trichoderma* strains. It can be concluded that *T. koningii* is an effective cellulase producing microorganism showing good yields of enzymes on both pretreated bagasse and rice straw.

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YEASTS ISOLATED FROM DIFFERENT EUROPEAN DRY-SAUSAGES IN THE COURSE OF RIPENING

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(Received: 10 January 2000; accepted: 18 September 2000)

The evolution in counts of yeasts from six lots of different dry-sausages produced in three European Union (EU) countries (France, Italy and Spain) is tested. Each lot was analyzed in three phases of their production: fresh product, first staged drying and final product. Three samples were analyzed in each test. Counts of yeasts during the ripening process showed an irregular evolution. Regarding the species isolated, the most frequent ones belonged to genera *Candida* (*C. albicans, C. famata, C. guillermondii, C. krusei, C. parapsilosis, C. stellatoidea, C. zeylanoides.*), *Trichosporon* (*T. capitatum, T. beigelli*), *Rhodotorula* and *Geotrichum* (*G. candidum*).

Yeasts are microorganisms widely distributed in the natural media (ground, air, water and plants). Also, they are part of the normal biota of milk (sometimes counts of 10^4 cfu g^{-1} can be found) and some species can grow on cheeses reaching 10^8–10^9 cfu g^{-1} counts (BOISSONNET et al., 1994).

Occurrence of yeasts in fermented meats has been scarcely studied in spite of being one of the microbial groups that, together with lactic acid bacteria (LAB), micrococci and moulds, participate in the fermentation processes (BACUS, 1986; LÜCKE, 1988). Yeasts are indispensable to complete the normal ripening process (LEISTNER, 1992), because of their important lipolytic and proteolytic capacity that leads to the development of the flavour (FOURNAUD, 1976). Furthermore, yeasts have a high O_2 consumption which suppose a redox potential decrease. This contribute to a rapid

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reddening of dry-sausages. Also, yeasts produce catalase, inhibiting the rancidity process (LEISTNER, 1995). Because of this reason organisms such *Debaryomyces hansenii, Debaryomyces klockeri* and *Candida famata* are included in sausages as starters (HUGAS et al., 1992; ROCABAYERA, 1992). Generally, the growth of this fungal biota occurs during the first three weeks, with counts of 10^3–10^4 cfu g^{-1} (LARPENT-GOURGAUD et al., 1993).

The enzymatic activity of yeasts contribute to the production of aromatic compounds that are desirable in dry-sausages. These compounds are by-products of the lipid and protein degradation. However, an error in controlling the growth of these microorganisms can lead to the development of undesirable flavours (METAXOPOULUS et al., 1996).

In the raw meat both molds and yeasts are always present with low numbers but they can compete with bacteria if the meat surface is dried out (DILLON & BOARD, 1991). In the same way, counts of yeasts in meat can increase and, eventually become the predominant organisms during the storage period under refrigerated conditions. Yeasts can be found both in the meat surface as in the interior, although their main location is the exterior of the product (JESSEN, 1995) due to the high oxygen requirement of these microorganisms. However, at the beginning of the ripening period they can be inside the product (LEISTNER, 1992).

In processed meats (as dry-sausages) the yeasts most frequently isolated belong to the genera *Debaryomyces, Trichosporon* and *Candida* (VILJOEN et al., 1993). Regarding to the first, species such *D. hansenii*, capable of growing under conditions of 24% NaCl and a_w 0.65 are included (JAY, 1996). *D. hansenii* have no activity on nitrates and it is aerobic. The activity of this organism in dry-sausages depends in a great measure on the presence or absence of other starter cultures. When directly inoculating to the meat 10^6–10^7 cfu g^{-1}, the activity of yeasts is preferably observed at the surface of the sausage. This activity expedites the oxygen consume and it has repercussion on the coloration of external surface. Also, the appearance of a characteristic flavour that is especially desirable in Italian dry-sausages is favored (CORETTI, 1977). Other expected advantage is the catalase production which can delay the appearance of rancidity (LEISTNER, 1995).

According to GEHLEN and co-workers (1991) when yeasts are used as starters in combination with micrococci and lactic acid bacteria, better results are obtained. The yeasts effect depends on a great extent on the occurrence or absence of other starters. Only yeasts lead to an increase in the ammonia content and a pH decrease (JENSSEN, 1995). On the other hand, it is necessary to add microorganisms with a strong nitrate-reducing activity, because of the inhibitory effect of yeasts on staphylococci which occur in meat in a natural way (GEHLEN et al., 1991).

The aim of this paper is to study the evolution of the counts of yeasts and to identify which species participate in the ripening process of six types of dry-sausages produced in three countries belonging to the European Union (EU): Italy, France and Spain.

1. Material and methods

1.1. Sampling

Fifty-four samples from six lots (Table 1) corresponding to six types of dry-sausages produced by manufacturers from three countries belonging to EU (Italy, France and Spain) have been tested. Each lot was analyzed in three phases of their production: at process start, at the completion of the first drying stage and as end product. Three samples were analyzed in each test. A number of colonies equivalent to the square root of the total counted were isolated to be identified.

1.2. Yeasts count and identification

The 10±1 g sample was homogenized with 90 ml of 0.1% bacto-peptone (Difco) in buffered sterile water (pH 7.2). Further dilutions were made as required. The yeast count was verified on acidified Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI) at 20 to 25 °C for 5 to 7 days, according to the Compendium of Methods for the Microbiological Examination of Foods (APHA, 1976). The results were expressed as log colony forming units (cfu) per gram of sample.

Table 1

Description of the types of the dry-sausages analyzed (n=162)

Types	A (n=27)[a]	B (n=27)[a]	C (n=27)[a]	D (n=27)[a]	E (n=27)[a]	F (n=27)[a]
External diameter (mm)	55–60	90	55–60	100	50–55	80
Casing	natural	artificial[b]	natural	natural	natural	artificial[b]
Grinding size (mm)	8	3.5	7	7	5	5
Duration of ripening[c] (days)	15	60	30	60	15	30

[a] 3 lots x 3 samplings x 3 replicates

[b] Collagen

[c] Different commercial starters were added

For identification, the yeasts-type microorganisms were primarily classified following simple microbial criteria, such as macro- and microscopic aspects, the formation of germinal tube and the production of chlamidospores (CASAL & LINARES, 1981). Further, the identification follows biochemical criteria such sugars assimilation, by using the API 20C Auxonogram System (La Balme Les Grottes-38390 Montalieu-Vercieu, France) or by urease test (BARNETT et al., 1990). Also, we used the chromogenic medium CHROMagar Candida (ODDS & BERNAERTS, 1994) which permits the differentiation between several species of yeasts of great importance in clinical microbiology. This medium is based on the contrast of colors of the colonies developed due to reactions between the species-specific enzyme and a substratum with chromogenic properties (LINARES et al., 1996).

2. Results and discussion

The counts of yeasts during the ripening process are summarized in Table 2.

A regular evolution in these counts during the period studied is not observed. Thus, the lowest numbers were found in the second and third test, for lots A and B respectively. In the cases of lots C and D, the extreme numbers were reached in fresh products. For lots E and F, this fungal biota increased during the ripening process, and the highest numbers were found in the final product.

Table 2

Counts[a] of yeasts in the course of ripening of three European dry-sausages

	Lot A (n=9)		Lot B (n=9)		Lot C (n=9)	
	Mean	Rank	Mean	Rank	Mean	Rank
Process start	3.81	3.68–3.89	2.36	2.32–2.40	3.94	3.48–4.20
First stage drying	4.74	4.57–4.85	2.29	1.00–2.64	3.77	3.61–3.88
End products	3.78	3.49–4.04	3.47	2.75–3.74	3.47	2.76–3.71

	Lot D (n=9)		Lot E (n=9)		Lot F (n=9)	
	Mean	Rank	Mean	Rank	Mean	Rank
Process start	4.00	3.60–4.28	4.16	4.00–4.30	3.89	3.60–4.04
First stage drying	2.00	1.00–2.93	5.20	4.96–5.30	5.14	4.72–5.31
End products	2.55	2.32–2.85	5.33	5.03–5.51	5.49	4.74–5.85

[a] Expressed as log cfu g^{-1}

According to LARPENT-GOURGAUD and co-workers (1993) and BOISSONET and co-workers (1994), in sausages yeasts multiply during the ripening process (three weeks) up to counts of 10^3–10^4 cfu g^{-1}, what we have found in most of the samples studied. Also, SAMELIS and co-workers (1993; 1994) suggest that yeasts, which exceed 10^5 cfu g^{-1} during late ripening, may contribute in a decisive way to the good flavor and aroma of certain batches of sausages. In our work, this counts only occur in two lots (E and F).

In a work dealing with Italian dry-sausages, a similar variability in yeasts counts to that found in our work was reported, and the maximum was log 5.6 cfu g^{-1} (SSICA, 1992).

JAY and MARGITIC (1981) reported counts of yeasts similarly high in raw meat (log 7 cfu g^{-1}). SAMELIS and co-workers (1993; 1994) consider the yeasts counts higher than log 5 cfu g^{-1} as contributing in a decisive way to the characteristic flavour of this type of products.

Most of the consulted references (SMITH & PALUMBO, 1973; PALUMBO et al., 1976; DOMÍNGUEZ et al., 1989; SAMELIS et al., 1994 and DAOUDI et al., 1995) reported that yeasts are always present in high numbers (>log 5 cfu g^{-1}) in these products. Regarding to this aspect, MCCARTHY and DAMAGLOU (1996) think that high yeasts counts in dry-sausages can be a hazard, above all if sulfites are used as additive. These compounds inhibit the growth of most of the bacteria, except for Gram-positive ones and yeasts, becoming the last as dominant biota.

In spite of the important role of yeasts in this type of dry-sausages biota, neither of the legislation of the countries involved (Italy, France and Spain) include it as microbial criterion.

The species isolated belong to four genera: *Candida, Trichosporon, Rhodotorula* and *Geotrichum*. The greater incidence corresponds to the genus *Candida* from which a higher number of species was isolated. From lots A and B, *C. albicans, C. famata, C. guillermondii, C. krusei, C. parapsilosis* and *C. zeylanoides* were isolated; *Trichosporon capitatum* and *Rhodotorula* were detected in a low percentage. From lots C and D, besides of some *Candida* spp. (*C. famata, C. parapsilosis, C. stellatoidea* and *C. zeylanoides*), *Trichosporon beigelli* and *Geotrichum candidum* were also identified in a high percentage. In the samples correspondent to lots E and F, the predominant genera were *Candida* (*C. famata, C. guillermondii* and *C. krusei*) and *Rhodotorula*. *T. beigelli* was also identified (Table 3).

Table 3

Classification and distribution[a] of yeasts isolated from six lots of sausages

	Lot					
	A	B	C	D	E	F
Candida albicans	0	18	0	0	0	0
Candida famata	26	27	40	34	25	25
Candida guillermondii	17	9	0	0	0	25
Candida krusei	33	0	0	0	25	25
Candida parapsilosis	0	9	20	0	0	0
Candida stellatoidea	0	0	20	0	0	0
Candida zeylanoides	8	27	20	0	0	0
Trichosporon beigelli	8	0	0	33	25	0
Trichosporon capitatum	0	9	0	0	0	0
Rhodotorula rubra	0	0	0	0	0	25
Rhodotorula sp.	8	0	0	0	25	0
Geotrichum candidum	0	0	0	33	0	0

[a] Expressed as percentage (%)

On opposite to some authors (VILJOEN et al., 1993; METAXOPOULOS et al., 1996) we have not isolated species of the genus *Debaryomyces* in the teleomorphic form, which is abundant in this type of products. On the other hand, *C. famata*, considered an anamorphic species of *D. hansenii* (BARNETT et al., 1990) has been detected. LARPENT-GOURGAUD and co-workers (1993), on different types of french sausages, isolated yeasts belonging to the genera *Pichia* and *Candida*, but they did not detect (like us) *Debaryomyces*. These authors report *D. hansenii* as the most frequent flavouring yeast, although they consider that in sausages that role can be done by other species. Also, other authors reported *C. famata* as one of the most frequently isolated yeasts in German (SMITH & HADLOK, 1976), Italian (GRAZIA et al., 1989) and Greek (METAXOPOULOS et al., 1996) sausages. BOISSONNET and co-workers (1994) studied two types of sausages, one handmade and the other one of semi-industrial production. They noted that *C. famata* was absent in the last type of production, while in the handmade one the percentages were similar to those found in our work.

Other species belonging to *Candida* are also frequently associated with processed meats. Thus, *C. zeylanoides* has been isolated from pork sausages (DALTON et al., 1984; VILJOEN et al., 1993), ham (DILLON, 1988), dry-sausages (LARPENT-GOURGAUD et al., 1993; BOISSONNET et al., 1994) and salami (METAXOPOULOS et al., 1996). *C. guillermondii* and *C. parapsilosis* have been isolated from fermented meats (LEISTNER & BEM, 1970; COMI & CANTONI, 1980) and *C. krusei* from salami (METAXOPOULOS et al., 1996).

C. zeylanoides and *C. famata* have a high lipolytic activity. Although *C. zeylanoides* can produce disagreeable flavors, it can be sometimes interesting because of its greater and faster lipolytic activity. Both species are of interest in the development of aroma in sausages (BOISSONNET et al., 1994).

According to JAY (1996), the most ubiquitous genera of yeasts in raw meat belong to *Candida* and *Rhodotorula*, as well as *Debaryomyces, Saccharomyces* and *Trichosporon* in cured meats. In the present work we have isolated two species belonging to this last genus (*Trichosporon beigelli* and *Trichosporon capitatum*), although in low percentages. In a paper dealing with the proteolytic activity of different species and strains of yeasts isolated from cheeses, SCHMIDT and co-workers (1993) found a great heterogeneousness as far as proteolytic activity is concerned. On this way, *T. beigelii* has an important proteolytic activity but a lower lipolytic activity. On the other hand, DENNIS & BUHAGIAR (1980) consider yeasts as the main responsible for lipolytic activity in foods, but only a few of them show proteolytic activity, among them *T. beigelii* (EKLUND et al., 1965).

Regarding to genus *Rhodotorula* LARPENT-GOURGAUD and co-workers, (1993) among other authors have also detected it in French dry-sausages, in a low incidence.

Some authors (MEISEL et al., 1989; GEHLEN et al., 1991) reported that selected strains of *D. hansenii* have an inhibitory effect on *Staphylococcus aureus*. This effect was attributed to a decrease in the O_2 level due to yeasts, and enhanced by lactic acid bacteria and micrococci as starter cultures.

3. Conclusion

It is necessary to emphasize that the isolated species belonging to the genera *Candida, Trichosporon, Rhodotorula* and *Geotrichum*, are widely distributed in the environment and they can behave as opportunist microorganisms. From this point of view, they could origin human infections of different location and importance, generally associated with patients with reduced immune system, like the administration of antitumoral and inmunosuppressors medicaments, radiation treatments, diagnostic operations and aggressive therapies (catheters, probes...), parenteral feeding, organs transplanting, AIDS infection and drug addiction (CRISSEY & LANG, 1995).

*

The work reported here was carried out in the course of the DRIP project. This project is partially funded by the FAIR Programme of the Commission of the European Communities (project number 96-1220). This paper represents the authors' point of view and does not necessarily reflect that of the DRIP Consortium.

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Acta Alimentaria, Vol. 30 (1), pp. 25–35 (2001)

DIRECT EFFECT OF BILE ON COLONIC MUCOSA IN ALIMENTARY INDUCED HYPERLIPIDEMY IN RATS

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(Received: 7 February 2000; accepted: 18 September 2000)

An experimental surgical model was developed in rats after a short term alimentary induced hyperlipidemy to study the direct effect of bile on the colonic mucosa, with regard to the cancerogenic properties of lipid rich diet. The purpose of this study was to light on the role of fatty acid alteration and lipid peroxidation processes of bile in the epithelial cell damage. Animals were fed with normal (group A) and fat rich diet (group B) for 10 days and then bile samples were collected by the cannulation of the common bile duct in deep anaesthesia. The circulation preserved colons of control rats were treated either with bile from the control or hyperlipidemic rats. The treatment was carried out for 30 minutes.

The electronmicroscopic alterations of epithelial cells (both enterocytes and goblet cells) caused by bile from hyperlipidemic rats were significantly greater than that of controls.

Unfavourable changes of the redox state of the colonic mucosa were also detected both in the hyperlipidemic and bile treated groups. A significant increase was observed in the free-SH concentration of the two bile treated groups against the untreated animals. The changes could be explained among others by the modified bile fatty acid composition. The present study supports that the alimentary modified bile can influence the structure of the epithelium of colonic mucosa and it can be one of the inducing factor of carcinogenesis.

Keywords: hyperlipidemy, colonic mucosa, bile, electronmicroscopy, redox state

Colon cancer is the most common gastrointestinal neoplastic disease in the Western world. It is generally agreed that the composition of diet plays a fundamental role in the induction of this type of cancer (POTTER et al., 1993). Diet high in animal fat and low in fiber has a high incidence of colorectal cancer. High fiber diet may decrease

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0139-3006/2001/$ 5.00

the colonic transit time and thus can increase the bulk of stool, may dilute the carcinogens and decrease the contact time of the carcinogens. Diet can affect colonic carcinogenesis by increasing cell proliferation and it can alter the genotoxic effect of carcinogens or it can modify the growth and progression of cells initiated by carcinogens. Persons on high fat diet excrete three times as much bile acids compared to vegetarians and their intestinal flora is changed, which alters the transformation of primary bile acids. From the primary bile acids excreted by hepatocytes secondary bile acids are formed in the intestine by bacterial modification of the nuclear hydroxyl group (SEYMOUR et al., 1989).

The secondary bile acids are believed to be the promoters of colorectal cancer (BAIJAL et al., 1998a, PEIFFER et al., 1997). The change in the composition of bile acids can induce damage to the mucosa, the innermost layer of the colon (MARTINEZ et al., 1998) and can influence the proliferation rate of colonic mucosa cells.

Lipid peroxidation products were detected both in human bile after cholecystectomy, and in chicken gallbladder bile as well as in model bile, verifying the importance of free radicals in gallstone diseases (ABDEL RAHMAN et al., 1995; BLÁZOVICS et al., 1996; EDER et al., 1996; SIPOS et al., 1997) and these products can alter the composition of bile, thus inducing further reactions in the intestine.

Bile is normally present in the colon and it may modulate the structure of the epithelium, the innermost cell layer of the colonic mucosa. It is not known yet how these effects occur in vivo. Therefore the purpose of this study was to examine the direct effects of bile on colonic mucosa using histological and biochemical investigations.

1. Materials and methods

1.1. Animal feeding study

All experimental procedures used conformed to the "Revised Guide for the Care and Use of Laboratory Animals" (1996), as well as specific national laws (1998). (Permission Number: TUKEB 59/1996 Semmelweis University).

Twenty male adult Wistar albino rats weighing 150–200 g were used after short term diet. The animals were randomly divided into 2 groups: group A: 10 animals were fed with normal chow (Charles River Hungary Kft.), being the control. Group B: 10 animals had lipogenic diet consisted of 2% cholesterol, 20% sunflower oil and 0.5% cholic acid added to the normal chow. All of the animals had water ad libitum during the whole period (BLÁZOVICS et al., 1996).

After 10 day feeding period they were anaesthetised with Nembuthal 50 mg/b.w.kg dose, for deep narcosis. Then the common bile duct was cannulated and approximately 3–4 ml bile/rat was collected from both group (A, B).

In two other groups (5–5 rats fed with normal chow) the ascending colon was banded in order to protect the ileal backflow and 0.5 ml pooled bile sample from the controls (group C) and from the hyperlipidemic rats (group D) was injected in the ascending and descending circulation preserved colon. After 30 min incubation with bile samples, the colon was removed and gut lumen was washed with isotonic KCl solution. Small pieces of the colon were cut and put in fixative solutions for electronmicroscopy.

Small pieces of the colon were fixed in 4% neutral buffered formalin, embedded in paraffin and 5 μm thin slices were cut and stained with haematoxilin-eosin.

1.2. Electronmicroscopy

For electronmicroscopical investigations the materials were fixed with a fixative solution containing 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer (pH 7.2). The samples were postfixed in 1% osmium tetroxide and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, then examined with Jeol 100 electromicroscope.

1.3. Biochemical measurements

The reducing power property was determined by the spectrophotometric method of OYAIZU (1986) at 700 nm. Free SH-group concentrations were determined by the SEDLAK and LINDSAY (1968) method based on ELLMANN (1959) reaction, with the detection of 2-nitro-5-mercapto-benzoic acid at 440 nm. The proton donating ability was measured in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (BLOIS, 1958 and HATANO et al., 1988). Hyperlipidemy was checked by serum parameters (alkaline phosphatase, aspartate and alanine aminotransferases, γ-glutamyl transferase, total cholesterol, triglycerides) (BLÁZOVICS et al., 1996). Protein contents of homogenates of colonic mucosa were measured by the method of LOWRY and co-workers (1951).

Fatty acid analysis of bile juice was carried out by gaschromatographic techniques. Lipid fraction was isolated with chloroform:methanol 2:1 mixture. Chloroform phase (1 cm^3) was analysed after dehydration and esterification. The lipids were saponified by 5% sodium hydroxide in 50% aqueous methanol and the fatty acids were esterised by 10% boron trifluorid methanol reagent. The fatty acids were extracted by hexane and purified by silica gel column chromatography according to ALEXANDER and co-workers (1985).

1.4. Gas chromatography

Hewlett-Packard 5890 gas chromatography with flame ionisation detector and split capillary injector was used for the analysis. Column: Carbowax 20M (25 m×0.2 mm, ID. 0.25 μm film thickness), injector: split, split ratio:1:50, temperature column oven 180 °C, injector 280 °C, detector 205 °C, carrier gas: nitrogen 0.3 $cm^3 min^{-1}$, detector gas: hydrogen 30 $cm^3 min^{-1}$, air 400 $cm^3 min^{-1}$. The peaks were identified by comparison with behaviour of known standards of SUPELCO FAME mixtures (SIGMA, St. Luis).

Stable radical 1,1-diphenyl-2-picrylhydrasyl (DPPH) was obtained form SIGMA (St. Luis), serum bovine albumin from CALBIOCHEM AG (Lucerne). All other reagents were purchased from REANAL (Budapest).

1.5. Statistical analysis

All values are presented as mean ± standard error of the arithmetic mean. The differences between the 2 groups were examined by Student's *t* probe. Differences were considered statistically significant at $P<0.05$.

2. Results

The epithelial lining of the colon was disrupted (Fig. 1), the quantity of the goblet cells significantly decreased and a large number of inflammatory cells appeared in the tunica mucosa of the colon treated with bile from animals fed with fat rich diet (group D) comparing to the control and rats fed with fat rich diet (group A, B). Electronmicroscopic investigations showed that the epithelial lining of the colonic mucosa changed, the mucopolysaccharide surface coat disappeared in the animals fed with fat rich diet and treated with bile (group B, C, D). Figure 2 shows the enterocytes with a large number of microvilli from the control colonic mucosa. The microvilli of the epithelial cells (both the enterocyte and goblet cells) were reduced in hyperlipidemic rats. A slight inflammatory reaction was also observed in the tunica propria. After bile treatment the cell membranes of them were disrupted, bile was observed in the cytoplasma establishing lysosomes (Fig. 3). The changes of the epithelial lining were more evident in the animals treated with bile from the fat rich diet group (D) (Fig. 4). The number of the goblet cells/100 μm was decreased (group B, C, D) significantly compared to the group A. The epithelial cells disappeared showing ulcer like abnormality in some parts of the colonic mucosa of the D group.

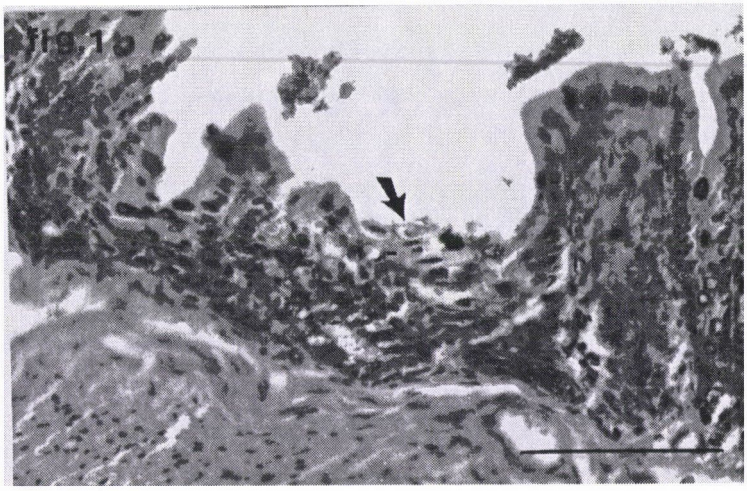

Fig. 1. Part of the tunica mucosa of bile treated rat after fat rich diet. Arrow points disruption of the epithelium infiltrated with inflammatory cells. Bar scale = 100 μm

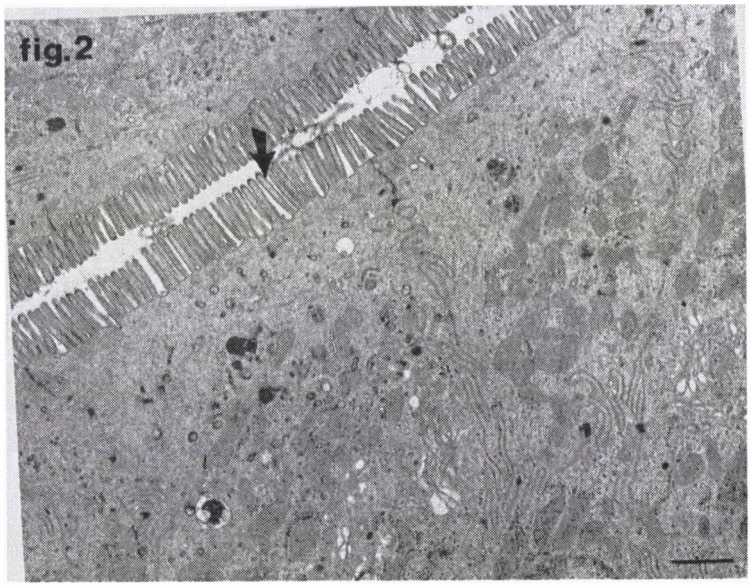

Fig. 2. A part of the enterocyte with a large number of microvilli (arrow) from the normolipidemic rat

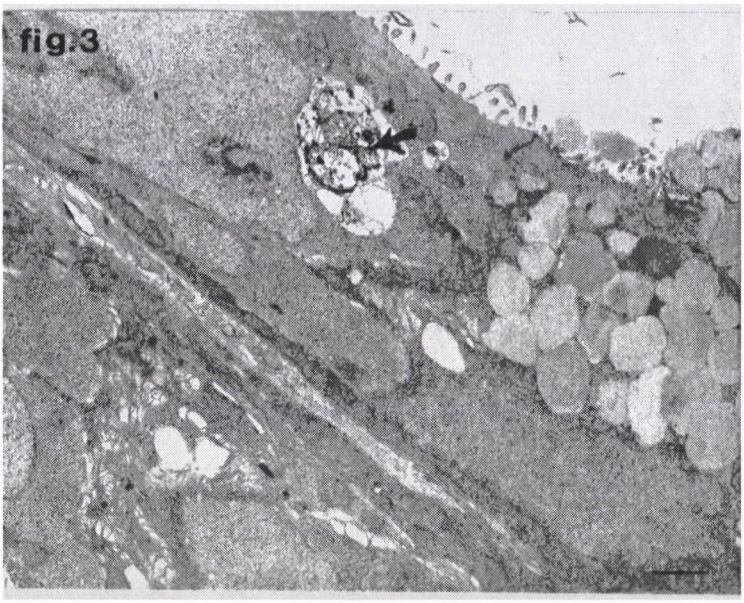

Fig. 3. A part of the colonic mucosa from the animal treated by bile (group C). Arrow points the lysosome having bile in it. Bar scale =1 μm

After fat rich diet the free-SH concentration of the colonic mucosa decreased significantly (group B) compared to control. The free-SH group concentration was elevated in group C and D in comparison with group A, which may be in connection with liberation of membrane bound proteins and/or enzymes from membrane structure as a consequence of bile treatment (Fig. 5).

A fall was observed after fat rich diet (B) in the proton donor activity and significant difference was measured between the bile treated groups (C-D) (Fig. 6). The reducing activity was significantly lowered in group B, and in both bile treated groups (C-D). There was no statistically significant difference among these groups (Fig. 7). Membrane destruction and inflammatory reaction (see the electronmicroscopic pictures) were supposed as the reasons for the changes in these parameters.

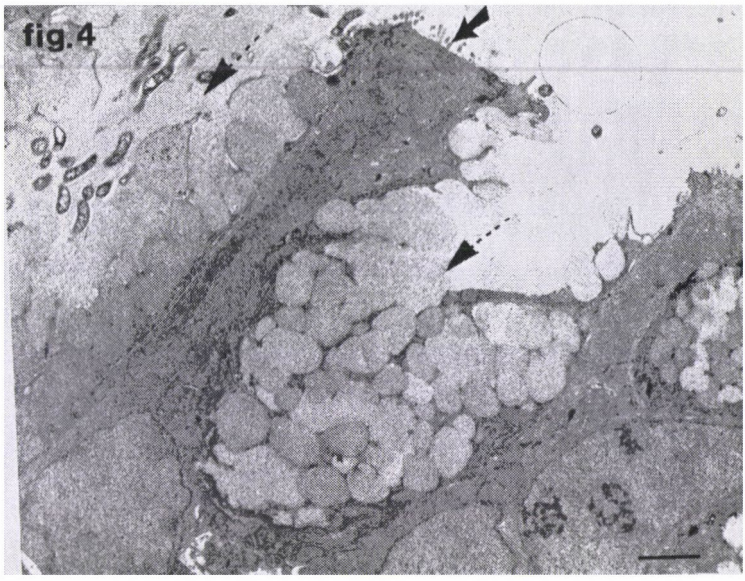

Fig. 4. A part of the colonic mucosa after bile treatment from the hyperlipidemic rat (group D). Arrows point the reduced microvilli of the enterocytes. Dotted arrows show the disrupted goblet cells. Bar scale =1 μm

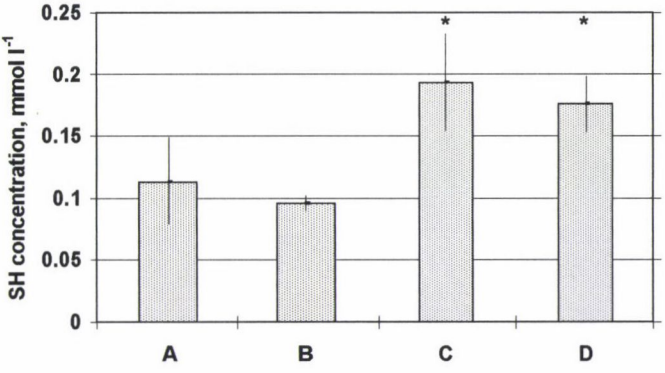

Fig. 5. Free SH group concentration (mmol l^{-1}) in group A, B, C, D. * shows the significant difference between A-C, A-D. Group A: control colonic mucosa, group B: colonic mucosa of rats fed with fat rich diet, group C: control bile treated colonic mucosa, group D: colonic mucosa of rats after bile treatment (from hyperlipidemic animals)

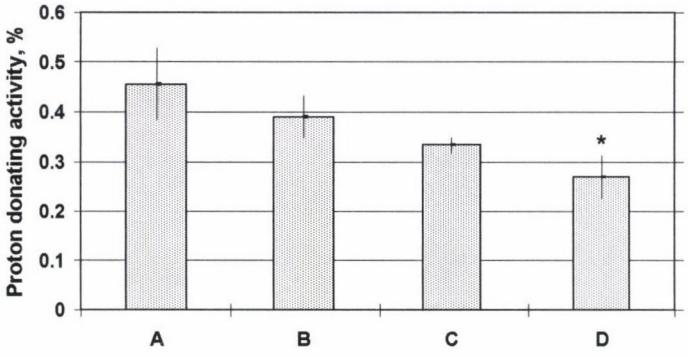

Fig. 6. The proton donating ability (%) in group A, B, C, D. * shows the significant difference between C-D. Group A: control colonic mucosa; group B: colonic mucosa of rats fed with fat rich diet; group C: control bile treated colonic mucosa; group D: colonic mucosa of rats after bile treatment (from hyperlipidemic animals)

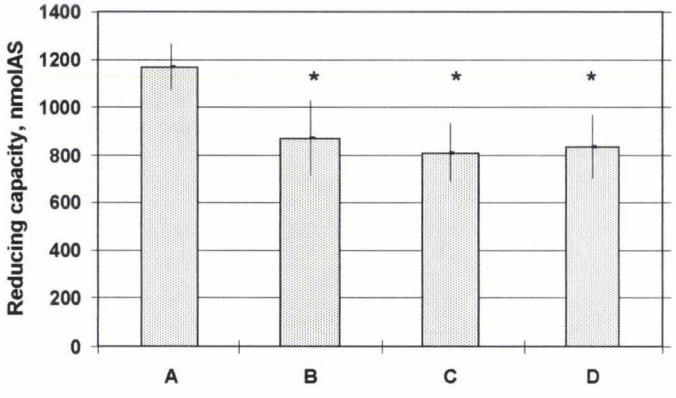

Fig. 7. The reducing power (nmolAS) comparing to group A, B, C, D. * shows the significant difference between A-B, A-C, A-D. Group A: control colonic mucosa; group B: colonic mucosa of rats fed with fat rich diet; group C: control bile treated colonic mucosa; group D: colonic mucosa of rats after bile treatment (from hyperlipidemic animals)

Table 1

Fatty acid composition of bile in control and in hyperlipidemic rats

Fatty acids	Normolipidemy (%±SD)	Hyperlipidemy (%±SD)
C12:0	1.45 ± 0.55	0.26 ± 0.04
C14:0	2.14 ± 0.29	3.34 ± 2.91
C15:0	0.88 ± 0.75	1.47 ± 0.65
C16:0	21.09 ± 2.06	19.58 ± 0.44
C16:1	0.70 ± 0.63	4.02 ± 3.35
C18:0	21.53 ± 4.17	8.10 ± 3.48
C18:1	34.96 ± 3.26	23.29 ± 8.71
C18:2	10.52 ± 2.36	17.54 ± 3.33
C18:3, C19:0	1.99 ± 0.34	1.87 ± 0.69
C20:0	0.54 ± 0.18	6.22 ± 0.68
C20:2	1.70 ± 0.87	3.01 ± 0.17
C20:3	0.63 ± 0.00	2.04 ± 0.85

The fatty acid composition of bile of hyperlipidemic rats was also changed and the rate of polyunsaturated fatty acid components were significantly higher in it. Consequently this modified bile served as a lipid peroxidation precursor and immune stimulants (Table 1).

3. Conclusions

In a healthy person only small amount of fat reaches the large bowel. The amount of free fatty acids, especially long-chain and saturated fatty acids reaching the colon is elevated in fat malabsorption for example due to pancreatic insufficiency, cholestatic liver disease, mucosal abnormalities. Colonic bacteria can metabolize dietary fatty acids to produce hydroxy-fatty acids, which are potentially toxic, but long-chain polyunsaturated fatty acids have a powerful antibacterial effect in the colon. Change in the composition of bile influences the metabolism of colonocytes as well (LING & WEAVER, 1997). After cholesterol feeding bile acid pool and cholic acid synthesis were increased (XU et al., 1998).

In this present study the alterations (e.g. reduction of microvilli and goblet cells, disruption of the cell membranes) of the epithelial lining caused by bile from hyperlipidemic rats were proved by electronmicroscopy as well as by biochemical investigations. Significant changes were measured in the redox state of normal colonic mucosa as a consequence of hyperlipidemic and control bile administration. These

effects may be in concordance with the changes in the composition of bile fatty acids and/or bile acids. The higher rate of polyunsaturated fatty acids could influence the prostaglandin biosynthesis and stimulate the immune responses, which can be seen in the electronmicroscopic picture (Fig. 3).

Changes in the composition of bile acids can alter the colonic mucin production and intestinal cytoprotection (SHEKELS et al., 1996). Bile acids in the colon can increase the release of prostaglandin E2 and nitrates plus nitrites via NO-synthese (CASELLAS et al., 1996). Secondary bile acid deoxycholate in pharmacological concentration induces hyperproliferation in vitro (VELAZQUEZ et al., 1997). In tumor cell lines chenodeoxycholic or deoxycholic acid caused morphological changes, that were characteristic of apoptosis, whereas incubating cells with ursodeoxycholic acid inhibited cell proliferation but did not induce apoptosis (MARTINEZ et al., 1998). Another possibility for the injury role of bile acids, that primary and secondary bile acids can inhibit xenobiotic metabolising enzymes such as glutathion S-transferase and UDP-glucuronyl-transferase (BAIJAL et al., 1998b). In rat model dietary restriction increased bile flow compared to age matched ad libitum fed rats, and it was associated with higher biliary GSH secretion rate and resulting in enhance GSH and GSH derived thiols supply to the intestinal lumen (BOUCHARD et al., 1998).

The present study supports that the hyperlipidemic diet can modify the structure of epithelium of the colonic mucosa (FEHÉR et al., 1993) as well as the composition of bile acid pool significantly. The cell proliferation inducing property of the hiperlipidemic diet can take part in adenoma formation and carcinogenesis.

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We would like to express our thanks to Mrs. S. BÁRKOVICS and Mrs. E. PINTÉR for their excellent technical assistance.

The research was supported by the National Scientific Research Foundation (OTKA F 029252) and Ministry of Welfare ETT 02-517.

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Acta Alimentaria, Vol. 30 (1), pp. 37–52 (2001)

A NEW METHOD FOR THE QUANTITATIVE DETERMINATION OF PROTEIN OF BACTERIAL ORIGIN ON THE BASIS OF D-ASPARTIC ACID AND D-GLUTAMIC ACID CONTENT

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(Received: 28 February 2000; accepted: 10 August 2000)

In the past years several methods have been developed for the determination of the proportion of the nitrogen-containing substances of microbial origin passed from the rumen into the abomasum or the small intestine. Recently, on examining the D-amino acid content of foodstuffs, particularly milk and milk products, it has been observed that, in addition to D-Ala, D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) can also be detected in similar quantities, primarily in products which have links with bacterial activity. This gave rise to the idea of examining the diaminopimelic acid (DAPA), D-Glu and D-Asp content of bacteria extracted from the rumen of cattle and that of chyme from the same cattle, in order to determine the type of relation existing among these three components, and to establish whether D-Asp and D-Glu can be used in the estimation of protein of bacterial origin. On determination of the DAPA, D-Asp and D-Glu content by means of amino acid analyser and high performance liquid chromatography of duodenal chyme from five growing bulls and of ruminal bacteria from the same bulls, the following values were established. For chyme (and, in brackets, for ruminal bacteria) r value calculated by means of linear regression was 0.78 (0.76) between DAPA and D-Asp, and 0.70 (0.81) between DAPA and D-Glu. The r values between the crude protein content of ruminal bacteria and the markers examined were found to be the following: DAPA, 0.74; D-Asp, 0.73; D-Glu, 0.61. In the model experiment performed for the re-obtaining of values for protein of bacterial origin the theoretical values were determined on the basis of D-Asp and D-Glu and values approximately 10% higher than the theoretical value on the basis of DAPA. It is therefore recommended that in addition to DAPA these other two amino acids be included among the bacterial protein markers.

Keywords: D-aspartic acid, D-glutamic acid, D-amino acids, protein of bacterial origin

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0139-3006/2001/$ 5.00

A common characteristic of the new protein evaluation systems introduced in cattle nutrition in a number of countries over the past decade is that the protein content of diets is judged on the basis of the quantity of amino acids absorbed from them in the small intestine of the animal. In addition to endogenous amino acids, which amount only to a small proportion, it comes, to a substantial extent, from two sources of amino acids, i.e., microbial protein synthesised in the rumen and dietary protein not broken down in the rumen (by-pass protein), which determine the quantity of amino acids absorbed. That is, on average, 70% of the protein content of the diet is broken down into amino acids in the rumen, this being utilised for the synthesis of amino acids or microbial protein or being broken down still further to provide ammonia for the construction of microbial body proteins. Ammonia not utilised for this is absorbed from the rumen and is converted into urea via the ornithine cycle in the liver. There is a further reason of importance for establishing the quantity of microbial protein produced in the rumen, since only when this quantity has been determined can the proportion of by-pass protein in the diet be established.

In order to define the quantity of microbial protein synthesised in the rumen, it is essential that microbial protein in the duodenal chyme be separable from the dietary by-pass protein proportion and from the protein of endogenous origin. This is possible only if components characteristic solely of microbial protein can be identified among the total protein.

In the past years several methods have been developed for the determination of the proportion of the nitrogen-containing substances of microbial origin passed from the rumen into the abomasum or the small intestine. Attempts have been made to estimate the proportion of the content of nitrogen-containing substances of microbial origin by means of determination with nucleic acid and tracing with vitamin B_{12} and sulphur 35 isotope. A critical evaluation and summary of these methods was given by STERN and HOOVER (1979).

CZERKAWSKI (1974) was successful in drawing a conclusion on protozoan nitrogen by means of the measurement of 2-amino-ethylphosphonic acid and on nitrogen content of bacterial origin by measuring 2-6-diaminopimelic acid (DAPA). That is, 2-amino-ethylphosphonic acid occurs to a great extent only in protozoa, while 2-6-diaminopimelic acid (hereafter referred to as DAPA) occurs exclusively in peptidoglycans in the bacterial cell wall. Despite the fact that the quantity of DAPA in the cell wall is strongly dependent on the species of bacterium in question, the ratio of DAPA in relation to total bacterial protein does not vary under constant dietary conditions; therefore, in comparative experiments DAPA can be used to good effect in the estimation of the proportion of protein of bacterial origin found in the contents of the intestine.

SCHLEIFER and KANDLER (1972) discovered that, like DAPA, D-alanine occurs also only in peptidoglycans in the bacterial cell wall and hence this compound is also suitable for use in the marking of proteins of bacterial origin and their quantitative determination. GARRETT and co-workers (1982) reported that, in the knowledge of this, they succeeded in determining nitrogen of bacterial origin by measuring the quantity of D-alanine in the ruminal fluid. GARRETT and co-workers (1987) subsequently performed comparative experiments using DAPA and D-alanine to investigate which compound enabled nitrogen of bacterial origin to be determined more precisely. They established that D-alanine proved to be a better indicator of nitrogen of bacterial origin, since the coefficient of variation resulting from the data obtained for D-alanine was substantially lower than that defined in the case of DAPA; in addition, greater accuracy was achieved in determination using D-alanine than with DAPA. In a series of experiments CSAPÓ and HENICS (1991) also established that both DAPA and D-Ala are suitable for use in the estimation of the quantity of protein of bacterial origin. No difference was observed between the two substances with respect to error either in analytical determination or in determination of protein of bacterial origin.

Several types of methods have been experimented with for the determination of DAPA in the ruminal fluid or the gut contents. HUTTON and co-workers (1971) used an automated amino acid analyser to determine DAPA, thus exploiting the characteristic of DAPA. In contrast to other amino acids but similarly to proline, it gives a yellow colour with acidic ninhydrin solution with the maximum light absorption value having been detected at a wavelength of 420 nm. In determining DAPA CZERKAWSKI (1974) hydrolysed the protein with acid, cleaned the hydrolysate in a charcoal column, separated the DAPA from the proline in an anion exchange column and determined DAPA with acidic ninhydrin.

PONGOR and BAINTNER (1980) developed a simple and fast method using ion exchange thin layer chromatography for the determination of DAPA combined with videodensitometry; however, due to the videodensitometric evaluation element this procedure has not become widely used in practice. EDOLS (1985) determined DAPA in a hydrolysate from the ruminal fluid by the application of a two-column method using an automated amino acid analyser. By the optimisation of the composition of buffers DAPA appeared between methionine and isoleucine on the chromatogram, well separated from these two amino acids, in the form of a sharp, easily quantifiable peak. CSAPÓ and co-workers (1986b) oxidised samples with performic acid prior to protein hydrolysis, as a consequence of which – the disturbing effects of neighbouring amino acids having been eliminated – DAPA, even if present only in trace quantities, could be determined accurately. Subsequently to this, exploiting the similar representation on

chromatograms of methionine and DAPA, the authors developed a fast method for the determination of DAPA alone by means of ion exchange column chromatography (CSAPÓ et al., 1995b).

Investigating the D-amino acid content of foodstuffs, particularly milk and milk products (CSAPÓ et al., 1995a; CSAPÓ et al., 1997a) it was observed that, in addition to D-Ala, D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) can also be detected in similar quantities, primarily in products which have links with bacterial activity. This gave rise to the idea of examining the DAPA, D-Asp and D-Glu content of bacteria extracted from the rumen of cattle and that of chyme from the same cattle, in order to determine the type of relation existing among these three components and to establish whether D-Asp and D-Glu can be used in the estimation of protein of bacterial origin.

Several methods have been developed for the separation and determination of amino acid enantiomers. Polarimetry was used for studying the racemisation of pure amino acids, and subsequently various enzyme techniques came into more general use. The flaw in such methods is that they cannot be used in the detection of trace quantities of D-amino acids, and contamination with amino acids of enzyme origin can also represent a very substantial source of error.

For the investigation of the optical purity of biologically active substances various methods using direct liquid chromatography have been developed. The essential elements of these procedures are the chiral column, consisting of chemically bound L-hydroxyproline-Cu^{2+} complex and the mobile phase, which is an aqueous solution containing Cu^{2+} ions. The application of the stationary phase creates the possibility for the investigation of the optical purity of all compounds which form chelate complexes with Cu^{2+} ions, for example the amino acids. The drawback of this method is that the D- and L-form of only one amino acid at a time can be determined.

WEINSTEIN and WEINER (1984) formed from amino acids the fluorescent 5-dimethyl-aminonaphthalene-1-sulphonyl derivative, and by using reversed phase liquid chromatography with the application of the chiral charge of N,N'-di-n-propyl-L-alanine and copper acetate succeeded in separating – from a sample – the D- and L-enantiomers of every amino acid involved in protein formation. Using 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, which contains a highly reactive fluorine atom, MARFEY (1984) created diastereoisomer derivatives, which were separable by means of liquid chromatography.

For the quantitative determination of amino acid enantiomers it is not sufficient to separate the enantiomers from each other; it must also be ensured that these enantiomers separate clearly from the other amino acids and derivatives of the latter. In addition, to achieve an appropriate degree of sensitivity amino acid derivatives detectable even in small quantities must be established. To this end there has recently been quite widespread application of pre-column derivative formation using fluorescent reagents

and reversed phase chromatography (RPC) for derivatives. With these methods the limit of verifiability for the amino acids to be determined is very low, and the flexibility of the analytical system conceals within it exceptional benefits (EINARSSON et al., 1987). Thus, automated methods, among others, have been developed for the determination of the α-amino acids with optically inactive o-phthalaldehyde/mercaptoethanol (OPA) (SMITH & PANICO, 1985), and for the determination of the α-amino acids and the imino acids together by means of 9-fluorenylmethyl chloroformate (FMOC-Cl) (CUNICO et al., 1986; BETNER & FÖLDI, 1988).

The reaction of the optically active (chiral) amino acids with chiral reagents results in a diastereoisomer compound, which in principle cannot be separated even in a chiral column. Should the chiral reagent be another amino acid, the isolation of the diastereoisomer peptides and their determination can also be performed by means of ion exchange column chromatography (HIRSCHMANN et al., 1967; MANNING & MOORE, 1968; IZUMIJA & MURAOKA, 1969; CSAPÓ et al., 1989a; CSAPÓ & CSAPÓ-KISS, 1989; CSAPÓ et al., 1989b).

Subsequent to the formation of derivatives with a chiral reagent it is possible to separate and determine the enantiomers of amino acids involved in protein formation in a single analysis by means of RPC. Since separation by chromatography may generally take 50 to 70 min, it is very important that any analytical method developed should be completely automated. Another precondition is that such a method should centre on a simple derivative forming reaction, which should run its course within a short period at room temperature. The reaction between the optically active thiols, OPA and the amino acids to be determined has been used for the separation and determination of amino acid enantiomers (ASWAD, 1984; BUCK & KRUMMEN, 1987; BRÜCKNER et al., 1994). The utilisation of chiral 1-(9-fluorenyl)ethyl chloroformate (FLEC) for the separation of enantiomers also entails the advantage that not only the α-amino acids but also the imino acids form stable derivatives (EINARSSON et al., 1987).

One of the fastest methods for the separation of D- and L-amino acids is gas chromatography. The enantiomers can be separated in the form of a diastereoisomer pair created by means of an appropriate asymmetrical reagent, or alternatively the volatilised derivatives can be separated in an optically active stationary phase. Gas chromatography has been developed to such perfection that error in the determination of enantiomers is below 5%, the level of repeatability being extremely high.

One of the major steps in the determination of D-amino acids is the hydrolysis of protein. It is also very important to establish whether racemisation occurs during protein hydrolysis, since, if it does, this may falsify the measurement data obtained. It has been reported in various studies that the degree of racemisation during hydrolysis is dependent upon the type of peptide or protein in question and also upon the other amino

acids present in the environment of the amino acid to be determined; it has also been established that peptide-bonded amino acids are generally racemised more rapidly than free amino acids (LIARDON & LEDERMAN, 1986; LIARDON & FRIEDMAN, 1987).

Recently many researchers have experimented with the application of microwave technology in protein hydrolysis and a number of them have reported excellent results obtained with hydrolysis performed for a short period at high temperature (CHIOU & WANG, 1988; CSAPÓ et al., 1994). If the objective is the separation and determination of amino acid enantiomers, a method of protein hydrolysis involving minimal racemisation should be selected, since should a substantial amount of racemisation occur during hydrolysis it would be impossible to establish whether a proportion of the amino acid enantiomers was originally present in the sample, or whether these enantiomers were only formed in the course of hydrolysis. Several methods have been developed for the suppression of racemisation during protein hydrolysis (SMITH et al., 1983; REDDY et al., 1989; D'ANIELLO et al., 1990), but these have proven rather lengthy and cumbersome. CSAPÓ and co-workers (1997b) have developed a protein hydrolysis procedure performed for a short period at high temperature; the authors established that any measure which accelerates protein hydrolysis reduces the degree of racemisation.

1. Materials and methods

1.1. Methodology for the animal experiment

The chyme and ruminal bacteria samples examined were taken from two experiments using five growing bulls of Hungarian Pied x Holstein Friesian cross origin of 480–500 kg body weight, each fitted with ruminal and duodenal fistulae; the objective of these experiments was to determine ruminal protein degradability for various diets, and also to establish the effect on the ruminal degradability of proteins of various types of feed additives (orthophosphoric acid, glyoxal, and a mixture of volatile oils). The diets examined included ones the protein of which is degraded in the rumen to a great extent (maize silage, extruded sunflower seed meal and molasses), to an intermediate degree (meadow hay), and only slightly (blood meal, feather meal and maize gluten).

Both experiments consisted of a ten-day adaptation period and a four-day trial period. In each trial period the degradability of the protein of a different diet and the effect exerted by a different additive was investigated. On days 1 and 4 of each experimental period samples of duodenal chyme were taken through the fistula every two hours from 6.00 to 16.00 h, giving a total of six samples for each treatment. The chyme samples subjected to examination were formulated from these partial samples. The quantity of chyme passing through the duodenum was determined by means of TiO_2

marker mixed into the diets. In order to establish the DAPA, D-Asp and D-Glu content of the ruminal bacteria, on day 2 of the experimental period samples of ruminal fluid were taken through the ruminal fistula three hours after morning feeding.

1.2. Preparation of samples for chemical investigation

In the preparation of the samples the ruminal fluid was centrifuged at 3000 rotation per minute in order to separate the feed particles from the infusorians. The bacterial mass was then separated by centrifugation of the fluid phase at 16,000 revolutions per minute; this mass was subsequently dried by means of lyophilisation. Aliquot parts of the chyme samples taken from the duodenum were also lyophilised.

1.3. Chemical investigation of samples

DAPA content was determined, by means of the procedure developed by CSAPÓ and co-workers (1986a) using Aminochrom-II and LKB 4101 type amino acid analysers, subsequent to protein oxidation with performic acid followed by 24-h hydrolysis with 6 mol hydrochloric acid containing 0.1% phenol.

Prior to determination of D-Asp and D-Glu the protein was hydrolysed with 6M hydrochloric acid for 30 min at 170 °C in order to permit the minimum possible degree of racemisation; separation and determination of the enantiomers were performed by means of high performance liquid chromatography in accordance with the method described by EINARSSON and co-workers (1987). For derivative formation o-phthalaldehyde (OPA) and 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside (TATG) were purchased from Sigma (St. Louis, Mo). Enantiomer separation was performed in a reversed phase (250×4.6 mm internal diameter, 5 mm particle size, Kromasil octyl (C8) charge) analytical column. In order to lengthen the life of the column a safety column (RP8, Newguard, 25×3.2 mm internal diameter, 7 mm particle size, Brownlee) was connected between the sample doser and the analytical column and a cleaning column (C18, 36×4.5 mm internal diameter, 20 mm particle size, Rsil) was fitted between the pump and the sample doser. A gradient system consisting of two components was used for enantiomer separation, the composition of this system being the following: A = in 40% methanol phosphate buffer (9.5 mmol, pH = 7.05); B = acetonitrile. The rate of flow used was 1 ml per min.

Since in this series of experiments only the quantity of D-Asp and D-Glu was of interest to the authors, determination was confined to these two amino acids and their enantiomers. By means of this procedure D-Asp and D-Glu present even in very small quantities can be detected and determined alongside with L-amino acid present in large quantities.

2. Results and discussion

The results for linear regression for the chyme and ruminal bacteria samples with respect to the relations to DAPA-D-Asp, DAPA-D-Glu and D-Asp-D-Glu are presented in Table 1. Table 2 shows the relation between crude protein content of the ruminal bacteria and chyme and D-Asp, DAPA and D-Glu for the same samples.

On analysis of the data presented in the tables it can be established that examination of both chyme and ruminal bacteria samples produced a very close correlation between DAPA and D-Asp and between DAPA and D-Glu content. The r value obtained on analysis of the relation between the DAPA and D-Glu content of the chyme was the lowest, at 0.70, and was the highest, at 0.81, in the case of DAPA and D-Glu in the ruminal bacteria. An even closer correlation was obtained on examination of the relation between D-Asp and D-Glu, both for the chyme ($r = 0.95$) and for the ruminal bacteria ($r = 0.84$).

Table 1

Linear regression parameters and statistical characteristics for chyme and ruminal bacteria with respect to DAPA-D-Asp, DAPA-D-Glu and D-Asp-D-Glu (Y=A+B×X)

Parameter, statistical characteristic	Chyme			Ruminal bacteria		
	DAPA-D-Asp	DAPA-D-Glu	D-Asp-D-Glu	DAPA-D-Asp	DAPA-D-Glu	D-Asp-D-Glu
A	0.31332	0.43877	–0.1682	1.12088	2.68554	2.34587
sd	0.08943	0.14195	0.07365	0.55976	0.43646	0.43684
B	0.65491	0.82992	1.33064	0.83955	0.77105	0.72862
sd	0.09351	0.14843	0.07825	0.18638	0.14533	0.11985
SD	0.11814	0.18752	0.08323	0.23758	0.18524	0.16914
N	34	34	34	17	17	17
r	**0.78**	**0.70**	**0.95**	**0.76**	**0.81**	**0.84**
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 2

Linear regression parameters and statistical characteristics determined between crude protein and DAPA, D-Asp and D-Glu for ruminal bacteria and chyme (Y=A+B×X)

Parameter, statistical characteristic	Ruminal bacteria			Chyme		
	D-Asp	DAPA	D-Glu	D-Asp	DAPA	D-Glu
A	–0.6983	–1.7455	1.4848	1.07286	1.26676	1.30856
sd	1.7043	1.1245	1.1521	0.3219	0.37937	0.45303
B	0.08822	0.09608	0.07013	–0.00608	–0.01364	–0.00394
sd	0.03448	0.02275	0.0233	0.01305	0.01536	0.01834
SD	0.32875	0.2169	0.2222	0.18739	0.22064	0.26348
N	17	17	17	34	34	34
r	**0.73**	**0.74**	**0.61**	**–0.08**	**–0.16**	**–0.04**
P	0.02181	0.00074	0.00881	0.6442	0.38112	0.83151

The very close correlations between DAPA and D-Asp, DAPA and D-Glu and the two D-amino acids (D-Asp and D-Glu) led the authors to perform further experiments in order to clarify how the two amino acids might function as markers of protein of bacterial origin, and what relation exists between bacterial markers and crude protein content. On analysis of the data presented in Table 2 it can be established that for the ruminal bacteria the closest correlation was obtained in the case of DAPA and crude protein content ($r=0.74$); the r value between D-Asp and crude protein content was only very slightly lower ($r=0.73$), while the closeness of the relation between D-Glu and crude protein content was seen to lag somewhat behind these correlations ($r=0.61$). However, the data obtained for the chyme samples produced no close correlation between the markers investigated and crude protein content; in fact, in the case of all three examinations linear regression analysis demonstrated a very weak negative correlation (r values varying between –0.04 and –0.16). This lack of correlation might be explained by the fact that only a proportion of the protein present in the chyme is derived from bacteria, the rest being comprised of dietary proteins which do not undergo bacterial degradation in the rumen, a small proportion of these being endogenous protein. Ruminal degradability of dietary proteins is on average 70%. However, this average conceals a very wide distribution of values. Results from experiments performed under in vivo and in vitro conditions corroborate that there are very great differences between the individual proteins with respect to ruminal

degradability (CHALUPA, 1976). There do exist feeds whose protein content undergoes almost total degradation in the rumen, while the ruminal degradability of other dietary proteins is only 15–20%. The great deviations encountered are connected with protein structure, primarily with the number of disulphide bonds present (MANGAN, 1972). The quantity and ratio of protein fractions influence ruminal degradability (WOHLT et al., 1976), as does protein amino acid composition (TAMMINGA, 1979) and the chemical and heat treatment of protein (KAUFMANN & LÜPPING, 1979; SCHMIDT et al., 1993). This implies that, dependent upon the proteins fed, there will always be variations in the proportions of microbial protein in the chyme and non-degradable dietary protein in the rumen.

As it was outlined in the section on methodology, in this experiment for the respective experimental periods the animals were fed diets of different ruminal degradability, which resulted in different ratios for the proportion of microbial protein and by-pass dietary protein in the chyme for the individual experimental periods. In the opinion of the authors this is the fundamental reason for the fact that in the case of chyme, in contrast to that of microbial protein, no relation was found between DAPA content and the quantity of the two D-amino acids.

By means of the linear regression parameters given in Table 2 a value of 49.05% was obtained for ruminal bacteria crude protein content on the basis of D-Asp, 49.26% on the basis of DAPA and 49.96% on the basis of D-Glu. The authors' calculations give an average value of 49.50% for ruminal crude protein content.

Subsequent to this, calculations were made with respect to the DAPA content of the ruminal bacteria obtained by means of the procedure applied, by which values of 0.606% for DAPA content, 0.740% for D-Asp content and 0.999% for D-Glu content were obtained. No possibility is available for the evaluation of the findings for D-Asp and D-Glu in the light of the relevant specialist literature, since as far as the authors are aware no other researchers have as yet carried out investigations into these components of bacterial protein. The value of 0.606% obtained for DAPA is lower than the value of 1.0±0.25% reported for bacterial protein by ORSKOV (1982) (data in the literature varying between 0.6 and 1.4%), which may be attributable to differences in the quality of the diets fed to the experimental livestock. Since the objective of these investigations was to identify a new marker for bacteria, it can be affirmed that the relative deviation in DAPA content observed, on comparison with data in the literature, does not influence the results obtained for D-Asp and D-Glu in this study.

After analysis of ruminal bacteria, and in the possession of data on crude protein content, multiplying factors were calculated to enable the proportion of protein of bacterial origin in an unknown sample to be estimated on the basis of DAPA, D-Asp and D-Glu content. The multiplying factor used for DAPA was 100/0.606 = 165; for D-Asp, 100/0.740 = 135; and for D-Glu, 100/0.999 = 100.

In order to establish the applicability in practice of the multiplying factors calculated in this study, two experiments were performed. In the first these multiplying factors were applied to analysis data obtained for various chyme samples. The results obtained are presented in Table 3. From these data it can be seen that, with two exceptions, values estimated on the basis of DAPA content are on average 10% higher than the microbial protein quantities determined on the basis of D-Glu or D-Asp content. The explanation for this may be that the DAPA content of ruminal bacteria calculated by the authors was somewhat lower than data reported in the literature. When protein content determined on the basis of D-Glu is compared with that for which D-Asp was the starting point, the level of concordance is immediately visible; in the majority of cases the respective values obtained concur almost entirely.

In the second part of the investigation performed to test the multiplying factors calculated a mean sample was produced from the 17 lyophilised ruminal bacteria samples available. The crude protein content of this mean sample was calculated at 49.5%, its DAPA content at 0.325%, its D-Asp content at 0.364% and its D-Glu content at 0.492%. By application of the multiplying factors obtained from the study crude protein content values of 53.63%, 49.14% and 49.20%, respectively, were estimated.

Table 3

Examples of the application of multiplying factors in the determination of protein of bacterial origin content of chyme samples

Chyme sample	Analysis results			Protein of bacterial origin (%) calculated on the basis of		
	D-Asp (%)	DAPA (%)	D-Glu (%)	D-Asp	DAPA	D-Glu
1	0.08546	0.07630	0.11513	11.54	12.59	11.67
2	0.06681	0.05814	0.08935	9.03	9.59	9.06
3	0.12546	0.11276	0.16415	16.96	18.61	16.64
4	0.07402	0.06560	0.10342	10.01	10.82	10.49
5	0.06519	0.05249	0.08644	8.81	8.66	8.77
6	0.10546	0.09933	0.13637	14.26	16.38	13.83
7	0.08564	0.07666	0.09952	11.58	12.64	10.09
8	0.08671	0.07041	0.11865	11.72	11.61	12.03
9	0.08591	0.08090	0.11730	11.61	13.34	11.89
10	0.09230	0.08189	0.12402	12.48	13.50	12.58

Table 4

Model experiment to investigate the accuracy of determination of protein of bacterial origin

1st sample (with 24.76% (calculated) protein of bacterial origin)

Parallel analyses	Analysis results			Protein of bacterial origin (%) calculated on the basis of		
	D-Asp (%)	DAPA (%)	D-Glu (%)	D-Asp	DAPA	D-Glu
1	0.182	0.162	0.247	24.60	26.71	25.05
2	0.181	0.159	0.239	24.47	26.22	24.24
3	0.179	0.155	0.251	24.20	25.56	25.45
4	0.184	0.164	0.241	24.87	27.04	24.44
5	0.185	0.166	0.246	25.01	27.37	24.94
Mean	0.1822	0.1612	0.2448	24.63	26.58	24.82
SD	0.0021	0.0038	0.0043	0.287	0.636	0.430

2nd sample (with 4.952% (calculated) protein of bacterial origin)

Parallel analyses	Analysis results			Protein of bacterial origin (%) calculated on the basis of		
	D-Asp (%)	DAPA (%)	D-Glu (%)	D-Asp	DAPA	D-Glu
1	0.037	0.037	0.047	5.001	6.101	4.766
2	0.035	0.035	0.049	4.731	5.772	4.969
3	0.038	0.032	0.051	5.136	5.277	5.171
4	0.034	0.031	0.046	4.596	5.112	4.664
5	0.037	0.033	0.047	5.001	5.442	4.766
Mean	0.362	0.0336	0.0480	4.893	5.541	4.867
SD	0.0015	0.0022	0.0018	0.198	0.355	0.181

Subsequently, the authors produced from beef by means of lyophilisation a meat meal of zero DAPA content and whose D-Asp and D-Glu content (racemisation testing being performed during protein hydrolysis) was, after hydrolysis of its protein using the method applied by the authors, found to be below 0.01% with respect to both glutamic acid and aspartic acid. For the first batch 1 g meat meal was mixed into 1 g bacterium sample and DAPA, D-Asp and D-Glu content were determined using 5 replicate analyses. This was then repeated using 9 g meat meal mixed with 1 g bacterium sample. The results obtained are presented in Table 4.

From the data given in the table it can be established that the dispersion percentages for the first sample, containing a higher quantity of bacterial protein, are in each case below 5%; thus the dispersion of the results meets the requirements for a reliable analytical method. For the second sample, in which the proportion of bacterial protein was only 20% of that in the first sample, with the exception of DAPA the dispersion percentage in each case remained below 5%, while that calculated for DAPA exceeded that value slightly. On comparison with the calculated values of the crude protein content obtained from the analysis data it can be ascertained that the results obtained for DAPA were, in the case of samples 1 and 2, approximately 10–15% higher than the anticipated values, while the values calculated on the basis of D-Asp and D-Glu practically concurred entirely with the expected values.

3. Conclusions

The investigations performed have provided evidence that both D-Asp and D-Glu may be appropriate for the determination of protein of bacterial origin. The results obtained using the two new bacteria markers proved to be approximately 10% lower than those obtained using DAPA, this being due not to error attributable to the new markers but rather to the unreliability of determination using DAPA. The analyses performed on samples of known bacterial protein content indicate that D-Asp and D-Glu on the one hand gave practically identical values for bacterial protein content, and on the other hand both provided results were very close to the theoretical (calculated) values.

The preparation process in the case of DAPA is lengthy due to the treatment with performic acid required. However, without performic acid treatment the determination of DAPA present in small quantities is unreliable, due to other amino acids in concentrations which may in some cases be higher by several orders of magnitude. In addition to the large amount of work required for the process, determination using DAPA also requires more time and more chemical agents, and is therefore fairly expensive. At the same time, an amino acid analyser operating on the principle of ion exchange column chromatography is suitable for analysis with DAPA, while separation of the D-amino acids cannot be performed with an amino acid analyser operating on the traditional principle.

Determination using D-Asp and D-Glu can be carried out with precision by means of high performance liquid chromatography. By the application of the fast procedure developed by the authors the determination of the quantity of D-Asp and D-Glu can be performed in a quarter of the amount of time required for ion exchange column chromatography analysis using DAPA. A different problem emerges in the determination of D-amino acids: that is, racemisation occurring during protein hydrolysis, which can falsify the experimental results obtained. Therefore, due to

racemisation greater quantities of D-Asp and D-Glu can be measured, leading to overestimation of the amount of protein of bacterial origin present. Two methods are recommended for the elimination of this source of error. One is the application of a hydrolysis procedure which allows only a very low degree of racemisation to take place (for example, the protein hydrolysis method developed by the authors, performed at high temperature and for a short period, i.e., 160–170 °C for 30–45 min). The other method is to determine by means of the method to be applied the D-Asp and D-Glu content of bacteria obtained from the rumen, following which, in a procedure similar to that used by the authors, multiplying factors should be calculated for the estimation of the quantity of protein of bacterial origin. In the case of the latter racemisation occurring during protein hydrolysis is to be regarded as a constant error, both in the calculation of multiplying factors and in the analysis of real samples, and therefore it exerts no substantial influence upon the accuracy of the determination procedure. Thus, method application is affected by the minimum of error if a protein hydrolysis procedure involving a low degree of racemisation is used, and if multiplying factors are determined by means of the method applied.

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The financial support of National Fund for Scientific Research (OTKA T 14916, OTKA 032411) and Ministry of Education (MKM-15) is gratefully acknowledged.

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Acta Alimentaria, Vol. 30 (1), pp. 53–61 (2001)

SUGAR AND ORGANIC ACID CONTENT OF TOMATO FRUITS (*LYCOPERSICON LYCOPERSICUM* MILL.) GROWN ON AEROPONICS AT DIFFERENT PLANT DENSITY

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(Received: 16 March 2000; accepted: 18 September 2000)

The quality of tomato fruits (*Lycopersicon lycopersicum* Mill.) depends on factors such as production conditions, the chosen cultivar, fertilisation, plant density, etc. The quality of tomato fruits cv. 'Arletta' grown on aeroponics has been analysed. The influence of plant density and pruning on yield quantity and quality was measured. Quality components were determined by high performance liquid chromatography (HPLC). The contents of sugars (sucrose, glucose, fructose and xylose) and organic acids (citric, malic, shikimic and fumaric acid) were analysed separately with regard to different plant density – 12, 16, 20 and 24 plants m^{-2} production surface. Evaluations showed that the differences in tomato quality, regarding the sugar (sucrose 0.46 g kg^{-1}, glucose 14.31 g kg^{-1}, fructose 12.06 g kg^{-1}, xylose 0.27 g kg^{-1}) and acid content (citric 7.79 g kg^{-1}, malic 0.68 g kg^{-1}, shikimic 4.36 g kg^{-1}, fumaric 14.34 g kg^{-1}) are not significant statistically with regard to plant density.

Keywords: tomato (*Lycopersicon lycopersicum* Mill.), quality, sugars, organic acid, aeroponics, plant density

Tomato as a vegetable variety is a food greatly appreciated by consumers throughout the year. The yield quality is closely correlated with growing conditions, chosen cultivars, plant care, production techniques, fruits' light exposure, etc. Horticultural market demands 'global quality' (BATTISEL & ENZO, 1999) both in the commercial (colour, durability, homogeneity, etc.) and in the alimentary aspect (taste, aroma, etc.). These qualities can be attained by proper growing substrates and with planned fertilisation. Tomato fruits produced by these measures are more tasty, even-sized, compact, resistant to damage and have a higher sugar content (BATTISEL & ENZO, 1999).

It has been established that the selection of production techniques is in direct correlation with yield quality (JANSE, 1994); the taste of the yield is dependent on the electric conductivity value (EC) and on the K^+ to Ca^{2+} ratio as well as the Na^+ to Ca^{2+} ratio. An insufficient supply of K^+ has the consequence of reducing the acid content,

0139-3006/2001/$ 5.00

and the tomato fruits acquire the characteristic aftertaste (JANSE, 1994). The high content of Ca^{2+} in the soil or in nutrient solution has a negative influence on fruit quality, because it increases the flouriness (JANSE, 1994). Phosphates and chlorides together with Ca^{2+} (at higher concentrations) also have a negative influence on the aroma features (JANSE, 1994). Greater concentrations of nutrient solution (higher EC-value) increase the tomato market value (the fruits are more juicy and aromatic and they contain higher amounts of sugars and acids (JANSE, 1994; SIRIGU et al., 1999)).

Higher concentrations of nutrient solution increase the osmotic pressure too and this way lower the consumption of nutrient solution (SIRIGU et al., 1999). This leads to signs of water stress (reduction of vegetative development, reduction of water content in plant tissue, increase of sugar and acid concentration as well as dry substance in the fruit juice). The nutrient solution concentration and its osmosis have a far greater influence on fruit quality than nutrient solution compound has. JANSE (1994) noted that by increasing the EC-value for 1 mS cm^{-1} content of sugars and acids increases by 4%.

The quality of tomatoes produced in protected areas (after SIRIGU et al., 1999) are appreciated by consumers (coloration and fruit shape, fruit size, increased content of sugars and acids) and is accepted on the Central European market. In traditional cultivation, these quality improvements are obtained by increased fertilisation and reduced watering.

1. Materials and methods

The research of plant density influence on the quality of tomatoes grown in aeroponics was done in a protected area – a greenhouse with double plastic coverings.

Aerponics is one of the soilless culture systems, where the plant roots are hanging in closed chambers. Periodically roots are sprayed with recycled nutrient solution.

The aeroponic system used in this trial was A-frame type, triangular in shape with equal sides of 1 m. The system was 15 m long, covering 15 m^2 of floor surface and having 30 m^2 of production area. Seedlings of tomato cultivar 'Arletta F1' were repotted into plastic net-pots with a diameter of 5.5 cm (02. 05. 1999). In the phase of developing the second pair of leaves, the plants were positioned (planted) in the holes of styrofoam plates on the system (24. 05. 1999). The seedlings were fertiirigated with a nutrient solution of higher concentration (EC 4–5 mS cm^{-1}).

The plants were planted in four different plant densities: A (24 plants m^{-2}), B (32 plants m^{-2}), C (40 plants m^{-2}) and D (48 plants m^{-2}) in three repetitions for each density. The trials were located in the 'Pilot farm' of the Biotechnical Faculty in Ljubljana.

The tomato plant roots were supplied with nutrient solution (Table 1) by fine sprinklers in programmed intervals of 5 min, and 60 s duration time of sprinkling. During the trial, the quality of nutrient solution was observed daily, its concentration was measured with a conductometer and kept at 3.5 mS cm^{-1}. The acidity of the nutrient solution was also measured daily and balanced in the range of pH 5.5 to 6.0 by the addition of sulphurous acid. If required the composition of the nutrient solution was corrected or replaced with a new stock (regarding the plant growth and temperature).

The plants were pruned regularly and cut back just under the second flower branch, leaving three leaves upwards from the first flower branch (29. 06.1999).

If required, the fruit branches were pruned leaving four fruits on each branch. Harvest was performed on all production area simultaneously (12. 08. 1999).

Estimation of the ripeness, development and quality of tomato fruits were carried out separately for each repetition and location of production area. The yield was determined by weighing the fruits per plant and m^2 of production area.

Fresh tomato fruits at technological ripeness were used for samples. The total soluble matter was determined by hand refractometer (Kübler) at standard conditions expressed in %. For each plant density four measurements have been carried out.

The content of sugars (sucrose, glucose, fructose and xylose) and organic acids (citric, malic, shikimic and fumaric acid) was analysed separately by high performance liquid chromatography (HPLC) at the Biotechnical Faculty, Institute for Fruit Growing, Viticulture and Vegetable Growing in Ljubljana (Table 2).

The HPLC system consisted of Thermo Separation Products (TSP) equipment with a pump model P2000, autosampler model AS1000, column heater and OS/2 Warp IBM Operating system (1994)-work station. The method was fully described by DOLENC-ŠTURM and co-workers (1999).

Table 1

List of salts required for preparation of 100.000 litres of nutrient solution

Salt - macroelements	(kg)	Salt-microelements	(kg)
KNO_3	42.0	HEDTA (Fe 12%)	0.7
NH_4NO_3	4.0	H_3BO_3	0.24
K_2SO_4	31.4	$MnSO_4$	0.17
KH_2PO_4	20.4	$ZnSO_4$	0.142
$MgSO_4 \times 7H_2O$	43.0	$CuSO_4$	0.019
$Ca(NO_3)_2 \times 4H_2O$	92.5	$MoCl_3$	0.012

Table 2

HPLC conditions for analysing sugars and organic acids

HPLC conditions	Sugar analyses	Organic acid analyses
Cartridge	Aminex HPX-87C	Aminex HPX-87C
Sample	20 µl	20 µl
Eluent	bidistilled water	4 mM H_2SO_4
Flow rate	0.6 ml min^{-1}	0.6 ml/min
Temperature	85 °C	65 °C
Detector	Sephadex RI-71	WellChrom K - 2500 UV

2. Results

Results of tomato production cultivar 'Arletta' on aeroponics with different plant density and with cut back are presented in Table 3.

The average fruit weight at all plant densities was within the limits of not statistically significant differences. Significant differences on an average yield, are from 12.6 to 21.2 kg m^{-2}.

Table 4 shows the results of total soluble solids (SS in %), amount of separated sugars (sucrose, glucose, xylose, fructose) and amount of total sugar.

The amount of total soluble substance is in the range from 4.0 to 4.8% with no significant differences caused by plant density.

The chromatogram of standard sugar solutions is shown in Fig. 1.

Table 3

Achieved yield of tomato fruits cv. 'Arletta F1' grown on aeroponics with different plant density (4 types of density – from 24 to 48 plants m^{-2}) with decapitation on single branch

Plant density Sign	No. plants m^{-2}	Average yield m^{-2} of floor surface (kg)	Average fruit weight (g)	Average yield per plant (g)
A	24	12.6	122	526
B	32	16.4	120	513
C	40	17.5	125	438
D	48	21.2	122	442

Table 4

Content of total soluble solids (SS) and sugars in tomato fruits grown at different plant densities on aeroponics

Plant density	No.	SS	Average content of sugars in g kg^{-1}				
Sign	plants m^{-2}	(%)	Sucrose	Glucose	Xylose	Fructose	Σ
A	24	4.1	0.35	11.68	0.42	11.32	23.77
B	32	4.3	0.39	11.73	0.17	11.15	23.44
C	40	4.4	0.63	12.14	0.29	11.31	24.37
D	48	4.2	0.49	12.95	0.22	14.46	28.13
Average		4.2	0.46	12.12	0.27	12.06	24.91
Min.		4.0	0.22	9.89	0.10	9.22	20.61
Max.		4.8	1.21	13.80	0.68	17.68	31.78
LSD 0.05			0.34	2.26	0.42	3.32	

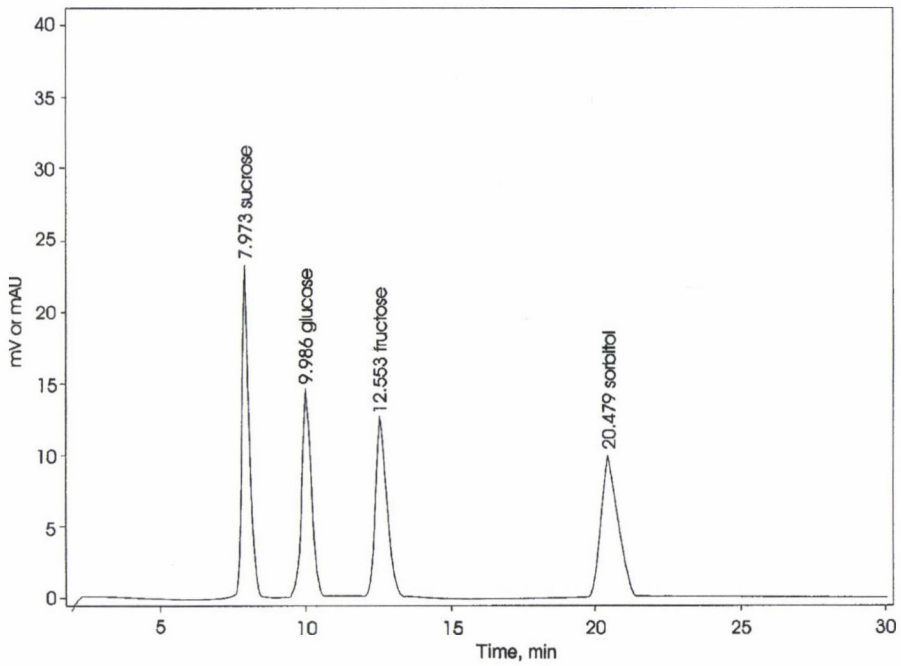

Fig. 1. Chromatographic separation of sugars in tomato fruits

Also, there are no significant differences in content of the sugars. The measured values of sucrose are in the range from 0.35 to 0.63 g kg^{-1}, glucose from 11.68 to 12.95 g kg^{-1}, xylose from 0.17 to 0.42 g kg^{-1} and fructose from 11.15 to 14.46 g kg^{-1}. The amount of total sugars is from 23.44 to 28.13 g kg^{-1}. The average amount of total sugars is 24.91 g kg^{-1}, significant decrease was noted at plant density of 48 plants m^{-2} of floor surface.

Changes in the organic acid content (citric, malic, shikimic, fumaric) as a function of plant density are shown in Table 5.

The chromatogram of standard organic acid solutions is shown in Fig. 2.

Significant differences in the average amount of organic acids were not found as a function of plant density. The highest values were measured for citric acid, ranging from 7.27 to 8.35 g kg^{-1}, malic acid from 0.60 to 0.74 g kg^{-1}, shikimic acid from 3.94 to 4.70 mg kg^{-1} and fumaric acid 13.67 to 15.51 mg kg^{-1}.

At the lowest plant density, content of citric acid was the highest (8.35 g kg^{-1}), while at the highest plant density the content of citric acid was the lowest (7.27 g kg^{-1}). In case of fumaric acid the opposite effect has been observed.

Table 5

Content of organic acids in tomato fruits grown at different plant densities on aeroponics

Plant density Sign	No. plants m^{-2}	Average content of organic acids			
		Citric (g kg^{-1})	Malic (g kg^{-1})	Shikimic (mg kg^{-1})	Fumaric (mg kg^{-1})
A	24	8.35	0.74	4.57	13.78
B	32	7.56	0.60	4.24	13.67
C	40	7.97	0.72	4.70	15.51
D	48	7.27	0.64	3.94	14.38
Average		7.77	0.68	4.36	14.33
Min.		7.15	0.46	3.61	7.81
Max.		8.59	0.89	5.28	19.32
LSD 0.05		1.11	0.17	1.20	5.78

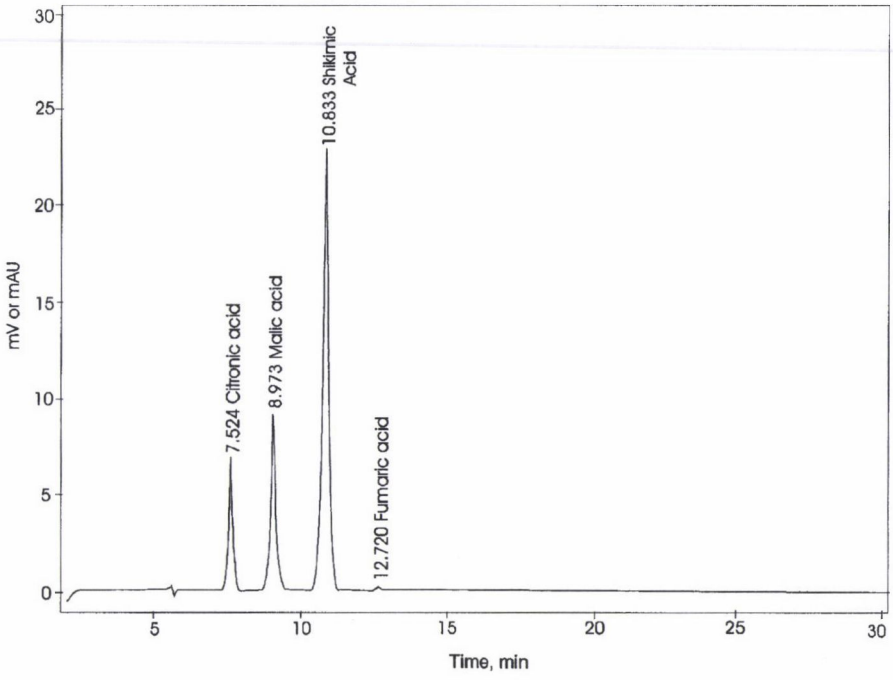

Fig. 2. Chromatographic separation of organic acids in tomato fruits

Table 6

The content of soluble solids (SS), total sugars (g kg^{-1}), glucose/fructose, total sugars/citric acid and citric/malic acid ratio in tomato fruits

Plant density Sign	Soluble solids SS (%)	Total sugars (g kg^{-1})	Glucose/ fructose (ratio)	Total sugars/ citric acid (ratio)	Citric/malic acid (ratio)
A	4.1	23.77	1.03	2.85	11.28
B	4.3	23.44	1.05	3.10	12.60
C	4.4	24.37	1.07	3.06	11.07
D	4.2	28.13	0.89	3.87	11.36
Average	4.25	24.91	1.00	3.20	11.36
Recommended value	4.8–6.6	24.3–52	0.7–0.95	1.87–7.65	2.96–6.5
Min.	4.1	10.61	0.74	2.34	13.57
Max.	4.8	31.78	1.13	4.85	9.38

Table 6 shows the values of sugars and acids in reciprocal ratios.

The measured average values between glucose and fructose are in a ratio from 0.89 to 1.07; sucrose and xylose from 0.83 to 2.29; total sugars and citric acid 2.85 to 3.87 and citric acid to malic acid from 11.07 to 12.60.

From the results of HPLC analysis and ratios of separated substances, it is noticeable that at the highest plant density (D) the total sugars as well as the ratio of sugar/acid are at the highest level.

3. Discussion

Quality is an important factor in the production and marketing. It is often strictly correlated with production circumstances, chosen cultivars, plant care, production techniques, fruits' light exposure, etc. These aims can be achieved by choosing proper growing substrates and by planned fertilisation (JANSE, 1994). By these measures, tomato fruits produced are more tasty, even-sized, compact, resistant to damage and posses higher sugar content (BATTISTEL & ENZO, 1999). The quality of tomato fruits depends on various factors, but the greatest impact is caused by sugars and organic acids (SHARMA & SHARMA, 1990; DOLENC-ŠTURM et al., 1999), as was detected in this research as well.

Quality, to a great extent, depends on the total sugar amount (24.91 g kg^{-1}), total soluble solids (4.15%), optimal glucose/fructose ratio (1.00), citric to malic ratio (11.36) and ratio between total sugar content and citric acid (3.2). These results imply that by increasing the concentration of the nutrient solution an impact can be achieved on the increase of sugar and organic acid content in tomato fruits, which leads to improved taste and quality.

Some authors assume (BATTISTEL & ENZO, 1999) that tomato quality is significantly affected by climatic circumstances, and less by the chosen production techniques. For quality improvement it is advisable to use proper cultivars (e.g. Mediterranean origin), that ripe simultaneously, posses intensive red colour and have high sugar content, etc. (BATTISTEL & ENZO, 1999).

In hydroponic tomato production, due to the small amount of substrate or its absence, regular and frequent nutrient solution supply is needed to prevent stresses and the consequent quality reduction (SIRIGU et al., 1999). With proper and regular nutrient solution supply towards plant roots, it is possible to achieve the planned tomato fruit quantity and quality with optimal sugar/acid ratio, as shown in Table 6.

4. Conclusions

The results of aeroponic tomato production combined with cut back and increased plant density are promising. In a short time high yields and marketable quality could be achieved by this method.

Yield analysis has proved that increased plant density per m^2 does not decrease the average fruit weight, while the total yield is increased.

HPLC sugar analysis of ripened fruits proved that sugar measures at different plant densities and locations do not show significant differences with regard to the average sugar content. Similar results were found considering the four most common organic acids in tomato fruits.

It would be advisable to measure the content of sugars and organic acids for the same cultivar but at different production systems and circumstances with various degrees of nutrient supply and simulations of water and nutrient stress.

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Acta Alimentaria, Vol. 30 (1), pp. 63–69 (2001)

BIOLOGICAL ACTIVITY OF FABA BEANS PROANTHOCYANIDINS

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(Received: 16 March 2000; accepted: 11 August 2000)

The objective of the experiment was to determine whether small amounts of proanthocyanidins (0.1 and 0.3%) may increase the antioxidative properties of the rat diet without exerting an antinutritional effect. Proanthocyanidins of faba bean seed coats were extracted with a mixture of acetone and water (70:30) and lyophilized. The amount of proanthocyanidins was two- or fourfold higher in the experimental diets as compared to the control diet. The addition of proanthocyanidin extract had no significant effect on the coefficients of digestibility of crude protein, daily nitrogen retention and the coefficient of biological value of diet protein. In the blood serum of rats fed diets supplemented with proanthocyanidin extract, there was a slightly higher content of vitamin E and alanine aminotransferase activity, while the content of vitamin A and aspartate aminotransferase activity were similar to those of the control group. In the contents of the rat gut (caecum), a lower activity of β-glucuronidase was found as compared to the control group, whereas β-galactosidase was unaffected. The addition of proanthocyanidin extract to diet caused a decrease in the malondialdehyde content in the heart, kidneys, erythrocytes and blood plasma of rats. The results obtained indicate that the amount of proanthocyanidins used did not exert any antinutritional effects, but extended the pool of diet antioxidants and beneficially affected the activity of the large bowel microflora.

Keywords: proanthocyanidins, nitrogen digestibility and retention, antioxidative activity, enzyme activity, rat

Polyphenols of seed coat, especially condensed tannins, are treated as the most important antinutritional components of faba bean seeds (JANSMAN, 1993). In polyphenol-rich diets, the availability of protein and aminoacids is decreased (ORTIZ et al., 1993; YU et al., 1996), and when such diets are consumed the activity of the digestive enzymes is lowered (YUSTE et al., 1992), and the absorption of food components from the alimentary tract is limited (JANSMAN, 1993; ZDUŃCZYK et al. 1996). On the other hand, those compounds are found to reveal antioxidative,

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0139-3006/2001/$ 5.00

bacteriostatic, anticarcinogenic and antimutagenic activity (BAGCHI et al., 1998; HAGERMAN et al., 1998; KAUL & KHANDUJA, 1998). There is, however, scarce information on what quantities of different groups of polyphenolic compounds are necessary for obtaining their beneficial or adverse biological properties.

The objective of the experiment was to determine whether a small amount of proanthocyanidins (0.1 and 0.3%) could increase the antioxidative properties of the rat diet without exerting an antinutritional effect.

1. Materials and methods

The experiment was conducted on 30 Wistar rats aged 40 days and weighing 110.1±2.2 g at the beginning of the test. The experimental groups were composed of 10 male rats. The rats were kept individually in metabolic cages at the temperature of 24 °C, 70% relative humidity and equal periods of dark and light. The composition of diets is presented in Table 1. The diets contained 150 g kg^{-1} crude protein (casein supplemented with DL-methionine) and standard amount of mineral mix (according to NRC, 1976) and vitamin mixtures (according to A.O.A.C., 1975). The experimental groups (II and III) were fed diets containing proanthocyanidin extract, 1 or 3 g kg^{-1}, respectively. The proanthocyanidin content in diet II and III was equal to their content in the diets containing 5 or 10% of seeds of colour-flowered faba bean. Proanthocyanidin extract was incorporated at the expense of potato starch.

Proanthocyanidins were isolated from the hulls of faba beans using method described by HUSSEIN and co-workers (1990). The hulls of faba beans were extracted with 70% aqueous acetone containing 2.8 mmol l^{-1} ascorbic acid as antioxidant. After semi-purification the extract contained 41% of proanthocyanidins in dry matter. The experiment lasted 10 days. Rats were anesthetized using urethane (140 mg×100 g^{-1} body wt.).

Blood was collected from the abdominal artery in order to obtain plasma samples and red blood cells (RBC). Blood was collected in heparinized tubes, plasma was prepared by centrifugation at 1500×g for 15 min at 4 °C and stored at –40 °C until analysis. The muscles of samples and internal organs (heart, lung, kidney and liver) were frozen in liquid nitrogen and stored at –40 °C until analysed. Samples were homogenized with 1.15% KCl until the assay of MDA. The malondialdehyde (MDA) concentration was determined using the colorimetric method described by UCHIYAMA and MIHARA (1978). The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was determined using the kinetic methods with an Alpha Diagnostics kit and an Epoll-20 photometer. The content of vitamins was determined by the HPLC method according to CUESTA SANZ and CASTRO SANTA-GRUZ (1986).

Table 1

Composition of diets without or with different content of tannin extract (g kg^{-1})

Ingredients	Experimental group		
	I	II	III
Casein	115.0	115.0	115.0
DL-methionine	2.0	2.0	2.0
Soya oil	100.0	100.0	100.0
Potato starch	100.0	99.0	97.0
Mineral mixture[a]	30.0	30.0	30.0
Vitamin mixture[b]	10.0	10.0	10.0
Proanthocyanidins extract	–	1.0	3.0
Maize starch	643.0	643.0	643.0

[a] Mineral mixture (NRC, 1976) containing in 100 g: 73.5 g $CaHPO_4$; 8.10 g K_2HPO_4; 6.80 g K_2SO_4; 3.06 g NaCl; 2.10 g $CaCO_3$; 2.14 g $NaHPO_4$; 2.50 g MgO; 558 mg ferric citrate; 81 mg $ZnCO_3$; 421 mg $MnCO_3$; 33.3 mg $CuCO_3$; 0.7 mg KJ and 705 mg citric acid

[b] Vitamin mixture (A.O.A.C., 1975) containing in 1 g: 2 000 IU vitamin A; 200 IU vitamin D_3; 10 IU vitamin E; 0.5 mg vitamin K; 200 mg choline; 10 mg p-aminobenzoic acid; 10 mg inositol; 4 mg niacin; 4 mg calcium pantothenate; 0.8 mg riboflavin; 0.5 mg thiamin; 0.5 mg pyridoxine; 0.2 mg folic acid; 0.04 mg biotin; 0.003 mg cobalamin; sucrose (supplement to 1 g)

The glycolytic activity was measured by the rate of release of *p*-(*o*-)nitrophenol from its *p*-(*o*-)nitrophenylglucosides. The reaction mixture contained 0.3 ml substrate solution (5 mmol) and 0.2 ml of a dilution 1:10 (v/v) caecal sample in a phosphate buffer (pH 6.4, 0.1 mol l^{-1}). Incubation proceeded at 37 °C and the *p*-nitrophenol concentration was measured as the optical absorbance at 400 nm (β-glucuronidase) and the *o*-nitrophenol concentration at 420 nm (β-galactosidase) after the addition of 2.5 ml 0.25 mol sodium carbonate. The enzyme activity (β-galactosidase and β-glucuronidase) was expressed as μmol of product formed per min (IU) per g of caecal sample (DJOUZI & ANDRIEUX, 1997).

The results of the experiments were analysed using one-way ANOVA, and significant differences between groups were determined by Duncan's multiple range test. Differences were considered significant at $P<0.05$ and $P<0.01$.

2. Results and discussion

The nitrogen balance for rats fed casein diets supplemented with proanthocyanidins is presented in Table 2. The addition of the proanthocyanidin extract reaching 0.1 or 0.3% caused a slight increase (by about 7%) in the amount of nitrogen

expelled with faeces, as compared to the control group. The coefficient of crude protein digestibility reached 94.7% in the control group and was insignificantly higher than that of the experimental groups (94.0%). The nitrogen losses in urine of rats given a diet supplemented with proanthocyanidins were higher by about 10% as compared to the control group. Subsequently, a small decrease in the nitrogen content retention in the organisms of rats from those groups was observed (68.3 and 68.7% of nitrogen absorbed) in comparison to the control group (71.6%). The biological value (BV) of diet protein was similar in all groups (from 84.8 to 87.5%).

The addition of proanthocyanidins did not have any antinutritional effects observed in the case of their increased content in a diet (ORTIZ et al., 1993; YU et al., 1996). It corresponded to the studies of LONGSTAFF and MCNAB (1991), who stated that a low proanthocyanidins content can even increase the digestibility coefficients of some components, e.g. fat.

In the blood plasma of rats fed diets supplemented with proanthocyanidins, a higher (by 20–20%) activity of alanine aminotransferase (ALT) was observed, in comparison with the control group (Table 3). The activity of aspartate aminotransferase (AST) was similar in all groups tested. The vitamin A content in blood plasma was similar in all groups, while that of vitamin E was higher in the experimental groups. The vitamin E content in blood plasma reached 838 mg l^{-1} for the control group, 902 mg l^{-1} for rats fed a diet supplemented with 0.1% proanthocyanidin extract, and 954 mg l^{-1} for rats given a diet with a higher amount of the proanthocyanidin extract.

Table 2

Digestibility and retention of nitrogen of diets

	Experimental group			SEM
	I	II	III	
Diet intake, g/week	66.9	67.3	67.0	0.15
Nitrogen intake, mg	1124.3	1130.6	1126.3	2.51
Nitrogen excretion:				
– faecal, mg	125.0	134.5	134.9	5.56
– urinary, mg	260.5	290.2	285.3	15.03
N digestibility, %	94.7	94.0	94.0	0.49
Nitrogen retention:				
– mg/day	114.9	110.3	110.4	1.96
– % of nitrogen intake	71.6	68.3	68.7	1.26
BV, %	87.5	84.8	85.7	1.39

Table 3

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity and vitamin A and E content in plasma

	Experimental group			SEM
	I	II	III	
AST (U l^{-1})	99.4	97.8	99.8	2.55
ALT (U l^{-1})	15.8 b	19.0 ab	20.4 a	0.83
Vitamin A, mg l^{-1}	368.6	350.6	358.8	18.8
Vitamin E, mg l^{-1}	838.3	901.8	953.6	29.0

a, b: significant at $P<0.05$

These results may indicate the antioxidative activity of the proanthocyanidin extract. Plant proanthocyanidins are natural biological antioxidants (HAGERMAN et al., 1998; PLUMB et al., 1998). When added to diet, they can limit the consumption of other antioxidants, including vitamin E.

The malondialdehyde (MDA) content in rat tissues is presented in Table 4. The addition of the proanthocyanidin extract to diet caused a decrease in the MDA concentration in heart, kidneys, erythrocytes and most of all in blood plasma. Only in the case of lung tissue, a significant increase in the malondialdehyde content was observed in groups fed a diet with faba bean proanthocyanidins. The decreased MDA content in tissues can be explained by the antioxidative activity of the proanthocyanidin extract. Polyphenols belong to the biologically active diet components which affect the oxidative status of tissues (DECKER, 1995).

The addition of the proanthocyanidin extract had a beneficial influence on the activity of the blind gut microflora, resulting in a decrease in the β-glucuronidase activity (Table 5). The hydrolysis of glucuronide bonds, caused by the presence of that enzyme, increases the content of substances revealing potential toxic and carcinogenic activity in the large bowel (REDDY et al., 1992). The addition of 0.1% and 0.3% tannin extract into a diet decreased the activity of β-glucuronidase by about 30% and 70%, respectively, as compared to the control group. No effect of the extract examined on the β-galactosidase activity was noted. The results obtained are in agreement with the studies of other authors (DE BRUYNE et al., 1999; CHUNG et al., 1998; TEBIB et al., 1996) indicating the beneficial effect of tannins on the microbiological activity of the blind gut and colon by having a limited growth of harmful bacteria.

Table 4

Malondialdehyde (MDA) concentration in internal organs and plasma

	Experimental group			SEM
	I	II	III	
Muscles, mg/100g	1.714	1.806	1.904	0.08
Lung, mg/100g	0.678 b	0.860 a	0.820 a	0.03
Kidney mg/100g	3.417 A	2.304 B	2.406 B	0.17
Liver, mg/100g	1.959	1.669	1.999	0.15
Heart, mg/100g	1.642 a	1.278 b	1.448 ab	0.06
Erythrocytes, mg/100g	4.377 A	3.001 B	2.998 B	0.22
Plasma, μg/100ml	133.2 A	44.3 B	47.28 B	11.35

A, B: significant at $P<0.01$
a, b: significant at $P<0.05$

Table 5

The activity of β-galactosidase and β-glucuronidase in the caecum content

Enzyme activity	Experimental group			SEM
	I	II	III	
β-Galactosidase, Ug^{-1}	2.48	2.58	2.19	0.18
β-Glucuronidase, Ug^{-1}	1.99 Aa	1.44 ABa	0.62 Bb	0.20

A, B: significant at $P<0.01$
a, b: significant at $P<0.05$

3. Conclusions

The results of the study indicate that a small amount of proanthocyanidins (0.1 and 0.3%) increases the antioxidative properties of diets without exerting antinutritional effects. The addition of proanthocyanidin extract had no effect on the digestibility and biological value of the proteins, it decreased the MDA content in most tissues and the β-glucuronidase activity in the blind gut contents, and increased the level of vitamin E in plasma.

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Acta Alimentaria, Vol. 30 (1), pp. 71–80 (2001)

CHARACTERIZATION OF BUCKWHEAT GRAIN PROTEINS AND ITS PRODUCTS

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(Received: 22 March 2000; accepted: 19 September 2000)

Immunological assays demonstrate that buckwheat flour proteins present no toxic prolamins to coeliac patients.

Our study proves that buckwheat has no homologous protein structure with wheat. Electrophoretograms showed that some protein bands of buckwheat proteins resemble papillionaceous (bean) proteins.

The allergenic character of buckwheat was measured by competitive indirect ELISA using anti-wheat germ lectin (Wga) immune serum. Properly hulled buckwheat flour did not react with Wga immune serum, and is therefore suitable to be used in the diet of coeliac patients.

Keywords: buckwheat, homologous protein structure, cross-reaction, sensitize

Major endosperm proteins of buckwheat *Fagopyrum esculentum* (MOENCH) are salt soluble globulins (TIARA, 1974, HILLER et al., 1975, MAKSIMOVIC et al., 1996). The main seed storage protein of buckwheat is the 13S globulin. It has a hexamer structure with subunits composed of dissolved bonded acidic and basic polypeptides, the structure common to all legumin-like storage proteins (MAKSIMOVIC et al., 1996). Vicillin-like proteins of buckwheat are 57–58 kDa polypeptides, which constitute 6.5% of total seed proteins (RADOVIC et al., 1999).

Protein content of buckwheat flour is ranging from 8.51 to 15.87% depending on variety (FRANCISCHI et al., 1994a). The main difference regarding protein fractions between buckwheat flour and wheat flour is that buckwheat is rich in albumin and globulin, but very low in prolamin (average 1.9% only) and glutelin content is also much lower than that of wheat flour. The quantity and ratio of individual protein fractions vary in a large range depending on varieties. This is valid for albumin and globulin (WEI et al., 1995). Compared with wheat flour protein, buckwheat proteins show a higher or similar content of nearly all amino acids except for glutamine and proline, the percentages of which are much lower than in wheat flour. Percentage of all

0139-3006/2001/$ 5.00

other essential amino acids is higher than or near to that of wheat protein (WEI et al., 1995). Particularly the content of the limiting amino acid lysine is 2.5 times higher than in wheat flour.

Buckwheat grains and buckwheat products have high nutritive value. Buckwheat noodles are favourite food in some countries and are also used for some diet due to their curative and healthy effects (WEI et al., 1995).

Limitations in terms of classification of buckwheat or other seed proteins based on solubility only to gluten proteins (JAVORNIK et al., 1981; IKEDA et al., 1991) is unfortunate and can lead to unnecessary exclusion of valuable sources of dietary protein in gluten-sensitive individuals (SKERRITT, 1986). Buckwheat flour is suitable for use in coeliac diet, since its prolamin content is low. In addition, immunological assays revealed that the buckwheat contains no toxic prolamins to coeliac disease patients (FRANCISCHI, 1994b).

The antinutritive and allergic characters of buckwheat have not yet been elicited entirely. KONDO and co-workers (1996) studied the allergic properties of protein. A 24 kDa buckwheat protein was shown by immunoblotting analysis to be the most frequently recognised allergic component.

The aim of research to be presented in this paper was to test the presence of homologous structure of wheat and legume proteins with buckwheat flour proteins; to study prolamin content of buckwheat with immunochemical method; to measure the quantity of homologous protein structure of buckwheat inner layer membrane with wheat germ agglutinin immune serum by competitive indirect ELISA. The cross-reaction of buckwheat with Wga immune serum showed similar groups (epitopes) which can sensitize cell receptors of patient and as a result can cause allergy, too.

1. Materials and methods

1.1. Materials

Samples. Whole meal flours of *Triticum aestivum* cultivar GK Öthalom (wheat variety), Novodur of durum wheat (semolina), *Triticum spelta*, Chinese spring wheat cultivar, *Triticum monococcum*. The investigated materials were: buckwheat flour, buckwheat products, pancake, bread from 100% buckwheat, buckwheat flour dehulled properly, wheat germ lectin, lentils lectin, soya, yellow pea, French buckwheat flour, buckwheat from Slovenia, pancake and muffin made of 100% buckwheat, muffin made of 100% wheat flour (AUBRECHT et al., 1998). The samples were commercial products, if variety or species names are not mentioned.

Sample preparation: The prolamin was extracted from ground sample (100 mg) with 1 cm^3 of 70% (v/v) ethanol and Wga was subtracted by PBS in centrifuge tube and extracted with vigorous shaking on a flash shaker (IKA-SCHÜTTLER MTS 4, 700 r.p.m. for 30 min at 4 °C). Following extraction, the samples were centrifuged (T 24 D, 5000 r.p.m. for 20 min at room temperature), the supernatant was separated and used for ELISA (SZAMOS et al., 1998).

Chemicals. N-acetyl-D-glucosamin, acrylamide, N-N'-methylene bisacrylamide, N,N,N',N'-tetramethyl-ethylenediamine, absolute ethanol, gliadin Fluka Co, wheat germ agglutinin lectin (Sigma Co.), 3,3',5,5'-tetramethyl benzidine (TMB) substrates were used. All other chemicals were of analytical grade.

Reagents for ELISA. Immune sera (anti-gliadin, anti Wga-immune serum) were developed in the Central Food Research Institute. Antigliadin-rabbit IgG and antigliadin-rabbit IgG-HRP conjugates were prepared.

Gel electrophoresis. SDS-PAGE was carried out according to LAEMMLI (1970), acid-PAGE by method of JANSEN and co-workers (1994).

Reagents for immunoblot. Methanol and other chemicals were bought from Reanal Co. The immunoblot membrane was purchased from Millipore Co.

1.2. Methods

Immunization protocol. It was carried out according to CUADRADO and co-workers (1998). Competitive indirect method to detect Wga concentration: Plates coated overnight at 4 °C with 0.5 μg ml^{-1} Wga (in sodium carbonate-bicarbonate buffer, pH 9.8) were washed three times with PBST (0.01M phosphate buffer 0.9% (w/v) NaCl 0.01% (v/v) Tween 20, pH 7.4). Then 0.2 μl/well of PBSG (0.01M phosphate-buffered saline containing 0.5% gelatine) was added and after incubation for 1 h at 37 °C the plates were washed three times with PBST. After this, 100 μl/well standard Wga diluted in PBS containing 0.1M N-acetyl-glucosamine or buckwheat or whole meal wheat flours samples (diluted in 0.1M N-acetyl-glucosamine in BPS) 50 μl/well of polyclonal anti-Wga IgG antibody (1:1000 in PBS) were prepared. After incubation for 1 h at 37 °C, the plates were washed with PBS. The anti rabbit goat IgG HPR-conjugatum 1:8000 was added. After at 1 h 37 °C incubation the plates were washed three times with PBS. The plates were dried, then 0.1 ml of a solution of OPD-H_2O_2 (0.34 mg ml^{-1} o-phenylene-diamine in 0.05M phosphate-citrate buffer, 0.03% (v/v) hydrogen peroxide, pH 5.0) was added to each well. After 5 min, the reaction was stopped by adding 0.05 ml of 2M H_2SO_4, and the optical density was measured at 492 nm using Dynatech plate reader. The lectin content of the samples was estimated by $Y = aln(x)+b$.

Sandwich ELISA (SKERRITT & HILL, 1990) modified by AUBRECHT and TÓTH (1995) was used.

SDS-polyacrylamide gel electrophoresis was carried out as described by LAEMMLI (1970). Acid-PAGE was performed according to JANSEN and co-workers (1994). Immunoblot method used was as of HERIAN and co-workers (1990).

2. Results

Protein bands of buckwheat flours were compared to papillionaceous plant seeds and wheat flour and studied by SDS-PAGE. The electrophoretograms of whole proteins of wheat flours and buckwheat flours can be seen in Fig. 1. lane 2 and 5, 6, 7.

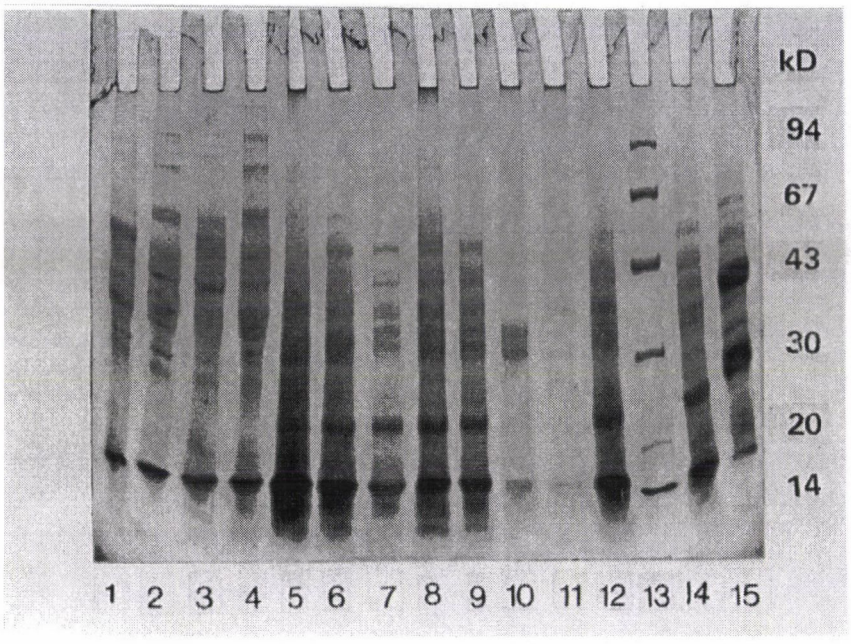

Fig. 1. SDS-PAGE of the total protein of buckwheat flours, their products and papillionaceous plants. Lane 1: bean; lane 2: GK Öthalom wheat variety; lane 3: soya; lane 4: yellow pea; lane 5: buckwheat flour; lane 6: buckwheat flour from France; lane 7: buckwheat from Slovenia; lane 8: pancake made of 100% buckwheat; lane 9: pancake made of 100% buckwheat; lane 10: muffin made of 100% buckwheat; lane 11: muffin made of 100% wheat flour; lane 12: pasta made of 100% buckwheat; lane 13: mol weight standard; lane 14: buckwheat seed; lane 15: oat bran

The electrophoretograms of buckwheat were compared to wheat and to papillionaceous (bean) plant proteins (Figs 2, 3, and 4). Wheat proteins compared to buckwheat do not contain similar protein bands with buckwheat as it can be seen in Figs 3 and 2. Electrophoretogram of bean resembles buckwheat protein, the fractions compared are marked on Figs 4 and 2.

Unlike wheat gliadin the corresponding buckwheat fraction was not electrophoretically resolvable neither into gliadin-like bands on lactate-buffered polyacrilamide gels nor on sodium dodecyl sulfate polyacrilamide gels, it only had minor components in the gliadin molecular size range. The protein bands of buckwheat were diffuse, and wheat gliadin gave three different protein bands.

The prolamin content of buckwheat and wheat flour was measured by ELISA using polyclonal anti-rabbit serum. Optical density of buckwheat flour protein was 0.353, while the optical density of products which were made of 100% buckwheat like bread and pancake were measured at 0.179 and 0.268, respectively. Wheat flour absorbance was 1.241. Ten samples were measured and the standard deviation was under 10%. Estimated prolamin content of buckwheat was ranging from 3.8 mg×100 g^{-1} dry material to 5.2 mg×100 g^{-1} on a dry material basis. The prolamin content of buckwheat flour and products (pancake and bread) made of 100% buckwheat flour was well below the permitted limit (100 mg kg^{-1} dry matter basis (FAO/WHO, 1998).

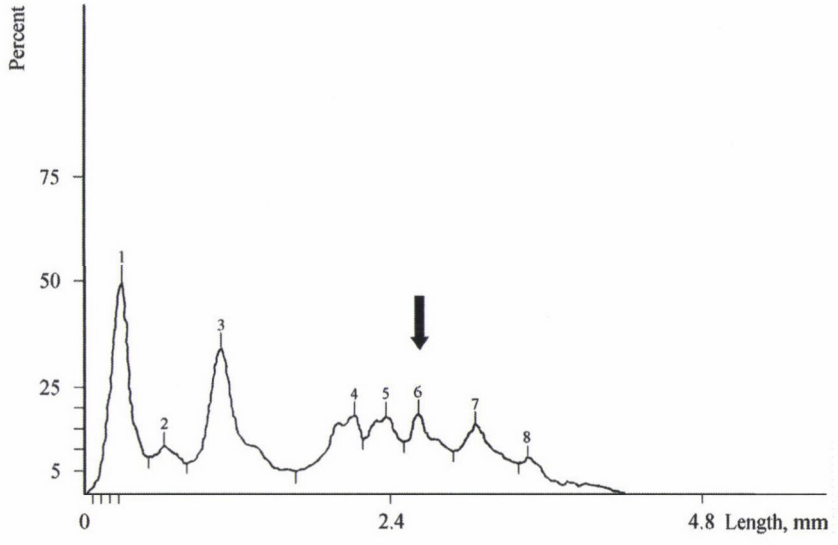

Fig. 2. Electrophoretogram of buckwheat

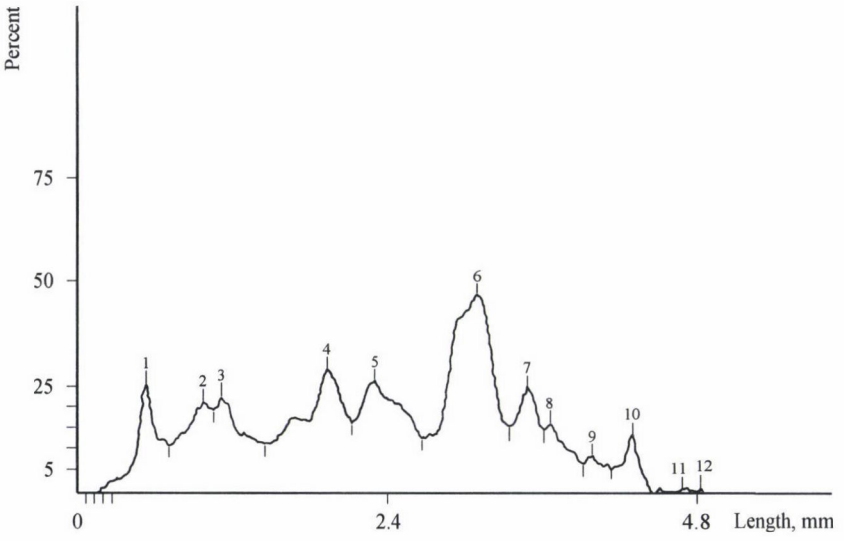

Fig. 3. Electrophoretogram of wheat flour

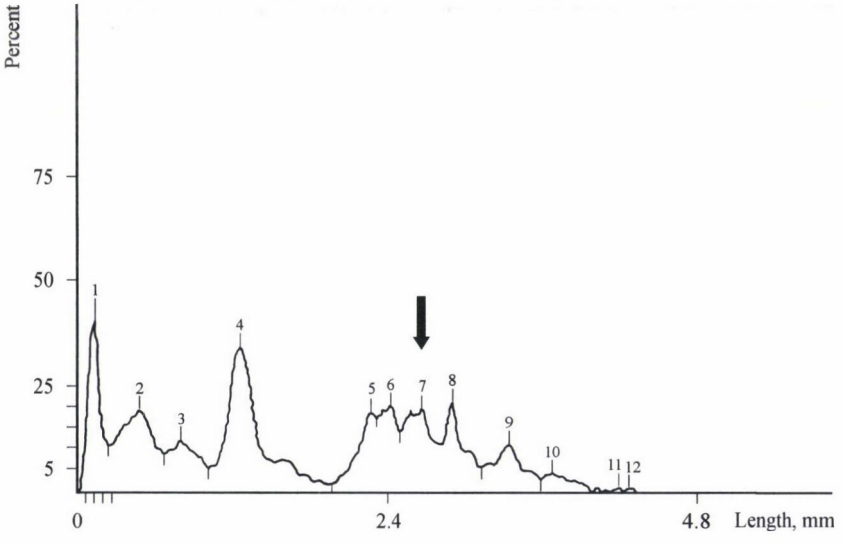

Fig. 4. Electrophoretogram of papillionaceous plants (bean)

Wga lectin can cause allergic reaction to susceptible patients, the allergic activity of buckwheat was characterised with cross-reaction of anti-Wga immune-serum.

With polyclonal Wga serum the presence of wheat germ lectin or its homologous structure in buckwheat flour was tested. Buckwheat grain with inner layer membrane reacted with anti-Wga immune serum. Properly hulled buckwheat, however, does not react to anti-Wga immune serum. The immunochemical reaction of positive sample (*Triticum monococcum*, durum wheat, *Triticum aestivum*) of grits of cereals can be seen in (Fig. 5 lane 7, 6, 4). The immunochemical reaction of gliadin fraction (Fig. 5 lane 2) with immune serum revealed three different bands. It proved that gliadin has homologous structure with wheat germ lectin. These lectin fractions are glycoproteins which proved that in vitro digested wheat gliadins can easily be separated by affinity chromatography of N-acetyl-glycosamine (DE-VINCENZI et al., 1995). Lentils (Fig. 5 lane 3, 8) have epitopes similar to wheat germ lectins.

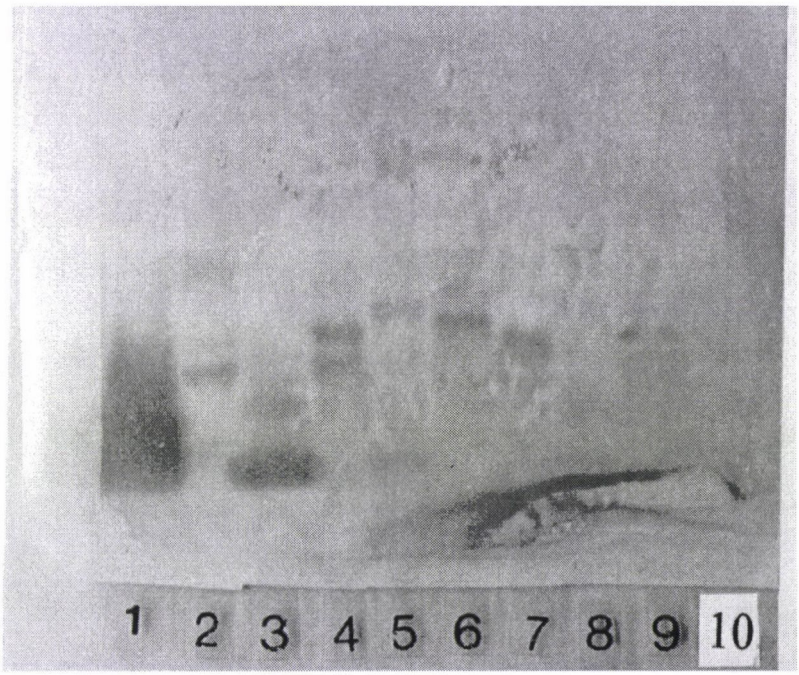

Fig. 5. Immunoblot of anti-wheat germ lectin serum with different plant proteins. The total grain run on 10% SDS-PAGE gel demonstrating specificity of anti-Wga serum and cross-reaction with homologous structure. Lane 1: wheat germ lectin; lane 2: wheat gliadin; lane 3: lentil lectin; lane 4: whole ground wheat gain GK Öthalom; lane 5: hulled buckwheat seed with inner layer membrane; lane 6: semolina Novodur; lane 7: *Triticum monococcum*; lane 8: ground lentil; lane 9: buckwheat flour; lane 10: buckwheat flour hulled properly

The quantity of Wga and its homologous structure were measured by competitive indirect ELISA with the results set forth in Table 1. Properly hulled buckwheat grain does not react with anti-Wga lectin serum or its lectin concentration is very low (Fig. 5 lane 10). The difference between the two type of hulled buckwheat was significant ($P = 0.05$). The wheat flours GK Öthalom and GK Tiszatáj did not contain any Wga-lectin (Table 1). Wheat grits and hulled buckwheat flour with inner layer membrane reacted with the immune serum. The inner layer membrane of buckwheat contains homologous structure with Wga since it gave cross-reaction with anti-wheat germ lectin immune serum (Fig. 5 lane 5 and 9, Table 1). No toxic concentration of Wga lectin or its homologous structure has been identified yet. It would be important to know the maximal non-toxic concentrations if buckwheat flour was regularly used in coeliac diet.

Table 1

Wheat germ agglutinin and its homologous concentration measured by indirect competitive ELISA with anti-wheat germ lectin immune serum

Plant varieties	Wga concentration in flour ($\mu g\ ml^{-1}$)	Wga concentration of grist ($\mu g\ ml^{-1}$)	Whole meal ($\mu g\ ml^{-1}$)
Gk Öthalom wheat variety	$1.413 \cdot 10^{-5}$	5.915	4.764
Barley	–	$3.535 \cdot 10^{-5}$	1.042
Gk Tiszatáj wheat variety	$1.03 \cdot 10^{-5}$	13.59	2.21
Buckwheat hulled properly	–	–	–
Buckwheat hulled containing inner layer membrane	0.089	nd	–
Wheat germ	110	nd	–

–: not investigated
nd: no detected

3. Conclusions

Immunological studies with anti-gliadin rabbit polyclonal antibodies showed little cross-reactivity between wheat prolamins and buckwheat proteins.

Results suggest that alcohol soluble buckwheat proteins bear little molecular similarity to wheat prolamins. While it is possible that components in buckwheat (such as polyphenols) may decrease extraction of gluten-like proteins from buckwheat, this is unlikely to account for the failure to observe such proteins, because a wide variety of extractans (acidic, alkaline, alcoholic and SDS-based) were used in these studies by SKERRITT (1986). In our study prolamin concentration or cross-reaction of buckwheat and their products were under the permitted limit 100 mg gliadin kg^{-1} on dry material

basis (FAO/WHO, 1998). RADOVIC and co-workers (1999) found that the buckwheat does not contain any prolamin, but has antinutritive effect and allergen activity for sensitive patients.

It is known that 24 kDa protein bands can cause allergic reaction (KONDO et al., 1996). Studies with heat-treated 100% buckwheat product showed that 24 kD protein band disappears and form 36 kD aggregate (AUBRECHT & BIACS, 1999).

The buckwheat grain hulled properly will be suitable for use in coeliac diet after clinical trial (FRANCISCHI et al., 1994a,b).

*

The authors wish to thank Dr. É. GELENCSÉR for her kind advice and for providing us with chemicals, and suggestions. The efficient technical assistance of Ms. E. SZERDAHELYI, Ms. É. VALENTIN and Ms. C. MOLNÁR are greatly acknowledged.

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Acta Alimentaria, Vol. 30 (1), pp. 81–87 (2001)

EFFECTIVENESS OF SOME CROWN COMPOUNDS ON INHIBITION OF POLYPHENOLOXIDASE IN MODEL SYSTEMS AND IN APPLE

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(Received: 28 March 2000; accepted: 19 September 2000)

Enzymatic browning is (in most cases) an undesirable reaction which usually impairs the sensory properties and chemical changes in raw fruits and vegetables after mechanical operations (such as peeling, coring or slicing).

A great emphasis is put on research to develop new methods to prevent enzymatic browning especially in fresh-cut (minimally processed) fruits and vegetables.

The inhibition effect of crown compounds, macrocyclic ethers, benzo-18-crown-6 with sorbic acid and benzo-18-crown-6 with potassium sorbate, on polyphenoloxidase (PPO) activity was studied. The effectiveness of these compounds was evaluated by using 3,4-dihydroxy phenylalanine (L-DOPA), and chlorogenic acid (3-*o*-caffeoyl-D-quinic acid), the most widespread natural PPO substrates in fruits and vegetables, as well as browning inhibition substances on the cut surface of apples.

Results showed that crown compounds used in this study were effective, both as inhibitors of the oxidation of phenolic compounds (PPO substrates) in model solutions and as inhibitors of enzyme discolorations of real systems (fresh-cut apples).

In the earlier published papers (VUKOVIĆ et al., 1999) the synthesis of crown compound used in this study was presented.

Keywords: crown compounds, enzymatic browning, inhibition, polyphenoloxidase

The colour of foods is one of the most important properties which can be crucial for acceptance of some products. During fruits and vegetables processing and storage of their products numerous reactions occur. Such reactions can have influence on food colour and in general on food quality. Among them, browning discolourations, both, enzymatic and nonenzymatic, are of great importance in food industry.

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0139-3006/2001/$ 5.00

Enzymatic browning

The half of the world's fruit and vegetable crops is lost due to postharvest deteriorative reactions (MARTINEZ & WHITAKER, 1995). Among reactions of deterioration of fruits and vegetables and their products is enzymatic browning (SAPERS, 1993). In most cases enzymatic browning is undesirable. Enzymatic browning is a reaction that results when phenolic compounds of plants, in the presence of polyphenol oxidase (PPO) and molecular oxygen, are oxidized to o-quinones (MARQUES et al., 1995; SAPERS, 1993).

Browning occurs rapidly when the cellular integrity of plant tissues is disrupted by either physical injury during peeling, coring and slicing or physiological disorders such as scald, bitter pit and watercore (LUO & BARBOSA-CANOVAS, 1996). When this happens the phenolic compounds interact with PPO and atmospheric oxygen.

Since the sulfites are limited in their use to certain categories of food products in some countries, because they have been associated with severe allergy-like reactions in some asthmatics (TAYLOR & BUSH, 1986), food processors have turned to a number of sulfite alternatives as new enzymatic browning inhibitors (MOLNÁR-PERL & FRIEDMAN, 1990; SAPERS, 1993). At least three different modes of action for inhibitors can be considered (proposed): (1) the direct inhibition of the enzyme PPO, (2) the chemical reduction of o-quinones back into o-diphenolic compounds, and (3) the manipulation or removal of the phenolic substrates of PPO (VÁMOS-VIGYÁZÓ, 1981).

The objective of this research was to study the effectiveness of new crown compounds, macrocyclic ethers, benzo-18-crown-6 with sorbic acid (BSA), and benzo-18-crown-6 with potassium sorbate (BPS) as potential inhibitors of enzyme discolouration in model solutions with PPO substrates, L-DOPA and chlorogenic acid, as well as inhibitors of enzymatic browning of fresh-cut apples (Idared variety).

1. Materials and methods

1.1. Chemicals

For this study crown compounds have been prepared following the method described in the earlier published paper by VUKOVIĆ and co-workers (1999). The macrocyclic ether benzo-18-crown-6 is a part of compounds used in this study. This macrocyclic ether with sorbic acid (BSA) and potassium sorbate (BPS) forms stable complexes which showed ability (possible practical use) as inhibitors of PPO activity.

Mushroom polyphenol oxidase (PPO; EC 1.14.18.1) product T-7755, with activity of 3000 U mg^{-1} of solid (Sigma Chemical Co., St. Louis, MO, USA) as a dry powder was dissolved in phosphate buffer (pH 6.5, 47 mmol).

PPO substrates, L-DOPA (Sigma), and chlorogenic acid (Sigma) were prepared as 2.5 mmol solutions by disolving in phosphate buffer (pH 6.5).

1.2. Activity assay

The enzyme activity was determined by spectrophotometer CECIL 2000 (CECIL INSTRUMENTS, England). Reaction mixture, in a total volume of 3 ml, included L-DOPA (2.5 mmol), enzyme (16 μg ml^{-1}) dissolved in phosphate buffer (pH 6.5, 47 mmol) and certain concentration of solution of complex compounds, BSA (0.34 to 7.5 mmol) or BPS (0.5 to 5.0 mmol). As reference ("blank") a solution without inhibitors addition was used.

Before measurements, reaction solution (PPO substrate and crown compound in phosphate buffer) were thermostated for 20 min at 25 °C. The enzyme solution was added to treatment solution before measurement.

The appearance of the brown colour, in the case of the L-DOPA was measured at 475 nm as a function of time (for 10 min), and in the case of the chlorogenic acid at 420 nm. The enzyme activity was calculated from the initial part of the curve of the absorption vs reaction time (ΔA/min) (WEEMAES et al., 1997). Measured values were transformed into % inhibition.

1.3. Apple preparation for browning measurements

Apples (Idared variety) were purchased from the local growers and kept at 4 °C until needed. Before sample preparation, apples were held at least one hour at room temperature. To prepare samples, apples were washed with water, peeled and cut into dices (1 cm×1 cm×1 cm) with a sharp knife. One hundred g samples were immediately immersed for 20 s in treatment solution, 0.5% w/v BSA and 0.5% w/v BPS. The excess solution was then blotted with adsorbent tissue and samples were packed in plastic boxes, covered with plastic film to prevent evaporation and stored at 4 °C.

1.4. Treatment evaluation

1.4.1. Inhibition of PPO substrates oxidation. The inhibition of oxidation of PPO substrates (L-DOPA and chlorogenic acid prepared as model solutions) with crown compounds, that was used in this study, was based on a large number of studies which have established that copper ions (Cu^{2+}) can be captured in the cavity of a crown compounds (PEDERSEN & FRENDSDORFF, 1972; HIRAOKA, 1982). The ionic diameter of Cu^{2+} has a value 1.44 Å and is therefore able to form stable complexes with BSA and BPS, which have the cavity diameter between 1.7 and 2.2 Å (PEDERSEN & FRENDSDORFF, 1972). The crown compounds that we used in this study showed their inhibitory effect on mushroom PPO.

To compare the effectiveness of these crown compounds in inhibition of PPO activity, sorbic acid and potassium sorbate, as a part of molecule of crown compounds, were also used.

Inhibition of PPO in model solutions was determined spectrophotometrically (spectrophotometer CECIL 2000, England) at 475 nm for L-DOPA for 10 min, and at 420 nm for chlorogenic acid for 10 min.

1.4.2. Browning assessment. The degree of browning of apple samples was monitored by reflectance measurements immediately after cutting, treatment with crown compounds solutions ("0" time), and during storage of apple samples (untreated and treated) on day 4, 7, and 11. L*, a*, and b* values were measured by the tristimulus colorimeter Minolta CR-300 (Minolta Camera Co., Osaka, Japan) using the averaging mode with fifteen replications. Based on the measured data the calculation of effectiveness of each inhibitor was performed by equation:

$$\Delta E_{ab} = ((\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2)^{1/2}$$

2. Results and discussion

The effectiveness of crown compounds BSA and BPS on inhibition of oxidation of substrates L-DOPA and chlorogenic acid is shown as the concentration of the inhibitors (I_{50}) that causes 50% inhibition of PPO activity (Tables 1 and 2).

The results showed that BSA (5.0 mmol) had an inhibitory effect of 89.7% on oxidation of L-DOPA (2.5 mmol) in the presence of PPO (16 μg ml^{-1}) (Fig. 1). When chlorogenic acid (2.5 mmol) was used as a PPO substrate, the BSA (5.0 mmol) had 79.3% reducing effect on its oxidation (Table 2).

Table 1

The effectiveness of different inhibitors on PPO activity in solutions of L-DOPA

Inhibitors	I_{50} (mmol)[a]
Complex of benzo-18-crown with potassium sorbate	0.720
Complex of benzo-18-crown with sorbic acid	0.870
Sorbic acid	1.350
Potassium sorbate	2.530

[a] I_{50}: the concentration of the inhibitors that causes 50% inhibition of PPO activity

Table 2

The effectiveness of different inhibitors on PPO activity in solutions of chlorogenic acid

Inhibitors	I_{50} (mmol)[a]
Complex of benzo-18-crown with potassium sorbate	1.520
Complex of benzo-18-crown with sorbic acid	1.760
Sorbic acid	2.650
Potassium sorbate	2.530

[a] I_{50}: the concentration of the inhibitors that causes 50% inhibition of PPO activity

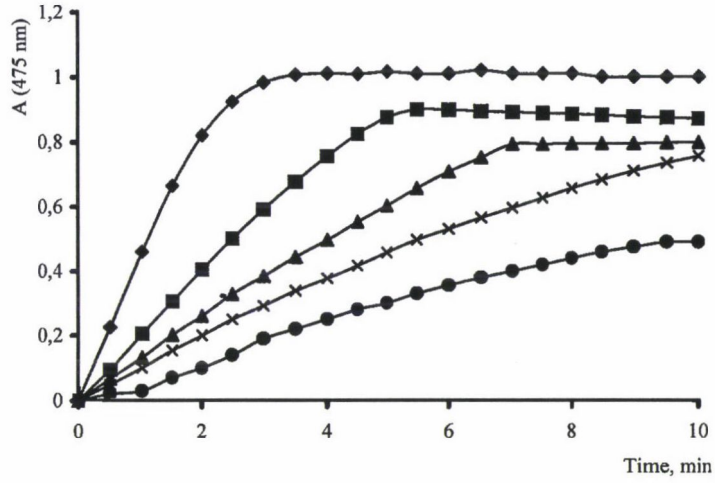

Fig. 1. Effect of different concentrations (0.5 mmol to 5.0 mmol) of complex crown compound benzo-18-crown-6 with potassium sorbate on PPO activity in solution of L-DOPA. ◆: Control; ■: 0.5 mmol BPS; ▲: 1.0 mmol BPS; x: 3.0 mmol BPS; ●: 5.0 mmol BPS

When the solution of BPS (5.0 mmol) was used as an inhibitor of oxidation of L-DOPA (2.5 mmol) it had 90.3% reducing effect on PPO activity. When chlorogenic acid was used as PPO substrate, BPS reduced PPO activity by 80.5%.

The effect of crown compounds (BSA and BPS), as potential browning inhibitors on apple dices is presented in Table 3.

Table 3

Effect of crown compounds, as potential browning inhibitors, applied to apple dices

Treatment	Day	Colour parameters		
		L value[a]	a value[a]	ΔE
Control	0	84.59 (1.84)	–4.21 (0.60)	
	4	80.01 (3.46)	–1.40 (1.19)	9.161
	7	77.88 (3.47)	–1.31 (1.10)	10.566
	11	77.53 (1.53)	–0.13 (0.75)	11.614
0.5% complex of benzo-18-crown with potassium sorbate	0	82.53 (2.10)	–4.33 (0.32)	
	4	81.34 (1.74)	–3.24 (0.31)	3.089
	7	81.41 (1.48)	–2.50 (0.40)	3.731
	11	79.50 (2.28)	–1.67 (0.58)	6.388
0.5% complex of benzo-18-crown with sorbic acid	0	83.40 (2.65)	–3.82 (0.85)	
	4	80.63 (2.90)	–1.68 (1.70)	4.482
	7	80.27 (2.71)	–1.50 (1.08)	4.954
	11	80.14 (3.14)	0.01 (1.02)	6.104

[a] L and a values (means of 15 replicates)
Values in parentheses are standard deviation (SD)

The browning reaction in apple samples was delayed after treatment with 0.5% (w/v) solutions of BSA and BPS significantly. Total colour change (ΔE_{ab}) in treated apples with both crown compounds was significantly lower than in untreated apples (control) during storage.

3. Conclusions

Novel crown compounds, benzo-18-crown-6 with sorbic acid, and benzo-18-crown-6 with potassium sorbate had significant inhibitory effect on mushroom PPO as a catalyst of oxidation of phenolic substrates L-DOPA and chlorogenic acid. Both compounds (BSA and BPS) had a slightly higher effect of enzyme inhibition in case of L-DOPA.

The results obtained in this study showed that the storage life of cut apples can be extended, by applying crown compounds (benzo-18-crown-6 with sorbic acid, and benzo-18-crown-6 with potassium sorbate) as browning inhibitors, for 11 days of storage at 4 °C.

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Acta Alimentaria, Vol. 30 (1), pp. 89–97 (2001)

THE EFFECT OF GROWTH PHASE, CRYOPROTECTANTS AND FREEZING RATES ON THE SURVIVAL OF SELECTED MICRO-ORGANISMS DURING FREEZING AND THAWING

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(Received: 4 May 2000; accepted: 27 September 2000)

The effect of growth phase of cells, cryoprotectant agents and freezing rate on the survival of selected micro-organisms *(Bacillus cereus, Lactobacillus plantarum, Escherichia coli, Pseudomonas fluorescens, Saccharomyces cerevisiae, Kluyveromyces lactis, Candida utilis, Mucor racemosus, Aspergillus niger)* during freezing and thawing was studied. In cases where significant differences were observed stationary phase cells always survived better than exponential phase ones, while of the two cryoprotectant agents studied, horse serum + inositol was always superior to skimmed milk powder + inositol. The effect of freezing rate was different among the studied micro-organisms.

Keywords: cryoprotectant, freezing, micro-organisms, survival

The inhibitory effects of reduced temperatures on physical, chemical and biochemical processes provide the bases of the long-term preservation of cells, tissues and organs (TAYLOR, 1987).

Parameters such as the age of the culture, the presence of cryoprotective agents, the rate of freezing are in many cases critical for the survival of micro-organisms. Although cryopreservation has been used extensively as a method for the maintenance of microbes for a long time, judgement of the effect of some parameters on survival during freezing is still subject to arguments.

According to HECKLY (1978), it is generally accepted that "cells from maximum stationary phase cultures are more resistant to damage by freezing and thawing than cells from the early or midlog phase of growth". Contrary to this, other authors found that some bacteria were more resistant against cold shock (MACKELVIE et al., 1968) and freezing (FRY, 1966), when cells were obtained from the exponential growth phase. From the usually applied cooling rates most authors consider slow cooling/freezing rates

0139-3006/2001/$ 5.00

more favourable in maintaining the viability of microbes (e.g. SMITH & ONIONS, 1994; DAVIS & OBAFEMI, 1985) than fast cooling rates, although SMITH and ONIONS (1994) demonstrated that the optimal cooling rate could be different for different fungi. However, there are other opinions as well. For instance DEÁK and BEUCHAT (1996) suggested fast freezing (coupled with fast thawing) for yeasts. Interestingly, despite of the abundance of results suggesting the opposite, INGRAM (1951) stated that the survival of micro-organisms directly after freezing is nearly independent of cooling rate and according to HECKLY (1978) freezing rate is not a critical factor for survival.

In the present paper we report the results of a study on the effect of the growth phase, cryoprotectants and cooling rates on the survival of some micro-organisms after freezing and thawing. All the selected strains belong to species with potential importance in the food industry either as starter cultures or as contaminants or spoilage organisms.

1. Materials and methods

1.1. Strains studied

The investigated strains with culture conditions are shown in Table 1.

Table 1

Microbial strains used

Name	Accession number	Medium	Incubation temperature
Bacillus cereus	NCAIM B 00076	TGE agar	30 °C
Lactobacillus plantarum	NCAIM B 01133	MRS agar	37 °C
Escherichia coli	NCAIM B 00200	TGE agar	37 °C
Pseudomonas fluorescens	NCAIM B 01154	TGE agar	26 °C
Saccharomyces cerevisiae	NCAIM Y 00200	GPY agar	26 °C
Kluyveromyces lactis	NCAIM Y 00231	GPY agar	26 °C
Candida utilis	NCAIM Y 00383	GPY agar	26 °C
Mucor racemosus	NCAIM F 00598	PDA agar	26 °C
Aspergillus niger	NCAIM F 00735	PDA agar	26 °C

NCAIM: National Collection of Agricultural and Industrial Micro-organisms, Budapest, Somlói út 14-16. H-1118, Hungary
TGE agar: Tryptone glucose yeast extract agar
GPY agar: Glucose peptone yeast extract agar
PDA agar: Potato dextrose agar

1.2. Experimental parameters

Except for the investigated mould strains (in which cases one week old cultures were used in order to have matured spores or conidia) cells from the late exponential growth phase (Ph1) and from the stationary growth phase (Ph2) were used. (In case of moulds the growth phase does not necessarily show direct correlation with the maturity stage of spores or conidia.) In order to determine the growth phase of the cultures grown on agar slants, a preliminary experiment was carried out. Sufficient number of slants of the given medium were inoculated with a loop of suspension made from young (overnight and 24 h in case of bacteria and yeasts, respectively) slant culture. Two slants of each strain were washed with 4–4 ml sterile water in regular intervals (in every hour in case of bacteria and in every second hour in case of yeasts). After homogenisation and dilution (if it was necessary) the absorbance of the suspensions was measured at 540 nm against the blind which was obtained by washing the same, but uninoculated medium. It was possible to fit a curve on the data in case of each strain with the aid of the modified Gompertz equation (ZWIETERING et al., 1990). The specific harvesting time for each strain was determined with the aid of these curves. The vast majority of stationary phase cells of the investigated *B. cereus* strain were spores. Comparing the survival of vegetative cells (Ph1) to spores (Ph2) had no theoretical but practical reason.

As cryoprotective agents two different suspending media were applied:

1) 8% skimmed milk powder + 4% inositol (Cr1)

2) inactivated horse serum + 5 g inositol/100 ml serum (Cr2)

Suspensions were made by pouring the suspending medium to the agar slant culture and stirring by vortex. They were homogenised before use. 0.2 cm^3 aliquots of the suspensions were distributed into sterile glass vials (about 48×6 mm with 1 mm thick wall). The vials were plugged with sterile cotton.

Three different freezing rates were applied. In the first treatment (T1) the suspensions were frozen on a centrifuge rack of a freeze-drying equipment (EDWARDS MODULYO) by the loss of latent heat (evaporative freezing) for 30 min.

In the second case (T2) the suspensions were kept in a commercial freezer at –25 °C for one hour, while in the third case (T3) the vials were submersed into liquid nitrogen (–196 °C) for one minute. The freezing rate for the T2 and T3 treatments were measured by copper-constantan thermocouple with the aid of a potentiometric recorder (RADELKIS OH-814/1) at 2 mV sensitivity. The freezing rates for T2 (freezing at –25 °C) and T3 (freezing in liquid nitrogen) were 2.1 °C min^{-1} and 233 °C min^{-1}, respectively.

Every experiment was done in duplicate. The experimental arrangement is shown in Fig. 1. Following every combination of the treatments described above, the samples were immediately thawed in a 37 °C water bath.

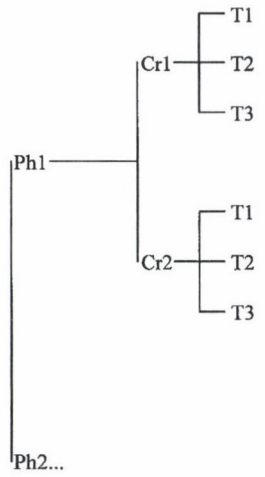

Fig. 1. Experimental arrangement. Ph1: Exponential growth phase, Ph2: Stationary growth phase, Cr1: 8% skimmed milk powder + 4% inositol, Cr2: Inactivated horse serum + inositol (5 g/100 ml), T1: Evaporative freezing, T2: Freezing at –25 °C, T3: Freezing in liquid nitrogen (–196 °C)

The viable count (colony forming unit, cfu) for every suspension was determined before freezing and after thawing. The viable count values obtained after freezing and thawing were normalised by dividing with the corresponding cfu values of the unfrozen suspensions. These normalised values are interpreted as survival ratio.

1.3 Mathematical-statistical evaluation

After logarithmic transformation of the normalised values multi-factor analysis of variance was applied to determine the significant effects of the treatments.

From the least significant difference (LSD) of the logarithmic values the critical survival ratios were calculated as follows:

$$LSD_R = 10^{LSD}$$

where:

LSD = the least significant difference between the logarithmic transformed survival ratios

LSD_R = the least significant ratio between the survival ratios

If the ratio of the survival belonging to the different treatments is higher than LSD_R, significant differences exist between the effects of the treatments. For the

simplest demonstration in tables the survival ratios together with LSD_R (critical ratio) values are summarised. For the mathematical statistical evaluation of the results STATGRAPHICS 5.1 program package (STATISTICAL GRAPHICS CORPORATION, USA) was used.

2. Results and discussion

2.1. The effect of the growth phase on the survival

The growth phase of the microbe had a significant effect on the survival in case of the *B. cereus, K. lactis* and *C. utilis* strains (Table 2). In all three cases the stationary growth phase (Ph2) resulted in a higher survival ratio than the late exponential one (Ph1). These results support the opinion of those authors who prefer stationary to exponential phase (e.g. HECKLY, 1978; OLASZ & TÖRÖK, 1988; STRANGE, 1976; KIRSOP, 1984) in order to ensure high survival ratio. In case of *B. cereus* the better survival of the culture from stationary phase may partly be explained by the spore formation of the species. (None of the investigated yeast strains formed spores under the culture conditions applied in this study.) The absence of significant differences in the survival in case of the other microbes can be – at least partly – considered to be the result of the high resistance to the freeze-thaw stress by some of the investigated micro-organisms. In case of the *S. cerevisiae* strain, for instance, the decrease in survival is within the accuracy of the applied pour plate method.

Table 2

Survival ratio (%) after freezing and thawing as a function of growth phases

Strains	Growth phase (%)		Critical ratio (LSD_R)
	Exponential (Ph1)	Stationary (Ph2)	
*B. cereus**	30.6	91.7	1.37
L. plantarum	78.9	89.7	1.19
E. coli	47.4	54.7	1.39
P. fluorescens	71.1	77.8	1.18
S. cerevisiae	99.8	96.4	1.14
*K. lactis**	57.8	71.3	1.19
*C. utilis**	21.2	34.6	1.33

* denotes significant difference at 95% level

LSD_R: the least significant quotient between the survival ratios

Table 3

Survival ratio (%) after freezing and thawing as a function of cryoprotectants

Strains	Cryoprotectants (%)		Critical ratio (LSD_R)
	Cr1	Cr2	
B. cereus	50.9	55.2	1.37
*L. plantarum**	76.2	92.7	1.19
*E. coli**	40.6	63.8	1.39
P. fluorescens	71.8	77.3	1.18
S. cerevisiae	97.1	99.5	1.14
*K. lactis**	54.8	75.0	1.19
*C. utilis**	22.2	37.6	1.33
M. racemosus	77.1	71.6	1.11
A. niger	75.3	81.2	1.31

* denotes significant difference at 95% level
Cr1: Skimmed milk + inositol
Cr2: Horse serum + inositol
LSD_R: the least significant quotient between the survival ratios

2.2. The effect of the cryoprotectants on the survival

The tested suspending media had significant effects on the survival in case of *L. plantarum*, *E. coli*, *K. lactis* and *C. utilis* (Table 3). In all cases the horse serum + inositol combination (Cr2) proved to be superior to skimmed milk powder + inositol (Cr1). These cryoprotectants were selected, because these or similar ones are widely used in the practice of microbial strain preservation.

2.3. The effect of freezing rate on the survival

In case of evaporative freezing from the time required for freezing (ca. 10 min.) the average freezing rate was estimated to be about 10 °C min^{-1}. This estimation was based on the following considerations. Taking into account the specific heat and latent heat of the water, the heat loss of the suspension during the cooling from room temperature (about 20 °C) to 0 °C is a quarter of the heat loss of the freezing at 0 °C. Counting with a constant heat transfer rate this ratio means that the time necessary for the cooling is about one fifth of the 10 min, required by the freezing of the suspension of 20 °C temperature. It would mean ca 10 °C min^{-1}. Although such calculation of the cooling rate from the time required for freezing is not suggested by MERYMAN (1966), we used it in the case of the other two freezing methods (T2 and T3) as well and in those cases we experienced good agreement with the data actually measured.

Table 4

Survival ratio (%) after freezing and thawing as a function of freezing rates

Strains	Survival ratio (%)			Critical ratio (LSD_R)
	T1	T2	T3	
B. cereus	52.8	46.7	60.5	1.47
*L. plantarum** (T1-T2, T1-T3)	71.3	94.0	88.9	1.24
*E. coli** (T1-T2, T2-T3)	33.3	92.0	43.2	1.50
*P. fluorescens** (T1-T3, T2-T3)	62.1	69.3	95.7	1.22
S. cerevisiae	92.5	101.6	100.9	1.17
*K. lactis** (T1-T3, T2-T3)	85.7	88.3	34.9	1.24
*C. utilis** (T1-T3, T2-T3)	100.6	82.8	2.9	1.42
*M. racemosus** (T1-T2, T2-T3)	78.2	63.4	82.8	1.14
*A. niger** (T2-T3)	87.1	66.8	97.7	1.39

* denotes significant difference at 95% level
T1: evaporative freezing
T2: freezing at –25 °C
T3: Freezing in liquid nitrogen (–196 °C)
LSD_R: the least significant quotient between the survival ratios

The freezing rate had a significant effect on the survival in the case of *L. plantarum*, *E. coli*, *P. fluorescens*, *K. lactis*, *C. utilis*, *M. racemosus* and *A. niger* (Table 4). In the case of *Lactobacillus plantarum* the evaporative freezing (T1: 10 °C min^{-1}) resulted in a significantly lower survival than the other freezing rates (T2: 2.1 °C min^{-1}; T3: 233 °C min^{-1}). This result does not support the "two factor hypothesis" (MAZUR et al., 1972) and may indicate that it is not generally valid for every cell type and every freezing condition. In case of *E. coli* the slowest freezing (T2: 2.1 °C min^{-1}), while in case of *P. fluorescens* the fastest freezing (T3: 233 °C min^{-1}) resulted in the highest survival ratio. The judgement of the effect of the freezing rate on the survival is therefore contradictory. One of the reasons must be that different microbes react differently to different freezing rate. This is clearly demonstrated by the results for the two Gram-negative bacteria (*E. coli* and *P. fluorescens*).

From the studied yeasts the fastest freezing rate (T3: 233 °C min^{-1}) resulted in a significantly lower survival in the case of *K. lactis* and *C. utilis* than the other two freezing rates. The difference for *C. utilis* exceeded one order of magnitude. Our findings on the effect of freezing rate on the survival of yeasts are in good agreement with the data of SMITH (1993) and SMITH & ONIONS (1994), while they differ from the findings of DEÁK and BEUCHAT (1996) according to which, for yeast cells, fast freezing (coupled with fast thawing) is less lethal than slow freezing.

From the studied moulds *M. racemosus* had the lowest survival when frozen with the slowest cooling rate (T2: 2.1 °C min^{-1}). In case of *A. niger* the slowest freezing rate (T2: 2.1 °C min^{-1}) resulted in a significantly lower survival ratio than the fastest one (T3: 233 °C min^{-1}). These results are not in agreement with the findings of those authors who consider slow freezing rates more favourable in maintaining the viability of fungi than fast freezing rates.

Our data also reveal that Ingram's opinion (INGRAM, 1951) which was accepted also by JAY (1992) can be negotiated, that is, it is not supported by these investigations that the survival ratio of micro-organisms after freezing is always nearly independent of the cooling rate. During our study out of the 9 investigated microbes the different freezing rates resulted in significantly different survival ratios in 7 cases. From the remaining two, in case of *S. cerevisiae* the small decrease in viability (less than 10%) may have masked the effect of different freezing rates (Fig. 2). We have to add that in the present study not only the freezing rates were different, but the final temperatures as well and this may also have influence on the survival ratio.

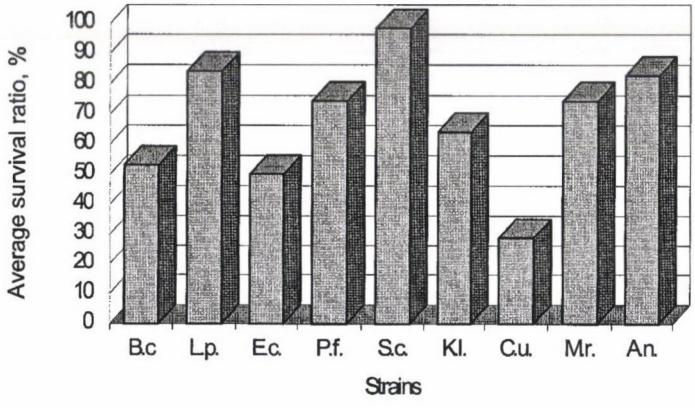

Fig. 2. The average survival ratio (calculated as the average of different treatments) of micro-organisms after freezing and thawing. B.c.: *Bacillus cereus,* L.p.: *Lactobacillus plantarum,* E.c.: *Escherichia coli,* P.f.: *Pseudomonas fluorescens,* S.c.: *Saccharomyces cerevisiae,* K.l.: *Kluyveromyces lactis,* C.u.: *Candida utilis,* M.r.: *Mucor racemosus,* A.n.: *Aspergillus niger*

Conclusions

Under the present experimental conditions the microbial cells from stationary growth phase showed usually higher survival ratios than cells from late exponential phase. This difference was significant in three cases out of the studied seven.

For four of the nine investigated strains horse serum and inositol proved to be better cryoprotectant than the skimmed milk + inositol combination.

The effect of freezing rate was different for the different microbes. As a consequence, if the aim is to achieve the possible highest survival rate, the above mentioned parameters should be optimised for each species.

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Acta Alimentaria, Vol. 30 (1), pp. 99–111 (2001)

EVALUATION OF THREE METHANOL FEED STRATEGIES FOR RECOMBINANT *PICHIA PASTORIS* MUT^S FERMENTATION

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(Received: 17 May 2000; accepted: 18 September 2000)

Methylotrophic yeast *Pichia pastoris* is an ideal host organism for recombinant protein production. However, adequate methanol feed is still a critical point of successful product formation in *P. pastoris* Mut^S fermentations.

Three methanol feed strategies were tested: an organic vapor sensor, a dissolved oxygen controlled methanol addition and a pre-determined model, as well.

The organic vapor sensor proved to be unsuitable for methanol concentration measurements when samples were taken from the head space of the bioreactor, but may have the potential to substitute expensive gas analyzers in methanol fed-batch with a suitable selector submerged into the fermentation media.

Dissolved oxygen and substrate consumption did not show strict mathematical relation. However, drop of dissolved oxygen tension for periodic methanol addition may be applied for the determination of the substrate concentration in the media. The rate of methanol consumption shows peaks at 0.45 and 0.95% (v/v) substrate concentrations.

The rate of the specific methanol consumption of our model organism was determined. Based on the value of 0.023 h^{-1}, which is significantly less than suggested by earlier experiments, a successful pre-determined methanol feed strategy was set up.

Keywords: methanol, fermentation, control, *P. pastoris*

The methylotrophic yeast, *Pichia pastoris* has great potential in recombinant protein production (BUCKHOLZ & GLEESON, 1991). Recombinant *Pichia* has been reported to express several proteins from various host organisms at high level, such as HIV-1 envelope glycoprotein, hirudin or human tissue factor (SCORER et al., 1993; ROSENFELD et al., 1996; AUSTIN et al., 1998). Most of the proteins up to 90 kDa are

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expressed extracellularly and give the majority (>90%) of the protein content of the cell-free media, which simplifies the purification procedure (CREGG et al., 1993). Unlike *Saccharomyces cerevisiae*, *P. pastoris* lacks α1,3-glycosidase activity thus does not hyperglycolisate the asparagine residues of proteins (GRINNA & TSCHOPP, 1989; MIELE et al., 1997). Furthermore, recombinant products from *P. pastoris* appear to keep the wild-type disulfide forms (IKEGAYA et al., 1997). These features result in mammal-like glycoprotein structure with full enzyme functions and potentially no immune reaction in humans (BIEMANS et al., 1998; OHTANI et al., 1998).

P. pastoris has two alcohol-oxidase genes, AOX1 and AOX2, however the latter gives only the 10% of its alcohol-oxidase activity (CREGG et al., 1989). Both genes are under glucose and glycerol initiated catabolic repression and are activated by methanol. Recombinant protein genes are cloned into the AOX1 gene (type Mut^S) or into the HIS4 gene of *P. pastoris* under the control of an AOX1 promoter (type Mut^+). Mut^+ grows faster on methanol. However, in many cases Mut^S *Pichia* showed higher functional protein production than Mut^+ (LOEWEN et al., 1997). Since Mut^S consumes methanol with extremely slow rate and is sensitive to methanol accumulation in the media, the appropriate control of methanol feed is of stringent necessity.

So far two distinct methods have been applied for methanol feed control in recombinant *Pichia* Mut^S fermentations. The simpler method works with a pre-determined methanol-feed strategy (Easy Select Pichia Expression Kit; CHEN et al., 1996). The second one includes a gas analyzer connected to the headspace of the bio-reactor to measure the methanol concentration on-line. A major drawback of this kind of methanol concentration measurement is the high cost of the analytical instrument. Thus attempts have been made to substitute the gas analyzer with less expensive units such as organic vapor sensors (GUARNA et al., 1997).

A third method to control methanol feed can be measuring a parameter, which depends on the methanol concentration in the media. Simple on-line computer control strategy for methanol feed, based on dissolved oxygen level readings has been performed with other methylotrophic species (RENARD et al., 1985). Similar strategy seems to be applicable for *P. pastoris* Mut^S strains, although only a single report has been published to our knowledge (RODRÍGUEZ HIMÉNEZ et al., 1997). Even in this case, the dissolved oxygen control was combined with a pre-determined methanol-feed strategy.

The purpose of our work was to establish the potentials of three different methanol-feed control strategies in the case of a human serum albumin producing Mut^S type *P. pastoris* as a model organism: a pre-determined, a dissolved oxygen controlled as well as an organic vapor sensor controlled methanol addition.

1. Materials and methods

1.1. Microorganism

Pichia pastoris GS115 Mut^S $His4^+$ with a single copy of human serum albumin V fraction (HSA) gene downstream of its AOX1 promoter (Invitrogen, USA) was maintained on YEPD agar slant (2% D-glucose, 2% peptone, 1%yeast extract {all media recipes are from Easy Select Pichia Expression Kit, (CHEN et al., 1996)}).

1.2. Media and cultivation parameters

One point two and 1.5 l stirred tank-reactors (Biostat Q and M, respectively, B. Braun, Germany) with B. Braun CSFV2 acquisition and control program was used in the experiments. In the 1.2 l reactor, the agitation speed was 1000 min^{-1} (magnetic stirrer of triangle-shaped cross-section), whereas it was 800 min^{-1} in the 1.5 l fermentor (flat blade turbine impeller). The 1.5 l bio-reactor was used for methanol-sensor experiments, while 1.2 l reactors were used for all other fermentations. Constant 1 l min^{-1} airflow was applied. pH control was applied in the methanol-fed phases. The temperature of the fermentations was 30 °C.

Seventy ml of a 24 h-old inoculum (BMGY media: 1% glycerol, 2% peptone, 1% yeast extract, 1.34% yeast nitrogen base, 0.00004% biotin, 0.1 mol l^{-1} KH_2PO_4/K_2HPO_4 at pH 6.0; 28 °C, 280 r.p.m.) was used to inoculate the 800 ml final volume fermentors resulting in a 1 g l^{-1} dry weight initial cell concentration (SIEGEL & BRIERLEY, 1989). The pH of the fermentation media (4% glycerol, 2.67% cc. H_3PO_4, 1.82 g l^{-1} K_2SO_4, 1.49 g l^{-1} $MgSO_4.7H_2O$, 0.413 g l^{-1} KOH, 0.093 g l^{-1} $CaSO_4$) was set to 5.0 prior to inoculation. The glycerol batch phase lasted for 16–24 h and its end was indicated by the raise of dissolved oxygen tension (DO). As the DO reached 85%, the methanol feed was started (100% methanol with 12 ml l^{-1} PTM_1 solution (g l^{-1}): 65.0 $FeSO_4.7H_2O$, 20.0 $ZnCl_2$, 6.0 $CuSO_4$. $5H_2O$, 3.0 $MnSO_4$. H_2O, 0.5 $CoCl_2$. $6H_2O$, 0.2 $NaMoO_4$. $2H_2O$, 0.2 biotin, 0.08 NaI, 0.02 H_3BO_4, 5 ml l^{-1} H_2SO_4). The methanol was added periodically by sterile syringes through a silicone membrane or continuously by infusion pumps (MTA KUTESZ, Hungary). In the methanol-phase, the pH was maintained at pH 3.0 or 5.2.

1.3. Assays

Cell concentration was determined by optical density (at 600 nm, 1 cm light path, in appropriate dilution; Ultrospec Plus, Pharmacia LKB, Sweden) and by dry weight measurements (filtration on Millipore 0.42 μm membrane, dried at 105 °C for 5 h). Glycerol concentration was determined by iodometry (HORWITZ et al., 1975), methanol was measured by gas chromatography (Laboratorni Pristoje, Czech R.). Cell-free

filtrated supernatant from fermentation media was used for total protein measurements and electrophoresis. The samples were stored at –20 °C in a buffer with 0.37 g l^{-1} Na_2EDTA, 1.21 g l^{-1} TrisHCl final concentration, at pH 8.0. Total protein concentration was determined by bicinchoninic acid reagent (Pierce, USA) or by the coumassie blue method. SDS electrophoresis was used to analyze the protein content of cell-free samples with gradient pre-fabricated poly-acrylamide gels (Pharmacia Phast System, Sweden, Phast Gel Gradient 8–25, Phast Gel SDS Buffer Strips). The gels were developed by silver staining, which has the capability to detect 5 μg protein per band as a minimal concentration. Crystallized HSA V (Sigma, USA) was used as positive standard for electrophoresis in 200 μg ml^{-1} solution. The scanned pictures of the gels were evaluated by Kodak Digital Science 1D Image Analysis Software. The set-up of the semiconductor on-line methanol sensor experiments with SnO_2 organic vapor sensor (Figaro Engineering Inc., Japan), air pump (KNF Neuberger, Germany), heating ribbon (Barnstead/Thermoline, USA) and recorder (Radelkis, Hungary) is described in the experimental part.

2. Results and discussion

2.1. Test of semi-conductor organic vapor sensor for P. pastoris *fermentation*

To improve recombinant *P. pastoris* AOX1 fermentation, three strategies for methanol feed control were tested. As a first approach, we used a semi-conductor methanol-sensor to measure the methanol concentration on-line during the fermentation (Fig. 1). The exhaust-air was connected to the methanol sensor through an oil-free air pump. The air-pump forwarded a constant air-flow through the methanol-sensor.

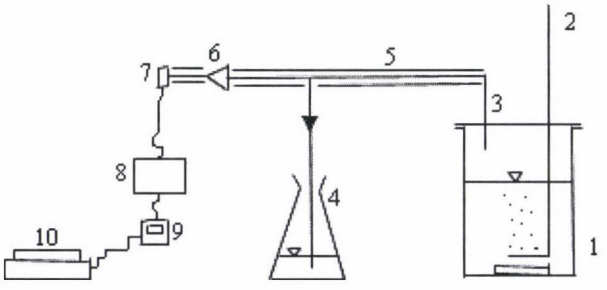

Fig. 1. Experimental set-up with semi-conductor methanol sensor. 1: fermentor; 2: air supply; 3: exhaust air; 4: extra air outlet; 5: heating ribbon; 6: air pump; 7: methanol sensor; 8: amplifier; 9: digital multimeter; 10: recorder

The extra air was led into a water bath. An adjustable heating ribbon ensured that the exhaust-air would be at 160 °C. The tubing between the fermentor and the sensor was as short as possible and composed of glass. An amplifier received the signal from the sensor. The amplifier had a built-in voltamater to set the zero level at the beginning of the fermentation. The amplifier was connected to a recorder and a multimeter, which showed the signal in mV.

To establish the capabilities of the sensor, its characteristics were determined in a water/methanol model system. We added the methanol to the water stepwise, increasing the concentration from 0 to 1.1% (v/v). We always waited with the following methanol addition until stable electrode-signal was given, which took 10–20 min. The semi-conductor methanol sensor gave similar results in our model system than in earlier experiments (AUSTIN et al., 1992). Figure 2 shows both the measured and the calculated values from a hyperbolic fit.

The sensor was used during a 1.5 l fermentation (Fig. 3). The measured values do not show accordance with the points calculated from the hyperbolic equation gained in the model system. Moreover, there could be no significant mathematical connection set up between the methanol concentration and the signal of the sensor. Also, the signal/noise ratio was large at higher measured voltages probably due to the low specificity of the sensor (data not shown). An additional problem was that the volume of the fermentation media decreased almost to its half during the 6-day-long fermentation, since the exhaust cooler had to be turned off.

To decrease disturbances and volume loss, a submerged unit was applied. The stainless steel unit had a 2 cm^2 silicon membrane surface submerged into the fermentation media. A stable oil-free air-flow washed the membrane's inner surface axially, then the air was led to the sensor. This set-up could not ensure sufficiently high methanol concentrations for the sensor to detect. The two different experimental set-ups showed that this kind of sensor can not be used for on-line methanol measurements during fermentation without a suitable selector for methanol.

2.2. Dissolved oxygen as a possible factor to control methanol feed in recombinant P. pastoris *fermentation*

A second way to control the methanol feed during recombinant *Pichia* fermentation can be measuring a parameter, which depends on the methanol concentration of the fermentation media and/or the activity of the yeast's alcohol-oxidase system. The most common parameters of this kind are the acid releasing rate of the microbe and the dissolved oxygen level in the fermentation media.

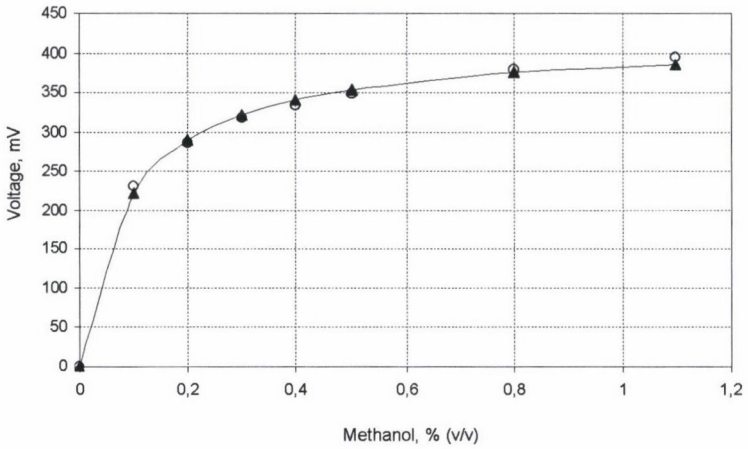

Fig. 2. Test of methanol sensor in methanol/water model-system. O: measured output signal from amplifier (mV); ▲: 415.43c/(0.09+c) R=0.9976; c: methanol concentration (%, v/v)

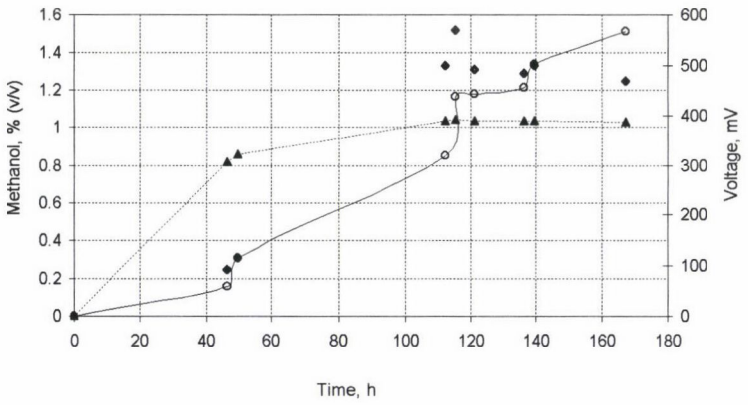

Fig. 3. Test of methanol sensor during 1.5 l fermentation. ◆: methanol concentration in media (%, v/v); O: measured output signal from amplifier (mV); ▲: 415.43c/(0.09+c); c: methanol concentration (%, v/v)

However, the pH does not change during the methanol-fed phase of *P. pastoris* fermentation, in contrast to the glycerol-phase, in which the pH decreases from 6.0 to 2.8. For this reason the dissolved oxygen level seemed to be the sole measured parameter other than the methanol, suitable to control the substrate feed during the product formation phase.

The oxygen utilization is more directly connected to the activity of the alcohol-oxydase system, since methanol is the sole carbon source during product formation. The activity of the alcohol-oxydase system depends on the methanol concentration in the media. Also, the cell mass determines the amount of catabolized oxygen. The dissolved oxygen level of the media divided by the dry cell mass can show the activity of the alcohol-oxidase 2 system, if constant aeration is applied. Nevertheless a major problem is that the *P. pastoris* Mut^S can utilize methanol only at an extremely slow rate and changes during the slow substrate consumption may not be noticed on the bases of dissolved oxygen level. To test, if dissolved oxygen is suitable to control methanol feed, first we measured how the alcohol utilization of the recombinant yeast depends on the methanol level in the media. We also determined if the dissolved oxygen level reflects the substrate utilization by the recombinant yeast. Finally, the influence of the methanol concentration on the dissolved oxygen level was measured.

The methanol concentration in the media was measured by offline gas-chromatography. The methanol consumption was counted as the difference of the methanol content of two following samples corrected with the amount of substrate supplied in this time interval. Unfortunately the rate of methanol consumption was commensurable to the methanol taken by the exhaust air flow, thus we had to estimate the methanol concentration decrease caused by the aeration. In our model, the methanol, leaving through the surface of the media in the bio-reactor was neglected. Only the mass transfer from the liquid phase into the air-bubbles was taken into consideration. A simple equilibrium between the two phases was supposed:

$$c_{liquid} \xleftrightarrow{K_I} c_{gas} \qquad K_I = \frac{c_{gas}}{c_{liquid}}$$

$$c_{gas} = K_I \cdot c_{liquid} \qquad \frac{dc_{liquid}}{dt} = -f \cdot c_{gas} = -f \cdot K_I \cdot c_{liquid}$$

$$\int_{c_{liquid,1}}^{c_{liquid,2}} \frac{1}{c_{liquid}} dc_{liquid} = -\int_0^t f \cdot K_I dt$$

$$c_{liquid,2} = c_{liquid,1} \cdot \exp(-f \cdot K_I \cdot t) = a \cdot \exp(-b \cdot t) \qquad \text{(I)}$$

where

K_I – equlibrium constant between gas and liquid phase
c_{gas}, c_{liquid} – methanol concentration in gas and liquid phase
t – time
f – air flow (constant)
a, b – constants

Based on equation I, the methanol concentration in the media can be defined as an exponential function of the time. This description shows a good correspondence with the values which we gained in a model experiment using a fermentation media without cells and glycerol (Fig. 4). Thus a correction can be taken into account on the basis of this model when calculating methanol consumption rates of *P. pastoris*.

Examining the methanol consumption by unit amount of biomass and the dissolved oxygen as the function of methanol concentration in the media at different fermentations, no strict correspondence was found between them (Fig. 5). However, the alcohol-oxydase system is virtually activated from 0.3% (v/v) of methanol, with a putative local methanol consumption maximum around 0.45% (v/v) substrate and a peak between 0.8–1.1% (v/v). This assumption is supported by earlier data.

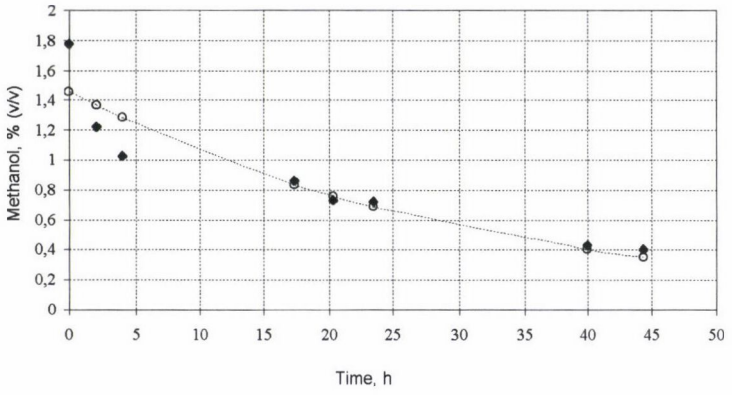

Fig. 4. Model experiment to determine the methanol concentration change in the media caused by constant air-flow. ◆: measured methanol concentration in media (%, v/v); O: 1.457 exp (–0.0321t), R=0.9322, t: time

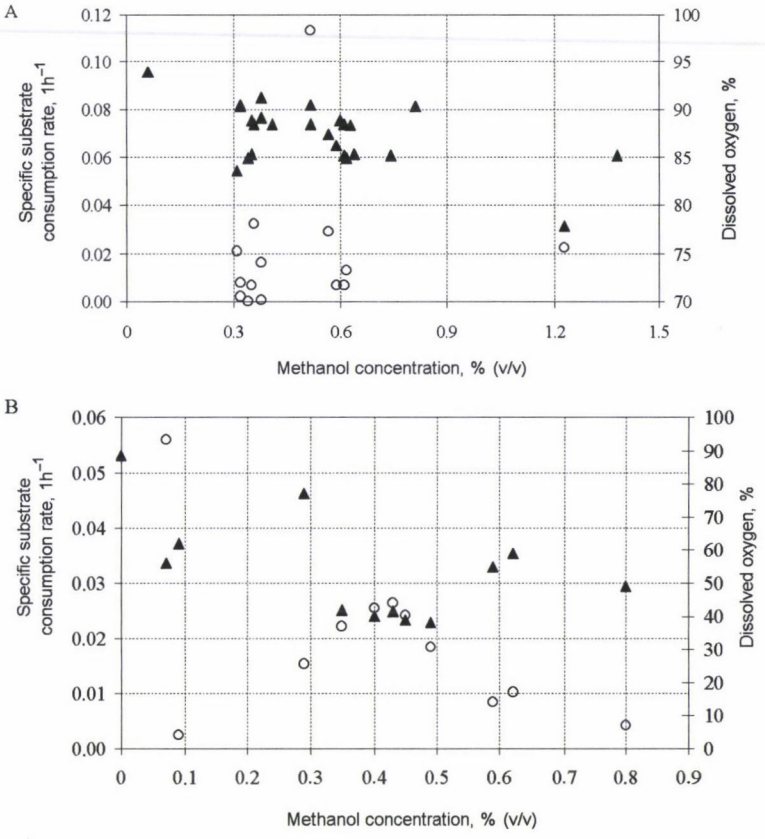

Fig. 5. Specific substrate consumption and dissolved oxigen levels at two different fermentations (A and B) as the functions of methanol concentration in the media. O: specific substrate consumption rate [g methanol (g cell dry weight)$^{-1}$ h^{-1} = h^{-1}]; ▲: dissolved oxygen level in the media (%)

We also examined, wheather the drop of dissolved oxygen level for periodic methanol addition reflects the methanol concentration in the fermentation media. It was supposed that the dissolved oxygen level alters the methanol concentration and the methanol concentration change in the media, since both of these influence the metabolic activity of the cells. Indeed, a maximum seemed to describe the dissolved oxygen drop as the function of the methanol concentration change/methanol concentration in the media (Fig. 6). The change of substrate concentration for periodic methanol addition can be counted from the amount of methanol added to the fermentor divided by the fermentor volume. This way, the actual methanol concentration can be determined from

the oxygen level drop, using the polynomial fit from Fig. 6. The problem with the equation is that it describes only dissolved oxygen changes lower than 9% and applies only to the 0.4–0.85% (v/v) methanol concentration range. Also, the changes of dissolved oxygen for the same methanol concentration change/methanol values showed differences with the age of the microbes in the product forming phase.

2.3. A pre-determined model based on methanol consumption measurements

Pre-determined methanol feed is widely used in recombinant *P. pastoris* fermentations. However, the most accepted substrate feed strategy, suggested by the developer of the Pichia Expression Systems proved to be unproper in case of our Mut^S HSA strain. Gradually increasing the continuous methanol feed rate from 1 to 3 ml l^{-1} h^{-1} and keeping this value during the rest of the fermentation proccess at around 200 g l^{-1} wet weight of cells resulted in undesired methanol accumulation in the media (data not shown). Furthermore HSA production was not observed in this fermentation. The calculated specific methanol consumption in our fermentations varied between 0.016 and 0.032 h^{-1} compared to the 0.056 h^{-1} dry weight suggested by Invitrogen manuals. Since limiting substrate concentration should be applied, when the alcohol consumption is relatively low, we suggest to count with 0.023 h^{-1} specific methanol consumption. To test if this methanol feed was adequate for recombinant protein production, we applied higher and lower continuous feed as well.

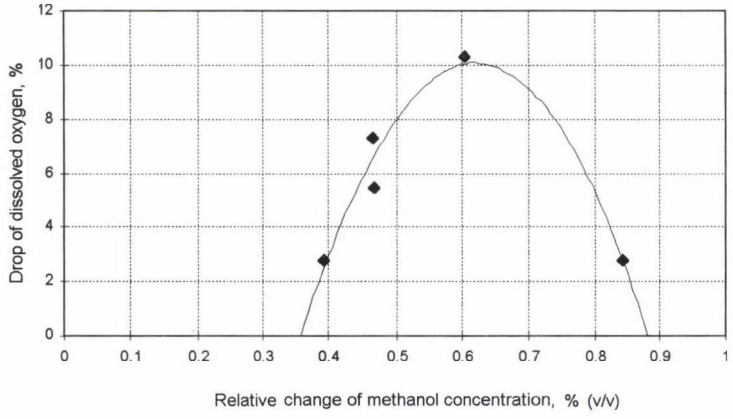

Fig. 6. Correlation between the drop of dissolved oxygen for periodic substrate addition and the relative change of methanol concentration (change of methanol concentration for substrate addition/methanol concentration after the addition). ◆: drop of dissolved oxygen; –: $y=-149x^2+181.65x-46.20$, $R^2=0.9451$ where y: dissolved oxygen, x: relative change of methanol concentration

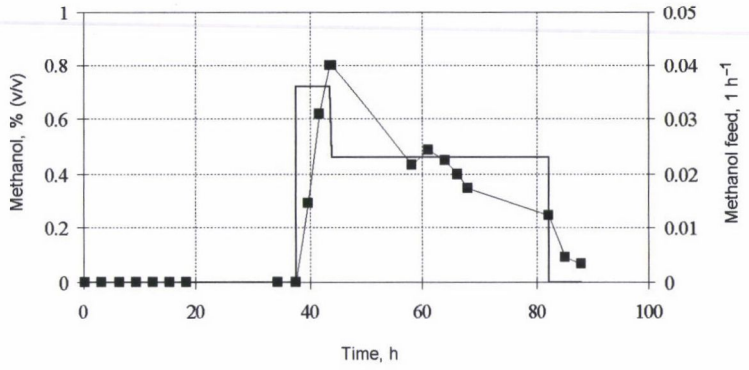

Fig. 7. Pre-determined methanol feed in *P. pastroris* Mut^S fermentation, based on substrate consumption measurements. ■: methanol concentration (%, v/v); –: specific methanol addition (h^{-1})

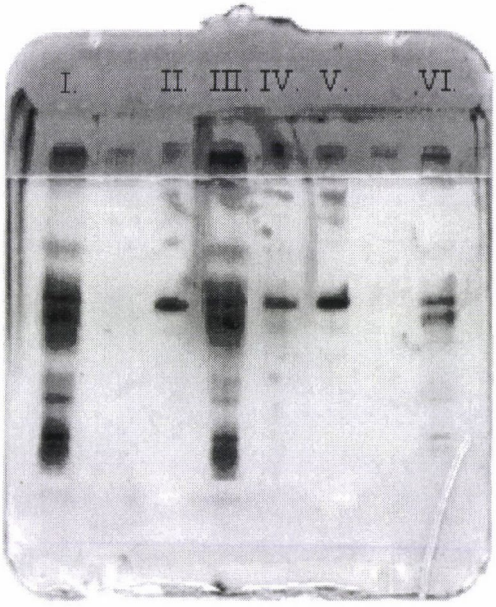

Fig. 8. Electrophoresis of samples from the fermentation with 0.023 h^{-1} pre-determined specific methanol addition. IV: 68th h of fermentation (149 mg l^{-1} HSA); VI: 90th h of fermentation (89 mg l^{-1} intact HSA); II, V: HSA standards (200 mg l^{-1}); I, III: samples condensed by ultrafiltration

After the adaptation period, the methanol feed was set to 0.036 h^{-1} which resulted in a substrate concentration increase to 0.8% in 6 h (Fig. 7). Then the methanol feed was reduced to 0.023 h^{-1}. Keeping this substrate feed, the methanol concentration stayed in the substrate limiting range in a 30-h-long period. Finally, turning off the methanol supply caused the rapid decrease of the alcohol concentration. The produced HSA concentration in the supernatant of the media peaked at the end of the 0.023 h^{-1} substrate feed period (70th h) with 149 mg l^{-1} HSA (Fig. 8). After the substrate supply was terminated, proteolytic degradation occured, decreasing the HSA concentration, showed by a second band on the gel. When 50% lower methanol supply was applied, after an initiative methanol concentration peak of 0.37%, the substrate concentration decreased below 0.1% and no HSA production was observed.

3. Conclusion

The organic vapor sensor applied has the potential to provide methanol concentration data during the fermentation of recombinant *P. pastoris*. However, a suitable selector for methanol with large contact surface submerged into the media is still required.

Dissolved oxygen level is not proper to control methanol feed, but drops in dissolved oxygen level for periodic methanol addition seem to correlate with methanol concentration in the media. This correlation is not strict enough to be used succesfully in *P. pastoris* Mut^S fermentations, but may be useful for *P. pastoris* Mut^+ cultivations.

A 0.023 h^{-1} specific methanol consumption rate was determined, which greatly differs from the 0.056 h^{-1} value suggested by manuals. Based on this specific substrate consumption value, a pre-determined methanol-feed strategy was tested and proved to be applicable for HSA producing *P. pastoris* Mut^S.

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This work was supported by OTKA grants T-002182 and T-029882.

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Acta Alimentaria, Vol. 30 (1), pp. 113–118 (2001)

Preliminary communication

COMPARISON OF TWO METHODS IN PURIFICATION OF MEAT-DNA FOR PCR

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(Received: 24 May 2000; accepted: 18 September 2000)

The Wizard Clean up System and a three-phase partitioning (TPP) method were used to purify genomic pork-DNA of various food samples for amplification. Quality of DNA purified by Wizard resin and partitioning was controlled by spectrophotometer and electrophoresis, respectively. A 108 bp fragment from the porcine growth hormone gene was applied, according to MEYER and co-workers (1994). Of all the samples prepared, amplicons were obtained by the pork-DNA specific PCR. Partitioning was found to be an efficient DNA purification step in preparation of PCR-grade DNA.

Keywords: DNA purification, three-phase partitioning, PCR

To meet requirements in the field of food analysis, application of specific, reliable, quick and sensitive methodologies are of increasing importance. One of the high sensitivity methods is PCR, the use of which for detection of food components has been successful even in the investigation of heat-treated foodstuff. Microorganisms, genetically modified organisms, raw and processed foods can be detected by PCR (MEYER & CANDRIAN, 1996). However, PCR is inhibited by a number of components originating from the foodstuff or chemicals used for purification of DNA (ROSSEN et al., 1992).

Three-phase partitioning is a protein purification method used primarily for crudes. It was found by LOVRIEN and co-workers (1987) that systems containing water, tert-butanol, dissolved proteins and ammonium-sulphate form a third phase or middle layer, on shaking and successive centrifugation. In case of enzymes, if the third phase is soluble, purification of small apolar molecules is achieved even though the specific activity of the given enzyme remains unchanged. It was found earlier that following proteinase K digestion

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0139-3006/2001/$ 5.00

of foods, wheat-DNA could be separated from other constituents by three phase partitioning and applied successfully to PCR (SZAMOS et al., 1998). The objective of the present work was to compare the Wizard DNA Clean up System and the method of interfacial partitioning in the purification of pork meat genomic DNA.

1. Materials and methods

Raw meat samples were obtained from slaughterhouse, foodstuff containing pork meat, Zala meat, pork sausage (red meat products) and winter salami (ripened, fermented product), were purchased in supermarket. All the reagents used were of analytical grade.

1.1. Extraction, purification and characterisation of DNA

DNA isolation was carried out by the method of MEYER and co-workers (1994). Minced meat (0.6 g) was transferred to a sterile 1.5 ml Eppendorf tube; 860 µl extraction buffer (10 mmol TRIS-HCl, 150 mmol NaCl, 2 mmol EDTA, 1% SDS, pH 8.0), 100 µl 5 mol guanidine hydrochloride and 40 µl proteinase K (20 mg ml^{-1} solution) was added. The mixture was incubated at 55 °C for 3 h. After digestion, the samples were centrifuged at 14.500×g for 10 min.

1.1.1. Wizard DNA clean-up procedure. Four hundred and fifty µl aqueous phase was purified with 1 ml of DNA Wizard resin following the instruction of the manufacturer.

1.1.2. Three-phase partitioning method. To prepare DNA for PCR, 450 µl of aqueous phase was pipetted into a 2 ml screw capped tube, then 312 µl of saturated ammonium-sulphate solution (sAS) was added. This solution was thoroughly mixed, then 236 µl of tert-butylalcohol was added. The system was vigorously shaken then let to stand for 15 min. Following low speed centrifugation, 500 µl of the lower aqueous phase was pipetted into a clean Eppendorf tube and 680 µl of sAS and 365 µl of tert-butylalcohol were added.

The system was treated as above, but the disc formed at the interface was separated and dissolved in 450 µl of distilled water.

In the case of Pastanaria sauce /Maggi/ and sausage 145 µl of proteinase-K reaction mixture was diluted to 600 µl then 450 µl of sAS and 310 µl of tert-butylalcohol were added. When partitioning was completed, 1 ml of aqueous phase was pipetted into a bio-vial Beckman (4.5 ml) and 1.36 ml of sAS and 730 µl of tert-butylalcohol were added. Following phase separation, DNA-disc (third phase) was dissolved in 700 µl of TRIS-EDTA buffer, pH 8.0.

1.2. Polymerase chain reaction

The primers of MEYER and co-workers (1994) were used. 250 ng of template DNA was added to 50 µl of reaction mix in a 0.5 ml MARSH tube containing 1× reaction buffer, 2 U Taq polymerase, 0.2 mmol of dATP, dCTP, dGTP and dTTP, 0.5 mmol of each primer and 2 µg ml^{-1} bovine serum albumin. The samples were denatured at 94 °C for 3 min (hot start), then 35 cycles of denaturation at 94 °C for 5 s, was performed, followed by annealing at 60 °C for 30 s and extension at 72 °C 40 s, with a final extension at 72 °C for 3 min (PDR-91, BLS thermocycler). The reaction was stopped by cooling to 4 °C. Ten µl of PCR mixture was separated using a 8% polyacrylamide gel-electrophoresis (AUSUBEL et al., 1989) with TRIS-boric acid-EDTA (0.89 mmol TRIS, 0.89 mmol boric acid, 2 mmol EDTA, pH 8) running buffer, for 3 h at 54 V. The bands were visualized by staining with ethidium bromide and photographed under UV transillumination.

2. Results

UV spectra of DNA-solutions illustrating product related differences are shown in Fig. 1 (JASCO 7850 uv/vis spectrophotometer). The highest DNA yield was obtained for winter-salami with high meat and low water content, while about half of the amount of DNA (compared to salami) could be prepared for heat-treated meat products and Maggi sauce. As it can be seen, purification with a good yield was achieved by the first stage TPP compared to spectrum of the crude DNA solution (Fig. 1). The amount of the DNA prepared by the second TPP amounts roughly one third of the first stage, while its quality proved to be suitable for amplification. Results of DNA purification are summarised in Table 1. In several cases a single three-phase partitioning was sufficient

Table 1

Comparison of Wizard and three-phase partitioning method of DNA purification

Meat products	Wizard DNA preparation technique		Three-phase partitioning (first)		Three-phase partitioning (second)	
	Σ DNS µg	A_{260}/A_{280}	Σ DNS µg	A_{260}/A_{280}	Σ DNS µg	A_{260}/A_{280}
Zala meat	13.4	2.0	57.9	1.6	25.4	1.9
Pork sausage	7.4	1.8	30.8	1.5	–	–
Winter salami	30.4	1.9	47.4	1.2	19.3	1.6
Luncheon meat	31.1	1.9	58.08	1.5	20.9	2.0
Pastanaria Maggi sauce	7.7	1.8	17.4	1.7	–	–

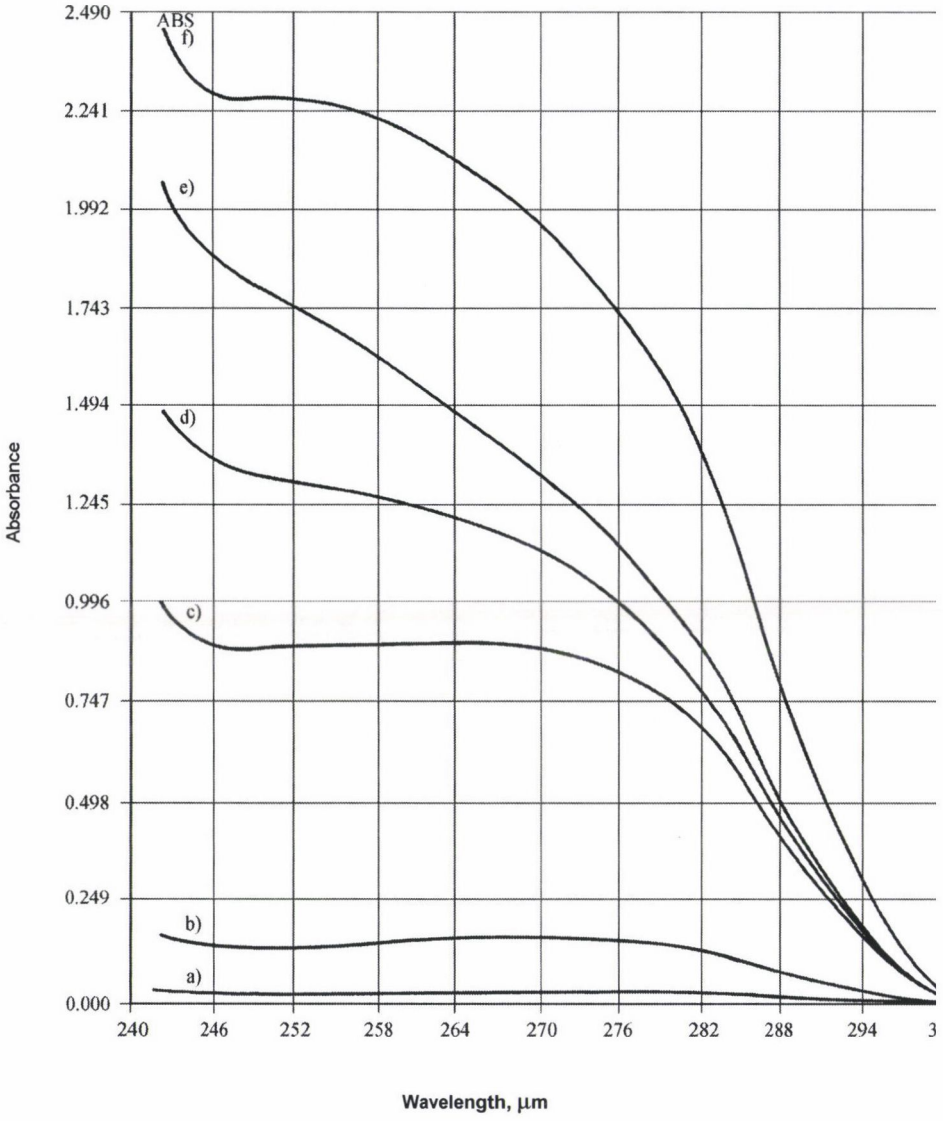

Fig. 1. The UV spectra of proteinase-K digested foodstuff samples. a: fat; b: lard; c: pork sausage; d: luncheon meat conserve; e: goliat salami; f: winter salami

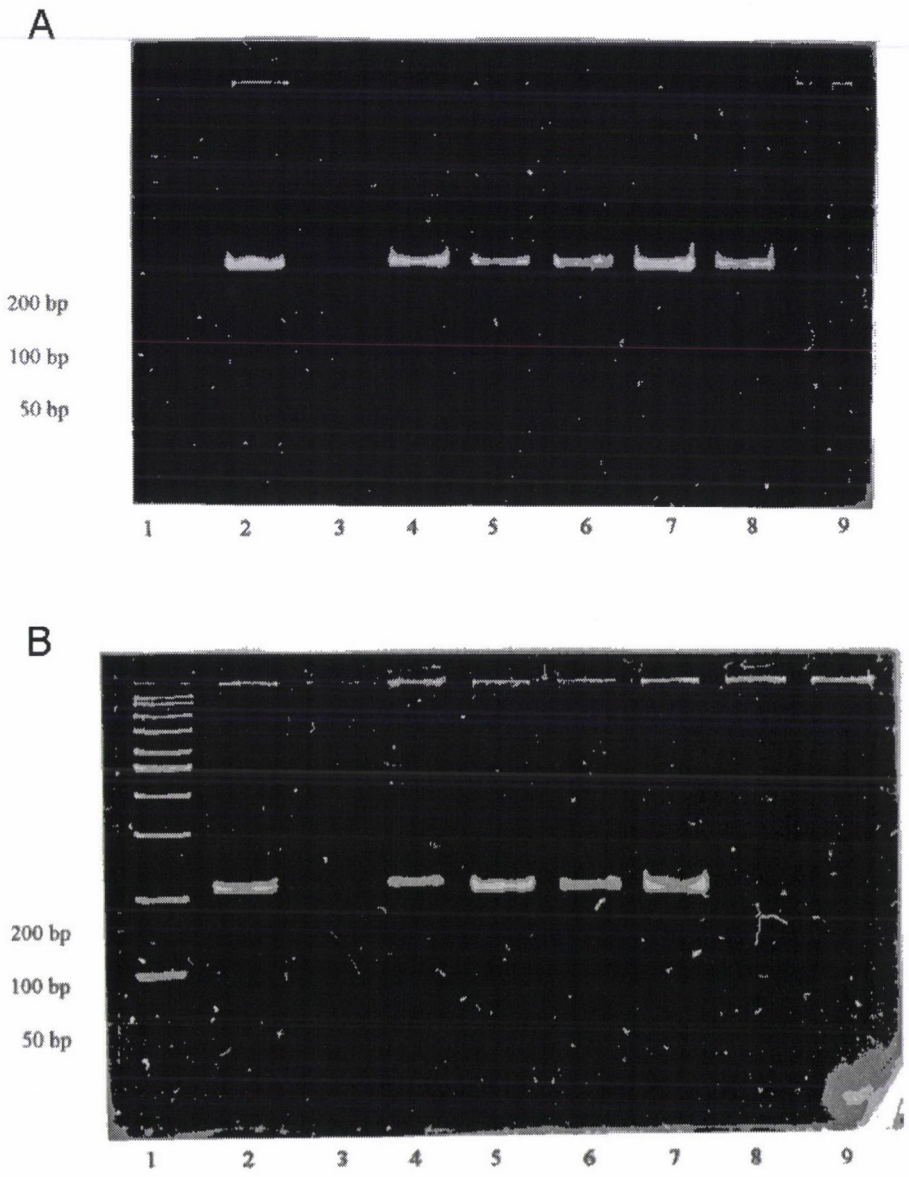

Fig. 2. PCR product of different meat and meat products DNA (DNA was purified with A-Wizard technique, B-TTP method). Lanes: 1: DNA ladder (50–2000 bp); 2: positive control (pork meat); 3: negative control; 4: pork sausage; 5: Zala meat; 6: luncheon meat conserve; 7:winter salami; 8: Pastanaria Maggi sauce; 9: beef meat

to prepare higher amounts of DNA, though accompanied by small amount of protein compared to that obtained by the Wizard system. Higher purity DNA could be obtained by a second partitioning, more over in case of salami, ratios of A_{260} and A_{280} for DNA partitioned are close to those of prepared by the Wizard system. DNAs prepared by both methods could be amplified by PCR at 250 ng 50 μl^{-1}. In Fig. 2 the electrophoretogram of PCR products of different food samples are shown. Signals (108 bp) were obtained for pork meat and all food samples investigated, while negative controls did not result in amplicons.

3. Conclusion

From different foods containing pork meat PCR-grade DNA could be purified by three-phase partitioning. Compared to purification method by Wizard resin, TPP is rapid and of lower cost, moreover it can easily be scaled up. This fact is of significance when inhomogenic materials are investigated. Compared to common DNA isolation methods (ZIMMERMANN et al., 1998) TPP can be primarily characterised by its low cost and short time of manipulations.

*

This work was supported by Ministry of Agriculture and Regional Development. The authors would like to thank Mrs. C. MOLNÁR for technical assistance and Mr. I. MAKK for photographs.

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Acta Alimentaria, Vol. 30 (1), pp. 119–121 (2001)

Book reviews

Polyamines in health and nutrition

S. BARDÓCZ & A. WHITE (Eds)

Kluwer Academic Publishers, Dordrecht, 1999, ISBN 0-412-82220-2, 352 pages

"Polyamines in Health and Nutrition" edited by S. Bardócz and A. White is a unique collection of papers discussing the true physiological role of polyamines. The importance of polyamines for all living cells has long been recognised and hundreds of papers and numerous books are published on this topic continuously. In spite of this the discrepancies between the biosynthetic capacity and polyamine content of cells remained unsolved. In this book the authors draw attention to polyamine uptake/transport and by identifying the endogenous and exogenous polyamine sources try to explain some of the failures of earlier research. The recognition that dietary polyamines contribute to the body pool and ways by which this pool can be changed using the diet is also investigated with the aim to benefit our health.

The main objective of this book was to collect information on the content, composition and bioavailability of polyamines in foods and to establish the mechanisms involved in the absorption, distribution, metabolism, bioavailability and storage of polyamines in the body.

In Part I (WHAT DO POLYAMINES DO? POLYAMINE SYNTHESIS, CATABOLISM AND HOMEOSTASIS) basic information is given on the structure, function and metabolism of polyamines. In Part II (THE POLYAMINE POOLS OF THE BODY) the readers' attention is drawn to the importance of the body pool and some of the ways it can be manipulated using either transgenic approach, inhibitors of polyamine biosynthesis or polyamine analogues. There are 4 main chapters within Part II: A: Manipulations of biosynthesis: transgenic approach, inhibitors and analogues; B: Contribution by diet and bacteria; C: Polyamines in the gut, uptake, distribution and bioavailability; D: Manipulation of polyamine body pools. Information on the polyamine composition of foods and contribution by the diet based on daily intake data by different age groups are given and the size of the body's polyamine pool is estimated. Information is provided on the mechanisms of polyamine uptake/transport, bioavailability and inter-organ distribution. The question whether luminal bacteria can contribute to the polyamine pools of the body is also addressed. Suggestions on decreasing the polyamine content of our food by using genetic modifications or by natural selection is also discussed. Finally, in order to influence tumor growth and improve our health novel dietary approaches are discussed for manipulating the inter-organ distribution of polyamines.

What are the novel information of this book in point of view of food science? The polyamine content of the most common foods consumed in the EU countries were measured and the daily intake of adults were calculated. It was found, that in Mediterranean countries, where people eat more fish, fruit and vegetables,

putrescine and spermidine intake is much higher and spermine intake is much lower than in Northern countries, where more meat, dairy products and cereals are eaten.

Storage and food processing can modify the polyamine content of different foods. If food is baked or fried or processed by microwave, its polyamine content remains unchanged. However, boiling reduced the polyamine content of foods through release into the cooking water. There is little change in the polyamine content during food preparation (if food is hygienically stored and safely handled). However, if microbial contamination occurs during storage and food processing, putrescine content of foods might increase as a result of microbial activity.

Though polyamine supplementation is not required for the healthy body, there are two special cases when polyamine supplementation would be beneficial: infants fed on artificial feed formulae, which have negligible amounts of polyamines, would benefit from spermidine and spermine supplementation to the levels naturally occurring in human milk. Similarly, patients on total parenteral nutrition for long periods of time, would benefit from spermidine supplementation.

Since the proliferation also of tumor cells is dependent on the sufficient supply of polyamines for their growth, removal of polyamines from the diet could have tremendous health benefits for cancer patients. Limiting dietary polyamine supply can contribute to slowing down tumor growth. Another possibility awaiting exploitation is the production of transgenic plants with low polyamine contents which could be used as the basis of special diets designed for cancer patients.

The editors and authors use the unique opportunity provided by the publishers to present novel information on the polyamines, some of which can be found in the literature, but with the freedom of interpretation.

GY. HAJÓS

Indirect food additives and polimers: migration and toxicology

V. O. SHEFTEL

Lewis Publishers 2000 CRC Press LLC, Boca Raton, 2000, ISBN 1-56670-499-5, 1320 pages

V. O. Sheftel's book is a very important handbook, it provides useful comprehensive information for specialists dealing with packaging of food, drugs and cosmetics, producing packaging materials, as well as dealing with the hygienic evaluation of the mentioned materials.

Because of the unique characteristics of plastics they have been applied in a wide range for packaging of these materials. Plastics are among the main source of chemical contamination of food products, because certain component unavoidably migrate from them during storage. In the USA according to the sanitary regulations (FDA) the plastics and their additives were qualified as indirect food additives.

The toxicity of plastics depends on the toxicity of migrable additives and their migration levels. The book gives information about nearly 2000 potential food contaminant additives on more than 1200 pages. It gives a view of the most frequently used starting materials, monomers, and groups of additives: plasticizers, stabilizers, antioxidants, auxiliary materials (catalysts, inhibitors, etc.), solvents, additives of rubber and other additives. It presents data for identification such as: chemical name, molecular formula, molecular mass, CAS number, synonimes and trade names; and typical data concerning the chemical properties, applications, expositions, migration data into food, water etc; as well as detailed toxicological data (acute-toxicity, short-term-, long-term toxicity, mutagenicity, carcinogenicity etc).

The book is useful also because it summarizes up-to-date toxicological data, data of similar books (e.g. Toxicology of plastics by René Lefaux, having been published also in Hungary in 1970) are nowadays for most part out-of-date and incomplete.

Sheftel gives in his book a review of the regulation, the use of additives in plastics, reviews concerning sanitary regulations, first of all EEC, FDA and the English regulations, as well as the less known Russian data.

After every reviewed material he presents the applied references. The book contains information from authentic and regarded sources. A wide variety of references are listed, but the author cannot assume responsibility for the validity of all materials or for consequences of their use.

It has been useful to assemble data from literature concerning the migration of additives next to the toxicological data. Beside summarizing toxicological data, collecting data from the literature referring to the migration of single additives bears special importance. The book contains all data referring to the plastics and being important from food hygienic point of view found in the literature and such as, it is a unique handbook for all specialists dealing with plastics, toxicology, food packaging.

G. SIMKOVITS

Typeset by
WEGATREND Kft., Budapest

PRINTED IN HUNGARY
PXP Ltd., Budapest

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ACTA ALIMENTARIA

An International Journal of Food Science

Editor
J. HOLLÓ

Volume 30 **June 2001** **Number 2**

Akadémiai Kiadó
Budapest

ISSN 0139-3006
CODEN ACALDI

ACTA ALIMENTARIA

An International Journal of Food Science

Sponsored by the Joint Complex Committee on Food Science of the Hungarian Academy of Sciences and published by financial support of the Ministry of Agriculture and Regional Development.

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Acta Alimentaria is a quarterly journal in English, publishing original papers on food science. The main subjects covered are: physics, physical chemistry, chemistry, analysis, biology, microbiology, enzymology, engineering, instrumentation, automation and economics of foods and food production.

Distributor:

AKADÉMIAI KIADÓ
H-1519 Budapest, P.O. Box 245, Hungary

Fax: (36-1) 464-8221
E-mail: kiss.s@akkrt.hu
http://www.akkrt.hu

Publication programme, 2001: Volume 30 (4 issues)

Subscription price per volume: US$ 236.00, including normal postage, airmail delivery US$ 20.00.

Acta Alimentaria is published 4 times per annum: March, June, September and December

Acta Alimentaria is surveyed by Chemical Abstracts, Current Contents/Agriculture, Biology and Environmental Science, ASCA, BIOSIS, Nutrion Abstracts and Reviews.

AAlim 30 (2001) 2

ACTA ALIMENTARIA

AN INTERNATIONAL JOURNAL OF FOOD SCIENCE

VOLUME 30
2001

AKADÉMIAI KIADÓ
BUDAPEST

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Acta Alimentaria, Vol. 30 (2), pp. 123–126 (2001)

Editorial

FOOD MICROBIOLOGY AND ITS PROSPECTS AT THE TURN OF THE MILLENNIUM

During its less than 150 years of history, food microbiology had to cope with an ever-widening range of problems and tasks, and it will have to do probably so in the forthcoming decades. In our age of globalization, all scientific problems and practical tasks become international. At the turn of the millennium, there is a "psychical" need to make a survey also on some of the key "drivers" of future food microbiological research, with particular reference to the *food quality* and *safety*. (The equally important area of food biotechnology will hopefully be considered by somebody else).

One of the evident tasks, which we have to deal with, relates to the complex problems rooted in a consumer demand for fresher, more natural foods, free of synthetic additives that possess a high margin of microbial safety. This somewhat contradictory demand stimulates the food industry to develop new, milder methods for food treatments: *minimal processing*, and still to control microbes without having a deleterious effect on food quality. Minimal processing is coupled with a long refrigerated storage life which can be achieved with vacuum or modified atmosphere packaging. However, it is a recent understanding that the reliance of refrigeration for storage of ready-to-eat, minimally processed foods, especially those packaged in modified atmospheres, does not preclude the growth of, and infection or intoxication of the consumer by, psychrotrophic pathogens. Consumers interest in chilled foods, raw or pre-cooked, but with extended shelf-lifes, may lead to products well suited for these hazards.

Current epidemiological data suggest that the frequencies of both illness and death associated with food-borne diseases are unacceptably high and that a broad array of potential pathogens contaminate our food supplies. Intense animal husbandry practices and major difficulties in controlling the spread of pathogens in the food chain, together with the acquired resistance of some foodborne ones highlight the importance of sanitary practices during the harvesting, processing and distribution of raw foods and food ingredients. It is increasingly realized that the technological development should progress "hand-in-hand" with an intense research on understanding of how *food-borne microbes adapt to and survive stresses* encountered within the food chain.

0139-3006/2001/$ 5.00

The emergence of newly identified or adapted pathogens with enhanced resistance to existing preservation systems and processes is a major concern in an industry to move toward milder processes. Various stress factors applied in the mild techniques induce *stress responses in microorganisms*, phenomena which should be well understood in safe application of mild preservation methods. We should understand better the selective pressures leading to *emergence of resistant strains*. Future food microbiology should then include a fundamental understanding of *microbial physiology* and *molecular biology of food-borne microorganisms*. A mechanistic understanding of microbial physiology and metabolism provides new approaches to food preservation and the foundation for genetic control of foodborne pathogens. We still have to know more about the mechanism and predictability of effects of even the traditional food processing techniques on microbes, and the *role of food structure in microbial growth*. Developments in *molecular biology* and *microbial ecology* will change or deepen our perspective about food microbiology. The "shock wave" from the explosion of knowledge in molecular biology is just now hitting food microbiology. Having made its mark on analytical food microbiology, it is only a matter of time before molecular biology provides new methods for ensuring food quality and safety.

A considerable amount of research into the development of non-thermal physical preservation methods and the use of biological preservation agents have been done already. Various newer non-thermal physical treatments appear to be promising tools for food preservation. However, conflicting data about their effects on bacterial spores and enzymes, and conflicting kinetic data need to be reconciled. The mechanistic understanding of the effects of these processes on foods, food components, and microbial cells, as well as of storage-dependent changes after non-thermal treatments, is limited. Thus, further research should concentrate on these questions. The remarkable resistance properties of *bacterial spores* will further stimulate research on better control of sporeformers. There is a considerable interest in using *biopreservatives* and understanding the mechanisms and specificity of their actions. Extensive research on sources and activities of natural antimicrobials is currently taking place. In the future, more information needs to be gathered on these natural compounds, including their activity, spectra, mechanims, targets, and interactions with environmental conditions and food components. Prior to their regulatory approval as food antimicrobials they have to be proven toxicologically safe. More natural may not necessarily mean more safe.

The requirements usually necessitate a specific blend of processing methods and aids for each category of product. The logical use of combination of techniques remains an attractive focus for the future work. However, more basic research is needed, which should focus on a better understanding of the homeostasis, stress reactions,

and especially on "multitarget" preservation of foods. Better understanding of both the traditional preservation processes and the application of novel preservation technologies will allow the development of further new and safe products.

Because of the recognition that quality and safety cannot be ensured by traditional inspection and product testing regimes, there are a global move toward product safety on process design and the control of processing by the application of *hazard analysis critical control point* (HACCP) concept and quality management systems. Introduction of more efficient *risk management* is needed to prevent the entry of unwanted microorganisms into food, prevent their growth, and to inactivate them where it is necessary. This again emphasises the need for further efforts on *predictive modelling of growth, survival, and inactivation* of microorganisms, which is an important relatively new development in food microbiology. Predictive microbiology is an evidently basic requirement for improving the scientific foundation for a better *risk quantification,* and also for risk management techniques such as the HACCP system. It is a danger, however, that the HACCP concept is sometimes only formally used, without good understanding the food-borne pathogens, and the potential hazards associated with the consumption of certain foods. Epidemiological data must be integrated with information from clinical and food microbiological studies and consumption data to produce credible *risk assessments.* At present, these data are generally more qualitative than quantitative, even in the richest nations, and they form an imperfect picture of the occurrence of food-borne disease. For a proper risk assessment in many cases a great deal of research data have to be generated in the near future, particularly in terms of *virulence* of food-borne pathogens, *dose-response relationships*, but also in relation to the use of *pre-* and *probiotics.* Having this knowledge, production of safe foods of proper keeping quality will increasingly be based on identification of possible problems before they occur and on the establishment of control measures at the optimal stages in processing.

The microbial food safety and stability requirements are inevitably bound with the need for introduction of n*ew methodical skills* for detection, qualification and quantification of microbial contamination. Among methodologies in food diagnostics, the *immunological* and *genetic techniques* have brought completely new potential to food testing. Rapid genetic and immunological methods for detecting food-borne microorganisms, predictive modelling and hazard analysis and critical control points are also key issues to the future of food microbiology. Further progress is expected and welcome in both conventional and *molecular biological methods*, particularly with respect to their speed, sensitivity and specificity. Development of food testing technology will continue to benefit from advances in clinical nucleic acid- and antibody-based diagnostics.

It is hoped that increasing awareness on the part of governments and industries of the importance of food microbiology will lead to infusion of more resources for research. All efforts in food microbiological research are furthered and facilitated by a better understanding of the physical chemistry and biochemistry of foods, and the functionality of their components. In this way, development of food microbiology will be ever-more in interaction with other sub-disciplines of food science, including also food engineering, releasing thereby the traditionally rigid separation of individual areas of scientific interest. Therefore, a multidisciplinary approach and an increased support for integration of food science and development of appropriate technologies are indispensable for the improvement of safety and preservation of the food supply. The key to this development is the cooperation between microbiologists and food technologists on the one hand and mathematicians and statisticians on the other. Thus, management of the complex problems characterizing our present epoch is a challenge for every food scientists, and alleviation of the emerging microbiological problems demand an increased co-operation both at national and international levels.

J. FARKAS

Acta Alimentaria, Vol. 30 (2), pp. 127–143 (2001)

SUITABILITY OF TEXTURAL PARAMETERS FOR CHARACTERIZATION OF TRAPPIST CHEESE RIPENING

O. BARA-HERCZEGH[a], K. HORVÁTH-ALMÁSSY[a], J. FENYVESSY[a] and F. ÖRSI[b]

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(Received: 13 January 2000; revision received: 6 October 2000; accepted: 14 November 2000)

The objectives of this study were to monitor the changes in textural parameters of Trappist cheese during ripening and shelf-life, and to find correlation between the changes in instrumental texture parameters and the age or sensory properties of the product. The textural parameters of Trappist samples of 5 different manufacturing processes were determined with a QTS 25 Texture Profile Analyser. In addition to the mechanical tests, composition analyses and sensory tests were also conducted. The empirical values from the mechanical tests were evaluated with statistical methods (single-valued and multivariate analysis). The age and the quality of the product can be estimated from the textural parameters despite the inhomogeneity of the samples that is due to the manufacturing processes.

Keywords: Trappist cheese, ripening, instrumental texture parameters, Texture Profile Analyser

Most rennet cheeses are ripened before consumption to achieve the desirable organoleptic qualities. Ripening involves a series of complex biochemical processes, which can be grouped broadly into proteolysis, lipolysis and lactose/lactate metabolism. The extent and type of ripening depend on storage time and temperature, cheese composition (especially moisture and salt levels) and the types and activities of enzymes and micro-organisms present. The physical and chemical changes that occur during ripening determine the overall organoleptic qualities of cheese (FARKYE & FOX, 1990). The body of a cheese is closely related to its microstructure which may be regarded as gel-like and is modified by proteolysis and other biochemical changes during ripening (FOX & MULVIHILL, 1990). The structure development in cheese is of particular interest because the development of structure takes place quite slowly and this can be followed by the accompanying changes in the rheological properties. Cheese is viscoelastic in nature, meaning that it exhibits both solid (elastic) and fluid (viscous) behaviour (KONSTANCE & HOLSINGER, 1992).

0139-3006/2001/$ 5.00

It would be advantageous to apply easily applicable, rapid physical methods which are suitable for routine work for the characterisation of the consistency and texture of cheese based on well-defined textural parameters.

The aim of this study was to find correlation between measured textural or mechanical parameters and the age of Trappist cheese in order to be able to trace and determine cheese ripening. We supposed that the textural parameters measured accurately under well-defined conditions might provide supplementary information for cheese grading.

Trappist is the most popular cheese in Hungary. It is of French origin, an invention of the friars of the "Notre Dame de Port du Salut" monastery. It appeared in our country at the end of the 19th century. It is a salted, ripened semi-hard cheese with characteristic sensory properties, which is made from pasteurized cow's milk and added lactic acid forming starter cultures (*Streptococcus lactis, Streptococcus cremoris, Leuconostoc citrovorum, Leuconostoc dextranicus, Streptococcus diacetillactis*), rennin, calcium chloride, and potassium nitrate. The weight of the product is about 1–1.5 kg, the form is disc-shaped and it is packed in a plastic foil that is shrinking for heat treatment. Its cutting surface is consistently pale yellow, dry or slightly wet to the feel. Some shiny round holes can be seen, whose diameter is 3–4 mm. Trappist cheese can be sliced well, easy to shape, elastic and it crumbles in the mouth. Its taste and smell are characteristically aromatic, mellow, acidulous, slightly salty (KLIERMEIER & LECHNER, 1973, CODEX ALIMENTARIUS HUNGARICUS, 1995).

Viscoelasticity may be demonstrated with rheological multicomponent models. These models consist of elastic springs obeying Hooke's law, and viscous dashpots which obey the Newtonian law of viscosity. The simplest possible models consist of a single spring and dashpot in series (Maxwell model) or in parallel (Voight or Kelvin model) connection. In the Kelvin body the deformation and the recovery are not instantaneous. This is in line with practical experience. The detailed investigation of the Maxwell and Kelvin models shows that exactly these ones do not demonstrate the viscoelastic form-change, though the Kelvin model gives the better approximation. With the extension of the Voigt and Maxwell bodies, the Burgers body is produced in a way that a Voigt body is placed end-to-end with a Maxwell body. In this model both the deformation and recovery are retarded to various degrees and the recovery is incomplete (PRENTICE, 1984a). As the number of elements increases it becomes disproportionately difficult to analyse the data. Only the simplest models have practical importance.

The rheological properties of cheese depend upon its structure. The three major constituents of cheese are casein, fat, and water, each contribute to the structure and therefore to the rheological properties in its own specific way. At room temperature, the casein is solid, the fat is a mixture of solid and liquid fractions, giving it what may be described as plastic properties, and the free water is liquid. Casein forms and open

mesh-like structure (CREAMER & OLSON, 1982). Fat is entrapped in this mesh. The aqueous phase contains the soluble constituents of the milk serum and some salt that was added during cheese making. Some of the water is bound to protein so it is largely immobilised. The remainder is free and fills the interstices between the casein matrix and fat.

The primary structure of the cheese curd is a three-dimensional grid whose sides consist of chains of casein molecules. This provides a structure of considerable inherent rigidity. During the clotting process these chains join together the individual casein particles in the serum. The serum surrounds the fat globules so that each cage encases at least one globule or cluster of globules. The complete cheese curd after the clotting process consists of an aggregate of these cells of casein plus fat and the whole is pervaded by the aqueous phase. If a force is applied to this structure, the deformation will be primarily controlled by the rigidity of the grid, modified by any elasticity in its structural members. The fat within it limits the deformation of the cells. At very low temperatures the fat would be solid and this would only add to the rigidity. At normal temperature, at which cheese is ripened and consumed, the fat has both solid and liquid constituents and has its own peculiar rheological properties. Any deformation of the casein matrix would also require the fat to deform. The water between the fat and the casein acts as a lubricant. As a result, the rigidity of the fat is added to that of the casein in a complex manner and it is this, which gives rise to the peculiar viscoelastic properties of the cheese.

The end product is not a continuous aggregate of cells. During manufacturing, the curd is cut into small pieces at least once to allow any excess serum to drain away. As the serum drains away, the casein matrix shrinks onto the fat globules, making a more compact whole. The final cheese mass is an aggregation of these granules which forms a secondary structure having its own set of rheological properties. Some processes such as pressing distort the whole structure of the cheese. During manufacturing and the subsequent ripening, the basic structure may be modified by mechanical or thermal treatment, or bacteria and any residual enzymes may act upon the casein. These agents may change the organisation of the structure or they may cause continuous fat globules to coalesce. Finally, water may be lost by evaporation from the surface (PRENTICE, 1984b). During maturation, primarily its pH and the ratio of casein to moisture content determine the structural changing of a cheese (LAWRENCE et al., 1987).

Although protein, fat and water constitute by far the greatest part of any cheese mass, other constituents can not be ignored either. Salt in the examined Trappist cheese is not present in quantities large enough to make any sensible contribution to the rheological properties of the cheese. All of the salt is present only in solution in the water. Its effect on the properties of the whole cheese is minimal. Salt may have some

serious contribution to the rheological properties of the cheese by indirect action. Being present at a high concentration, it inhibits the proteolysis in cheese.

Some cheeses, like Trappist, also contain a significant quantity of gas, which is concentrated in the holes. Hidden cracks or holes give rise to irregularities when measurements are made by any type of penetrometer.

The application of rheology to cheese is of the same age as rheology itself. Empirical, imitative and fundamental tests were applied alike for measuring the texture of cheese. The development of methodology was reviewed by KONSTANCE and HOLSINGER (1992). Among the empirical and imitative tests the Ball Compressor as a mechanical thumb provided some information about the mechanical deformation of the surface of the cheese (VOISEY, 1971). Among the force-measuring instruments the different kinds of penetration tests had importance because of their simplicity. A good example is the penetrometer, which uses needle as a probe. The major disadvantage of these tests was the influence of local variations and the need of multiple measurements to obtain a representative average value due to the inhomogeneity of cheese. Other types of penetration tests are the puncture tests. The punch, which is cylindrical in shape, penetrates into the food, and the instrument measures the force required to push it, and plots a force-time or force-distance curve such as the imitative test (BOURNE, 1979). The technique of texture profile analysis (TPA) was developed with the improvement of imitative methods, which imitate the action of the human jaws. SHAMA and SHERMAN (1973a, b) applied this technique (TPA) first to cheese. In the fundamental tests a given stress is applied for a given time to a sample and the relative deformation caused is observed (PRENTICE, 1984a). The deformation was measured with force-compression tests (CASIRAGHI et al., 1985; TUNICK et al., 1991; YUN et al., 1994), with creep-recovery tests or stress relaxation tests (SHAMA & SHERMAN, 1973b; PELEG, 1979; NOLAN, 1987; YUN et al., 1994). The dynamic mechanical properties were investigated with small-amplitude oscillatory shear (NOLAN et al., 1989; TUNICK et al., 1990). Although the literature on the rheological analysis of cheese is abundant, no standardised testing methods have been developed as yet; and it is also difficult to obtain representative samples. The number of the investigated cheese varieties is limited.

1. Materials and methods

1.1. Cheese samples

Trappist cheese samples were purchased from the Szekszárd Cheese Plant of the Tolna Dairy Company. All crude samples were in the original form with respect to package and weight (approx. 1 kg). The crude samples were ripened at 7 °C according

to the parameters given by the factory. The required temperature was ensured in a thermostat. One sample was examined every week in the ripening procedure and then during storage, so that the changes that occur during these periods could be revealed. The ripening procedure of the Trappist cheese was conventionally three–four weeks. The age of tested samples in the ripening procedure and then during storage was 3, 7, 14, 21 days and 28, 42, 56, 70 days, respectively. Forty samples were taken from 5 different production lots. (The samples were manufactured twice a month from February to April.)

1.2. Qualification methods of cheese samples

The main chemical components of the samples – dry matter, fat content in dry matter and salt content - were determined according to the HUNGARIAN STANDARDS (1989a,b,c), the protein content determination was made by Kjeldahl Tecator method (A.O.A.C., 1990). The HUNGARIAN STANDARDS (1989a,b,c) are analogouse to the ISO standards.

Besides the determination of the main chemical composition of the samples (water, fat, protein, and salt), the quantitative descriptive test of the HUNGARIAN STANDARD (1987b) (20 scores, with weighted factors) was applied during storage (21, 28, 42, 56, 70 days) of the cheese. The standard sensory test evaluates five characteristic groups of properties: appearance (shape), interior (colour, structure and hole formation), consistency, smell and taste, with maximum scores of 5. The sensory analysis of the cheeses was performed by a permanent panel of five panellists. They were trained, and the sensory suitability tests were carried out in accordance with the recommendations of MOLNÁR (1991b) and HUNGARIAN STANDARDS (1982a,b,c,d), identification of basic tastes: MSZ 7304/1, discrimination of taste: MSZ 7304/1, olfactory-test: MSZ 7304/10, and colour test: MSZ 7304/12.

The results of the sensory evaluation of ripened samples done by the panellists of the dairy company were also sent to us.

1.3. Determination of texture parameters

In recent years multifunctional texture test instruments have been developed which are easy to use and suitable for both imitative and empirical tests. Such an equipment is the QTS 25 Texture Analyser System (CNS Farnell, England), which evaluates the measured values with the software of Texture Profile Analysis (TPA).

The puncture test consists of measuring the force required to push a probe or punch into the food to a depth that causes irreversible crushing or flow of the food.

The puncture test is characterised by the shape of the probe (its area and perimeter) and the depth of penetration, which is usually held constant. The rate of strain is constant if the probe is driven into the sample at a constant speed.

The interaction between the probe and the sample is complicated. At least four factors have to be taken into consideration, the compression that is applied by the face of the punch to the area under the punch, the shear that is caused by the perimeter of the punch and the edge of the sample, the frictional resistance between the surface of the probe and the cheese; and the force required to create a lateral flow within the sample.

The puncture test is made with a small flat-faced cylinder two times in a reciprocating motion which imitates the action of the jaw. The first cycle is the "first bite", the second one is the "second bite". The constant speed of the probe leads to both force-time and force-distance curves. The curves as analogues of TPA were evaluated with the software of TPA (Fig. 1).

1.3.1. Testing conditions. When decision is made on the operating conditions, the sample should be large enough to be representative of the whole (PELEG, 1977) but it must not contain hidden cracks or irregularities.

Block shaped pieces from the inside of the cheese were analysed, the surface was 14×7 cm and the height was 4 cm. Chewing was imitated with penetration and was carried out twice. The investigation was made on two parts of the sample, on one surface the depth of penetration was 4.00 mm, on the other one it was 7.00 mm. The parameters of test are in the Table 1.

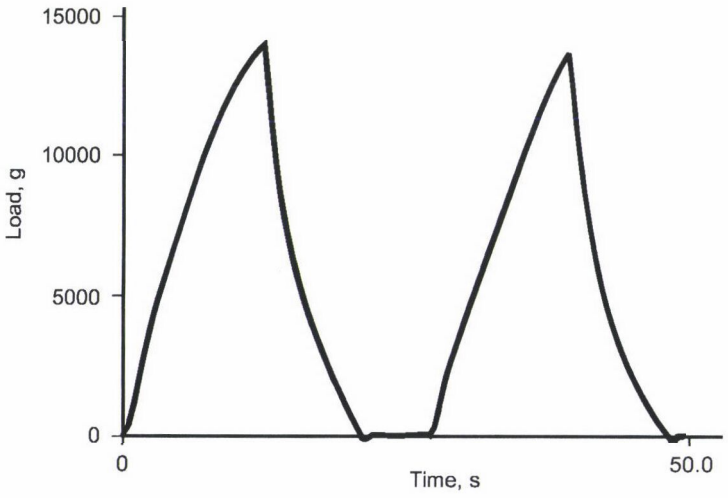

Fig. 1. TPA force-time curve of Trappist cheese

Table 1

Parameters of test

Probe:	1.2 cm Ø plastic cylinder
Type of test:	TPA
Speed of the probe:	30 mm min^{-1}
Trigger:	5.0 g
Depth of penetration[a]:	4.00 mm or 7.00 mm
Number of cycles:	2
Sample temperature:	20–22 °C
Number of analyses:	Each sample was analysed 10 times on different parts of the surface. At evaluation only the average of the replicates was taken into consideration.

[a] The determination of parameters with 4.00 mm depth of penetration was named 1st mode of operation, and the determination of parameters with 7.00 mm depth of penetration was named 2nd mode of operation.

1.3.2. Mechanical parameters investigated. The next 22 parameters (Hardness (1,2); Cohesiveness, Gumminess, Chewiness, Chewiness index, Modulus, Adhesive force, Adhesiveness, Springiness, Springiness index, Area (1,2); Stringiness length, Stringiness work done, Work done to hardness (1,2); Recoverable deformation (1,2); Recoverable work done (1,2); First peak) were determined by texture analysis with ten replicate measurements. From the 22 parameters 13 primary qualities and derived parameters by importance of parameter and reliability of measurements were selected.

Primary qualities: Hardness (1,2), Gumminess, Chewiness, Chewiness index, Modulus, Area (1,2).

Secondary (derived) parameters: Stringiness length, Work done to hardness (2), Recoverable deformation (1,2); Recoverable work done (1).

The interpretation of the primary and secondary parameters can be seen in Table 2.

1.4. Mathematical statistical evaluation of data

Sensory scores were evaluated after determination of the average scores and the rejection of outlying data in accordance with the HUNGARIAN STANDARD (1987a) "Sensory analysis of milk and dairy products" (MOLNÁR, 1991a). Data from the sensory tests and instrumental texture profile analyses (TPA) were evaluated using statistical methods (analysis of variance, principal component analysis, principal component regression and regression analysis) with the help of Statgraphics 5.0. (SVÁB, 1979).

Principal component analysis (PCA) is a multivariable method that helps to study and visualise the total structure of relationships and helps to express different characters by a few artificial variables calculated from their actual correlation (SVÁB, 1979).

Table 2

Explanation of the measured and evaluated texture parameters

Primary results	
Hardness (1 & 2)	Maximum load during compression Cycle 1 or 2
Gumminess	Hardness (Cycle 1) multiplied by the ratio of the positive force area in the second compression and that of the first compression
Chewiness	Gumminess multiplied by the distance the beam travels when compressing the sample in cycle 2
Chewiness index	Gumminess multiplied by the distance the beam travels when compressing the sample in cycle 2 divided by target distance value
Modulus	Gradient of curve between 20 and 80% of Hardness (Cycle 1)
Area (1 & 2)	Total positive area in cycle 1 or 2
Secondary (derived) results	
Stringiness length	Distance to 1st negative peak load from where load crosses the 0 g point on decompression of cycle 1
Work done to Hardness2	Positive area to Hardness 2
Recoverable deformation (1 & 2)	Distance travelled by the beam whilst sample decompression from Hardness (1 or 2) to zero in cycle 1 or 2
Recoverable work done (1)	Positive area between Hardness 1 and where load crosses the 0 g point on decompression of cycle 1

2. Results

2.1. Qualification of samples on the basis of chemical composition and sensory tests

The quality of ripened cheese samples was "appropriate" according to the HUNGARIAN STANDARD (1987b). It was based on the chemical composition and the weighted scores of sensory tests. The products had "excellent" quality (average weighted scores of sensory tests were 17.85 in the Dairy Company and 18.06 was given by our panellists). Tables 3a) and 3b) show the chemical composition of the samples and the results of sensory tests that aimed to determine shelf-life.

2.2. Selecting the textural parameters

Out of the 22 rheological parameters determined, 13 directly measured values or derived data were selected which were determined as reproducible (average variation coefficient is smaller than 40%) (COCK, 1994) and which correlated significantly ($P<0.05$) with the age of product in the case of both modes of operation.

Table 3a

Major chemical components of Trappist cheese samples

Manufacturing	Constituent			
	Dry material g/100 g	Fat content in dry material g/100 g	Salt content g/100 g	Protein content g/100 g
I	57.46	45.25	1.71	24.90
II	58.06	46.50	1.55	23.14
III	59.32	46.36	1.60	24.85
IV	57.46	45.25	1.81	19.89
V	57.75	45.83	1.68	21.74
Acceptance range	***58.0±2.5***	***45.0±2.0***	***1.5±0.5***	

n = 8 samples per manufacturing

Table 3b

Results of sensory tests

Manufacturing	Weighted total scores					
Age (days)	*21*	*28*	*42*	*56*	*70*	*Data from the Company (21 days)*
I	17.9	18.8	19.3	16.6	16.9	*18.04*
II	18.6	18.4	17.6	16.4	16.8	*18.2*
III	–	18.6	19.0	19.2	18.4	*17.2*
IV	17.1	16.9	16.3	14.2	12.3	*18.0*
V	17.2	17.6	16.8	14.7	14.4	*17.8*
Average	***17.7***	*18.06*	*17.80*	*16.22*	*15.76*	***17.85***

n = 5 samples from different production lots

The average value of the test results of 40 products, 10 measurements per product, the average values of the variation coefficient of replicate measurements (10), the correlation coefficients between the age of the product and each texture parameter are shown in Table 4. Most of the selected parameters (Hardness (1,2), Gumminess, Chewiness, Chewiness index, Modulus, Area (1,2), Work done to hardness (2), Recoverable deformation (1,2); Recoverable work done (1)) are negatively correlated with the age of product. They show a downward tendency as a function of time.

Table 4

Measurement of textural parameters and the correlation between the age of cheese and the parameters

Textural parameters (Units)	Average value	VC[a] (%)	Average value	VC[a] (%)	The values of correlation coefficients between age of product and each textural parameter	
depth of penetration:	*4 mm*		*7 mm*		*4 mm*	*7 mm*
Hardness1 (g)	2969	**16**	6031	**10**	**–0.4308****	*–0.3299**
Hardness2 (g)	3139	**16**	6224	**10**	**–0.4502****	*–0.3807**
Cohesiveness (none)	1.08	**2**	0.999	**4**	–0.2874	**–0.6297*****
Gumminess (g)	3190	**15**	6025	**11**	**–0.4683****	**–0.4329****
Chewiness (g×mm)	10872	**17**	35573	**11**	**–0.4707****	**–0.4064****
Chewiness index (none)	2719	**17**	5082	**11**	**–0.4708****	**–0.4064****
Modulus (none)	305	**16**	360	**10**	**–0.4027****	*–0.3103**
Adhesive force (g)	112	40	393	43	**0.5627*****	**0.6460*****
Adhesiveness (g×mm)	137	52	726	70	**0.6961*****	**0.6804*****
Springiness (mm)	3.33	2	5.89	2	*–0.3280**	0.1238
Springiness index (none)	0.83	2	0.84	2	–0.2805	0.1938
Area 1 (g×s)	23652	18	82488	11	**–0.4149****	*–0.3247**
Area 2 (g×s)	25396	17	82372	11	**–0.4536****	**–0.4294****
Stringiness length (mm)	0.41	23	0.79	31	**0.6612*****	**0.7321*****
Stringiness work done (g×s)	65	51	582	72	**0.6292*****	**0.6653*****
Work done to hardness 1 (g×s)	16280	**18**	59506	**11**	*–0.3907**	–0.2862
Work done to hardness 2 (g×s)	17205	**16**	58157	11	**–0.4366****	*–0.3995**
Recoverable deformation 1 (mm)	2.49	**4**	4.37	**2**	**–0.6449*****	**–0.7269*****
Recoverable deformation 2 (mm)	2.67	**4**	4.57	**3**	**–0.6404*****	**–0.7705*****
Recoverable work done 1 (g×s)	7367	**19**	22955	**12**	**–0.4557****	**–0.4071****
Recoverable work done 2 (g×s)	8190	19	24212	12	0.1765	0.0662
First peak (g.)	25	60	39	43	*0.3280**	*0.3355**

[a] Variation coefficient (VC): average values of variation coefficient of replicate measurements

*P<0.05, **P<0.01, ***P<0.001

The definitions of Units are not common. These were defined by the Company. The software of the QTS25 uses ones. The work type Units have to take the speed of the probe into account to get correct Units.

2.3. Evaluation of textural parameters with principal component analysis

Principal component analysis (PCA) was carried out on 13 variables of all samples. It was found that on the basis of the correlation of the original variables, variables could be reduced to one single and two principal component variables in the

case of the two modes of operation. The eigenvalue of the factors and the cumulative percentage (h^2) are shown in Table 5. The first principal component covers more than 87% of the variance of the original variables determined with both modes of operation. The importance of the first principal component is expressed by the high eigenvalue (JACKMAN & YADA, 1989).

From the values of principal component weights the relative importance of the original variables can be seen compared to each of the respective principal components.

The first principal component is determined by 10 variables to an approximately equal extent in both cases (Hardness1,2; Guminess; Chewiness; Chewiness index; Modulus; Area 1,2; Work done to hardness 2; Recoverable work done 1).

The second principal component is determined by three variables (Stringiness length and Recoverable deformation (1,2)) in the case of the 2nd. mode of operation.

Table 5

Results of principal component analysis (n=40, p=13)

1st mode of operation				2nd mode of operation			
No. of PC	λ	variance %	h^2	No. of PC	λ	variance %	h^2
1	11.83	91.03	**91.03**	1	11.35	87.29	87.29
				2	1.30	9.99	**97.28**

2.4. Comparison of samples from various manufacturing time on the basis of values of first few principal components

Samples from different manufacturing time were compared with analysis of variance to show whether there were significant differences between the manufacturing processes on the basis of the first principal components that were determined with various modes of operation (4 and 7 mm penetration). The analysis of variance of the first few principal components carried out with different modes of operation can be found in Fig. 2.

If the subgroups of the different manufacturing processes are compared, it can be concluded in the case of both modes of operation that the average values of the first three manufacturing lots were greater than those of the last two, but there are no significant differences between them.

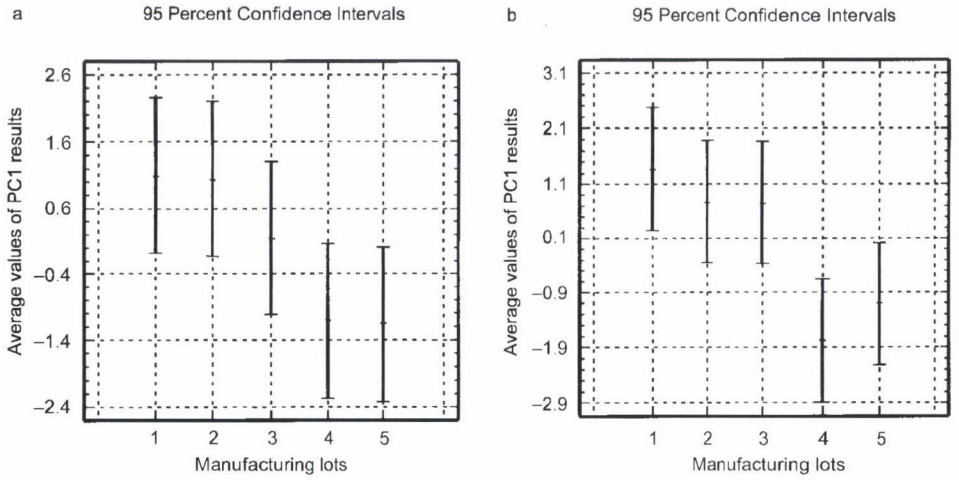

Fig. 2. Analysis of variance of textural parameters, carried out with two modes of operation. a: 4 mm penetration depth; b: 7 mm penetration depth

2.5. Estimation of the product age

The textural properties depended on the composition of crude cheese and the extent of ripeness. The developments of textural properties during ripening and subsequent storage of cheese were different, therefore, it is reasonable to take all properties into the evaluation of time dependence.

2.5.1. Estimation with principal component regression. The age of the product (i.e. period of time that has elapsed since the product was manufactured; actually the sum of ripening time and storage time) can be estimated from the principal components with principal component regression according to the $y=a_1x_1+a_2x_2+...+a_nx_n+b$ equation, where y is the age of the product (days) and x_1, x_2, ... x_n are the principal components. When all principal components (13) were used in the formula, the age of the product could be estimated with 12 days accuracy. (Standard errors of estimates at both modes of operation are 12.02 (a = 4.00 mm depth of penetration) and 11.85 (b = 7.00 mm depth of penetration), r_a=0.9016; r_b=0.9045). These values were compared to the full periods (ripening and storage) (70 days) that had a 17% accuracy. These principal components

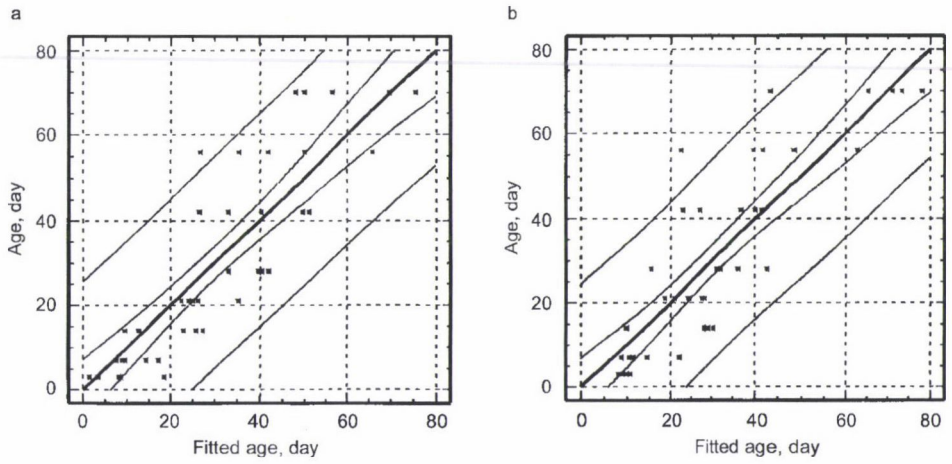

Fig. 3. Estimation of the product age by principal component regression, carried out with two modes of operation. a: 4 mm penetration depth; b: 7 mm penetration depth; Y: age of the cheese, PCX-Xth Principal component SE: standard error. a) Y = –3.35×PC1 – 14.92×PC2 + 9.36×PC3 – 102.59×PC7 + 1201.60×PC12 + 30.12 SE = 12.88 R = 0.848 n = 40 P<0.001; b) Y=–3.21×PC2 + 49.02×PC6 – 112.66×PC9 + 30.12 SE = 12.12 R = 0.863 n = 40 P<0.001

were selected by stepwise variable selection that estimated the age with the same significance. At the 5 mm depth of penetration the age of the product could be estimated with five (PC1, PC2, PC3, PC7, PC12) and at the 7 mm depth of penetration with four (PC1, PC2, PC6, PC9) principal components. The equations between the observed and fitted values of age are on the Fig. 3.

At the 7 mm depth of penetration the number of principal components were less and inaccuracy in estimation was better than at the 4 mm depth of penetration. The first principal component occurred in both formulas with negative coefficient.

2.5.2. Estimation with original variables. The age of the product was estimated with stepwise variable selection and multiple regression analyse involving all the original variables because in the first two principal components all the 13 variables occurred significantly. The standard equation of the estimating formula: $y=a_1x_1+a_2x_2+...+a_nx_n$, where y = age of the product, x_1, x_2, ... x_n = texture parameters. The equations between the observed and fitted values of age are on Fig. 4.

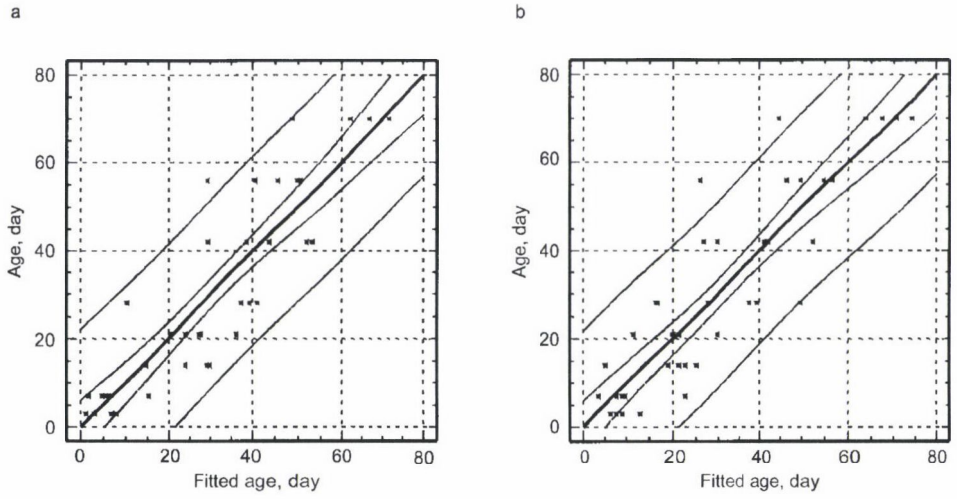

Fig. 4. Estimation of the product age by original parameters, carried out with two modes of operation. a: 4 mm penetration depth, b: 7 mm penetration depth, Y: age of the cheese, x: textural parameter SE: standard error. a) $Y = -1.102x_1 + 1.026x_2 + 0.525x_3 + 0.162x_4 - 0.122X_5 + 107.954x_6 - 0.0247X_7 - 16.45x_8 - 0.0602x_9$ SE = 11.59 R = 0.962 n = 40; b) $Y = 0.112x_1 - 0.0164x_2 - 0.0117x_3 + 28.192x_4 + 0.0119x_5 + 56.247x_6 - 59.808x_7 + 0.00938x_8$ SE = 11.23 R = 0.963 n = 40

At the 4 mm depth of penetration product age could be estimated from 9 variables (Hardness (1), Gumminess, Modulus, Area (1,2), Stringiness length, Work done to hardness (2), Recoverable deformation (1), Recoverable work done (1); and at the 7 mm penetration with 8 variables (Gumminess, Chewiness, Stringiness length, Work done to hardness (2), Recoverable deformation (1,2); Recoverable work done (1) with near the same accuracy. The estimation inaccuracy at the 7 mm depth of penetration was better to a minimum extent, e.g. 11.2 days, which was of 16% accuracy related to the whole period.

2.6. Relationships between sensory tests and textural parameters

A correlation was tried to be established between sensory test based on product standards and textural parameters measured with instrument, in order to establish whether it is possible to draw definite conclusions from the changes in textural parameters and the quality of sensory test.

Textural parameters were selected with stepwise variable selection to which the total sensory score correlated. The total sensory score at the 4 mm depth of penetration could be estimated with 1.205 point accuracy from the value of stringiness length according to the equation of Y=–13.735 × Stringiness length + 21.534 equation

(R=0.804, SE=1.205, n=24, P<0.001). At the 7 mm depth of penetration the estimation formula of the total sensory score Y=0.000522 × Gumminess –1.393 × Stringiness length +15.368 (R=0.802, SE=1.24, n=24, P<0.001). Both parameters related to the sample behaviour were observed during sensory test of the samples. The Stringiness length is connected with the rate of which a deformed sample goes back to its undeformed condition after the deforming force is seized. Gumminess is the quantity to simulate the energy required to disintegrate a semi-solid sample to a steady state of swallowing.

Since the total sensory score changes over the course of time according to the maximum curve while the textural parameters have a straight line correlation, the established relations are valid only after the optimal ripening condition, that is for the second part of shelf-life.

3. Conclusions

The changes in texture of Trappist samples from five different manufacturing processes during ripening time and storage were characterised by remarkable changes in various texture parameters. The ripened samples were of "excellent" quality according to the compositional analyses and sensory tests. There was a significant difference in the mechanical parameters for the first three and last two manufacturing processes. The following conclusions can be drawn from the results of our investigations, despite the inhomogeneity of samples, which was due to the manufacturing processes.

– The 13 chosen mechanical parameters revealed a close correlation. Because of this it was also possible to have a description with just one or two variables. On the basis of principal component weights, it was established from the two methods of measurement that the first main component was basically determined by 10 of the original characteristics, while the second main component was determined by the remaining 3.

– Between the values of principal components and the age of cheese a multiple linear correlation was observed. The values of principal components differred at the 4 and 7 mm depths of penetration, so the age of the product could be estimated with the use of a varied number of principal components (5, 4).

– Selecting those original variants, which had multiple linear correlation with the age of products an estimation formula was developed. Using this formula the age of cheese could be estimated using 9 variables at the 4 mm depth of penetration and 8 variables at the 7 mm depth of penetration with 11 days of accuracy.

– A correlation was found between the total sensory score indicating product quality and texture parameters related to the sensory test of samples (gumminess, chewiness) in the second section of shelf-life (after 42 days) in a way that the sensory score could be estimated from texture parameters with an accuracy of 1.20–1.24 score points.

– According to the data and processed data obtained from using the two measurement techniques, it can be said that, despite that both methods showed a correlation between product age and sensory score at a similar significance level and at a 7 mm penetration depth, but the number of variables used to estimate the time being less, so the accuracy of this estimation formula was better. Based on these results it is suggested that the 7 mm penetration depth be used for further tests on samples of Trappist cheese.

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Acta Alimentaria, Vol. 30 (2), pp. 145–157 (2001)

INFLUENCE OF DRYING TEMPERATURES ON CHEMICAL COMPOSITION OF CERTAIN CROATIAN WINTER WHEATS

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(Received: 19 January 2000; accepted: 18 December 2000)

Four Croatian winter wheat cultivars at 20% moisture were dried at temperatures of 50, 60, 70 and 100 °C until the moisture decreased to 14%. Two cultivars had improved quality, whereas two cultivars had lower quality characteristics. The examinations involved determination of seed germination, enzymatic activities and chemical indicators of grain properties. On the basis of the obtained results it could be concluded that the drying temperature of wheat should not exceed 50 °C in order to preserve biologically undamaged grain. Gluten of lower swelling ability increases sedimentation value at drying temperatures of 60 and 70 °C. For wheat samples with high quality gluten the wheat grain drying process at a temperature of 50 °C improved the wheat quality regarding the increased ability of gluten swelling. An increase in the drying temperature did not influence the characteristics of starch components. Diastatic and proteolytic activities of wheat decreased at a higher drying temperature.

Keywords: wheat, wheat drying, chemical composition, wheat quality

At the beginning of harvest wheat grain moisture content is often higher than 14%. The grain with moisture content higher than 14% is not suitable for quality storage. The preparation of those wheat for storage can be done by a drying process. The drying process must be provided under conditions which prevent deterioration of grain quality. Considerable research has been undertaken to investigate possible ways of drying, with alternative methods ranging from ambient air to high temperatures. Changes of grain properties by thermal treatment depend on the grain moisture, drying temperature and drying period (MÜHLBAUER, 1978).

Many authors reported about different optimal drying temperatures (WASSERMANN, 1978; LUPANO & ANÓN, 1987; CARDAS et al., 1988; RAGASIST, 1993). GHALLY and co-workers (1973) reported on heat damage of 15, 17 and 19% initial moisture content wheat dried with 60–188 °C air in a spouted-bed dryer. Germination,

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0139-3006/2001/$ 5.00

ash content, farinograph, extensiograph and baking tests were used as quality indicators. Changes of chemical and physical characteristics by thermal treatment have been discussed by NIERLE and co-workers (1978). All investigations showed that by drying temperature over 80 °C the wheat quality is impaired, i.e., thermal damage of the grain occurs.

Thermal damage implies mostly denaturation of proteins caused by thermal treatment. Thermo-stability of proteins depends on the grain moisture and gluten quality.

Many authors reported about quality changes in corn due to drying. MÜHLBAUER and CHRIST (1974), MÜHLBAUER and co-workers (1976). DEUBELIUS (1978) examined the effect of heated-air drying on the lysin content and protein quality of corn. PEPLINSKI and co-workers (1989) investigated the effects of hybrid and drying temperatures on the various properties and dry-milling response. PEPLINSKI and co-workers (1994) reported about changes in properties and physical quality in high-moisture corn, air-dried at 25–100 °C. They reported that drying under air temperature had little or no effect on any other physical or chemical characteristics studied, although these other properties had a very significant relationship with hybrid type.

HACKENBERGER & UGARČIĆ (1989) reported about specific behaviour of individual wheat varieties at various temperatures applied for wheat drying. They noticed that the drying process can be applied not only to avoid thermal damages but also to improve the technological quality of wheat (UGARČIĆ & HACKENBERGER, 1989).

Quality changes in grain due to drying can be determined by analytical methods. The aim of this study was to investigate the dependence of wheat grain quality on the drying temperature and to find out the possibilities of improving certain characteristics of wheat grain.

1. Material and methods

1.1. Kernel drying

Four samples of Croatian winter wheat cultivars (1997 harvest) were examined. Wheat samples were chosen in a way that two cultivars had improved quality (samples 1 and 2) whereas two cultivars had lower quality (samples 3 and 4) (Table 1). Criteria for quality determination were protein share, sedimentation value, gluten quality, rheologic and baking characteristics, etc, according to the Croatian wheat quality regulation (CROATIAN REGULATION for cereal and cereals products, 1992). Lots (1.5 kg) of cleaned wheat kernels at 10–13% moisture content were tempered to 18% moisture by adding water, held for 20 h, tempered to 20% moisture and held for 2 h. Then they were dried at the temperatures of 50, 60, 70 and 100 °C.

Table 1

Characteristics of wheat grain samples used in experiments

	Samples			
	1	2	3	4
Germination (%)	98	98	98	97
Yield of flour (%)	63.8	64.9	56.2	52.5
SDS – sedimentation value (ml)	69	55	42	39
Sedimentation value (Zeleny) (ml)	46	27	21	28
Protein content (%)	14.6	13.8	13.1	11.9
Starch content (%)	66.2	67.4	68.6	68.1
Damaged starch (%)	9.3	7.9	4.6	7.5
Diastatic activity (mg maltose)	4.61	5.06	2.13	2.69
Falling Number (sec)	366	358	326	419
Proteolytic activity (mg amino N)	2.45	4.21	2.12	2.82

The samples were dried in lab drier (Retsch, Type T61) in 6–8 cm thick stationary layer. The kernel temperature was measured with a temperature sensor (LM 335). The control samples were dried for 24 h at room temperature. Moisture was measured by moisture meter (Pfeuffer, HE 50) by taking samples every 30 minutes. Final moisture content of all samples ranged from 9.9 to 14.2%. The weight loss was determined by samples weighing before and after drying process. After the drying process, the warm samples were cooled at room temperature (25 °C). Cooled samples were then stored at 4 °C and brought up to 25 °C before testing was performed.

1.2. Analytical procedures

Germination was determined for 100 seeds in accordance with the Croatian regulation for seed testing. The flour yield was determined by milling using a laboratory mill (Brabender Quadrumat Junior). The kernels were prepared for examination by milling in a cyclone mill (Cyclotec) with 0.5 sieve mashes.

The nitrogen was determined by the Kjeldahl method (N×5.7). Capacity of gluten swelling was determinated by SDS-sedimentation values according to AXFORD and co-workers (1979) and by Zeleny method (ICC Standard method, 116/1, 1992).

The Ewers method was applied to determine the starch content while the quantity of damaged starch was calculated by the amylase number according to the findings of HAMPEL (1952). The total diastatic activity was defined by the WOODHEAT & WYATT (1960) method and the α-amylase activity was defined by the Hagberg Falling Number (ICC Standard method, 107/1). Proteolytic enzyme activity was determined by the formal titration (VAJIĆ, 1962). All results were expressed at moisture content of 14%.

2. Results and discussion

2.1. Physical properties

Germination ability is an important factor for the quality of seed wheat. Seed grain requires a high germination percentage. KREYGER (1972) determined the critical kernel temperature for a number of small grains as a function of equilibrium relative to humidity. Wheat germination is the least heat sensitive, followed by oats, corn and rye. Germination of all grains is more heat sensitive at high than at low moisture content. In our investigation the germination of the untreated control samples ranged from 97 to 98% (Table 1). It is indicated that all samples showed a high germination capacity. The rapid decrease of germination was observed at the drying temperature of 60 °C, which indicated that the grain was not biologically damaged by the drying temperature of 50 °C (Fig. 1).

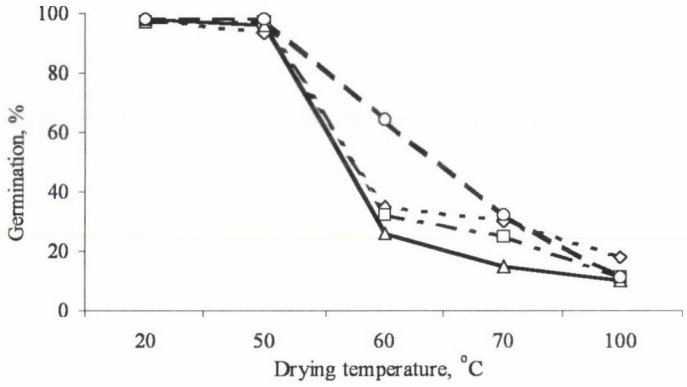

Fig. 1. Changes of grain germination with drying temperatures. ◊: Sample 1; □: sample 2; Δ: sample 3; O: sample 4

The investigated wheat samples contained 20% moisture at the start of drying. KÖKSEL and co-workers (1993) determined that most of the initial water was removed during the early stages of drying. The dehydration rate increased with increasing temperatures. They found that 12% of water was removed at 40 °C in the first 30 min. This percentage increased to 48% at 100 °C for the same period. The measurements of weight loss obtained for our wheat samples dried at different temperatures are given in Table 2. The weight loss ranged from 6.1 to 16.3%.

Table 2

Moisture content and weight loss at various drying temperatures

Parameters	Naturally dried kernels	Drying in laboratory drier			
Temperature	20 °C	50 °C	60 °C	70 °C	100 °C
Moisture content at the inlet (%)	20.1	20.3	20.0	20.5	20.8
Moisture content at the outlet (%)	11.5	14.2	11.1	9.7	9.9
Weight loss (%)	11.1	6.1	11.1	15.5	16.3

Table 3

Flour yield at various drying temperatures

Samples	Yield of flour (%)				
Temperature	20 °C	50 °C	60 °C	70 °C	100 °C
1	63.8	63.6	62.6	60.7	58.3
2	64.9	61.2	59.4	58.6	52.7
3	56.2	52.7	49.8	47.8	44.5
4	52.5	51.3	48.4	49.8	38.3

Flour yield is the most important technical and economical factor for milling. The samples had a low flour yield. Yield of flour decreased at drying temperatures higher than 70 °C (Table 3). It is especially pronounced at drying temperature of 100 °C for all wheat samples.

2.2. Effect of wheat drying temperature on chemical characteristics

The protein content of control samples of the blend winter wheat ranged from 11.9 to 14.6% in the dry matter while the SDS-sedimentation values ranged from 39 to 69 ml and the sedimentation value, according to Zeleny method from 21 to 46 ml (Table 1).

The protein content is used along with gluten swelling ability to establish the commercial value of wheat. With changes of drying temperatures protein content did not change significantly (Table 4). The obtained results are in accordance with CARDAS and co-workers (1988) and WASSERMANN (1978). Although they did not find differences in protein content of thermally treated wheat samples, it is considered that the differences in the protein content are the reflection of differences in morphological properties of

kernels across the individual varieties. Wheat gluten is the water insoluble complex protein fraction separated from wheat flours. Glutenin and gliadin, the two major protein components of wheat gluten, interact in an aqueous system to produce a viscoelastic mass. The viscoelastic behavior of hydrated wheat gluten is crucial to bakery technology in which it results in the ability to form adhesive and cohesive masses, films and three dimensional film networks. It is generally accepted that higher temperatures cause damage to gluten proteins, resulting in lower baking quality. When wheat at 14% moisture content was heated for 36 min, gluten was damaged by temperatures in 70–85 °C range (KENT 1975). BECKER and SALLANS (1956) reported that the denaturation of gluten in wheat flour directly affects the loaf volume. Heat brings about three important changes; it increases the degree of aggregation of glutenins, it decreases the extractability of large glutenins aggregates, and it decreases the hydrophobicity of insoluble glutenins. Changes in the functionality of heat-treated wheat gluten proteins may be attributed to disulfide/sulfhydril interchange reactions (WEEGELS et al., 1994; ELIASSON et al., 1991).

The increase of drying temperature of the wheat grain causes changes of SDS-sedimentation values (Fig. 2). These changes depended on the gluten quality. JEANJEAN and co-workers (1980) reported that heat treatment of gluten reinforced the strength of hydrophobic bonds and aggregation of gluten molecules appeared. The results obtained indicated specific changes of gluten for each variety. It could be confirmed that the bonds in the aggregation protein complex are weak and, moreover, the quality of the wheat flour is easily affected by the thermal treatment, unlike the samples of high sedimentation value (samples 1 and 2) whose ability of gluten swelling decreased at higher drying temperatures. The ability of gluten swelling decreased in all samples at the drying temperature of 100 °C.

Table 4

Changes of protein content with drying temperatures

Samples	Protein content (% dry mat.)				
Temperature	20 °C	50 °C	60 °C	70 °C	100 °C
1	14.6	14.3	14.7	14.1	14.1
2	13.8	13.1	13.5	13.3	12.0
3	13.1	12.7	12.6	12.4	12.6
4	11.0	11.7	11.7	11.7	13.1

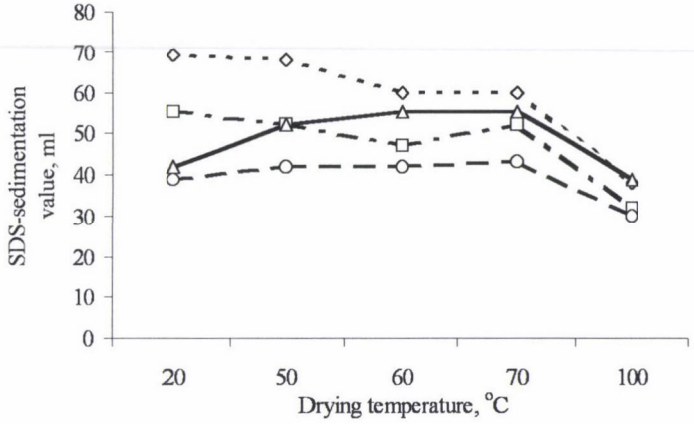

Fig. 2. Changes of SDS-sedimentation value with drying temperatures. ◊: Sample 1; □: sample 2; Δ: sample 3; O: sample 4

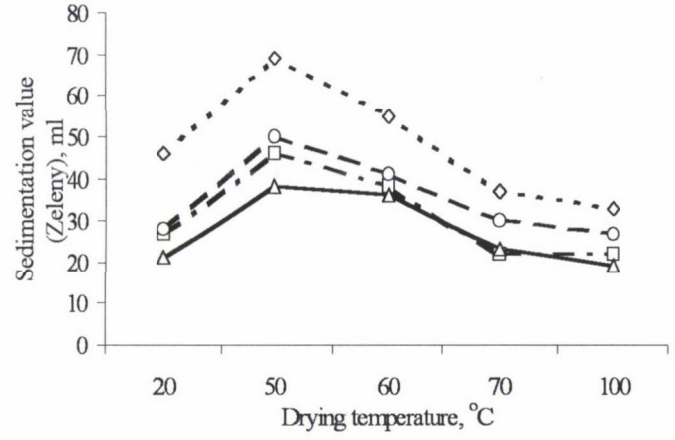

Fig. 3. Changes of sedimentation value (Zeleny method) with drying temperatures. ◊: Sample 1; □: sample 2; Δ: sample 3; O: sample 4

All samples dried at 50 °C indicated the rise in sedimentation value (Zeleny method) (Fig. 3). The sedimentation value for samples dried at 70 °C was similar to values for untreated samples. Since the sedimentation value is an indicator of the gluten swelling capacity it can be concluded that the impairment of gluten qualities was evident

at a drying temperature higher than 70 °C. The changes of gluten qualities were bigger by high quality gluten in relation to the low quality gluten. It is proved that the low quality wheat gluten was less hydrophobic than the high quality wheat gluten. Hydrophobic link increased by heating which resulted in different abilities of gluten swelling (LUPANO & ANÓN, 1987). These changes enabled improvement of wheat quality by grain drying up to 60 °C.

Starch, as the most represented component of the wheat grain significantly influences the wheat quality due to its structure and physical state. The starch content ranged from 66.2 to 68.6%. An increase of the drying temperature did not effect starch components.

KÖKSEL and co-workers (1993) investigated the influence of wheat-drying temperatures on the birefringence and X-ray diffraction patterns of wet-harvested wheat starch. With drying temperatures of 80 and 100 °C, 95% of the starch granules retained strong birefringence. They found out, that the amount of water in the wheat sample during higher temperature drying may not be sufficient to cause swelling and gelatinization. The physical constraints of the endosperm cells may also limit the swelling of the starch granules. They concluded that the retention of birefringence during wheat drying is due to the lack of water and to the physical protection of starch by the kernel and endosperm cells (Table 4).

During wheat milling, some starch granules are mechanically damaged. Lesions are quantitatively more or less important according to nature, conditioning, mechanical treatment, etc. The degree of starch damage of flour has technical consequences, differently appreciated in baking industries (bakeries, biscuit industries, pasta production), so it is important to control starch damage. The level of starch damage is related to kernel hardness or vitreousness of the parent wheat. The level of starch damage is low for soft wheat. Since proteins are tied to starch (attached proteins), together with starch damage, gluten grate on the surface of starch granules is also damaged. This leads to a reduced ability to retain gas during fermentation. The influence of starch damage on the rehydration rate of doughs and on the rate of enzyme activity and its effect on viscosity measurement has been observed previously by BAKKER and co-workers 1978).

The percentage of the damaged starch decreased by all samples due to temperature increase up to 60 °C (Fig. 4).

According to the flour yield, protein content and starch damage results it was evident that the two investigated samples might have been classified as hard wheat (samples 1 and 2), whereas two samples might have been classified as soft wheats (samples 3 and 4), (Table 1).

Table 5

Changes of starch content with drying temperatures

Samples	Starch content (% in dry mat.)				
Temperature	20 °C	50 °C	60 °C	70 °C	100 °C
1	66.2	65.6	67.4	66.3	67.8
2	67.4	67.0	66.4	66.1	68.2
3	68.6	69.4	68.7	67.7	65.1
4	68.1	68.9	69.0	67.7	65.1

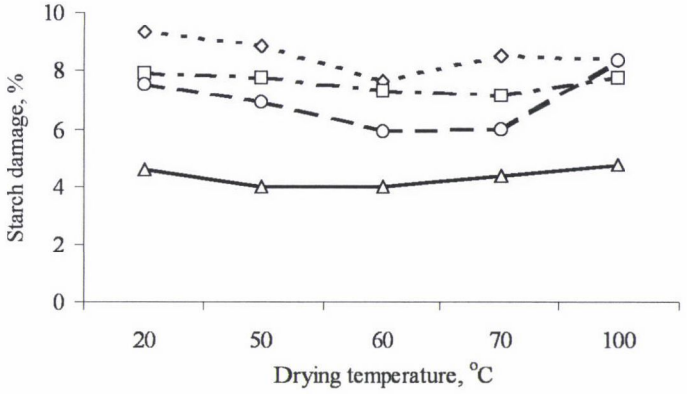

Fig. 4. Changes of starch damage with drying temperatures. ◊: Sample 1; □: sample 2; Δ: sample 3; O: sample 4

The enzyme levels in the mature, sound wheat grain are relatively low. Amylases and proteases have the highest influence on the technological properties. Increased enzyme activity, particularly of α-amylase is the main limiting quality factor in processing flour into bread and other baked goods. Degrading effects of enzymatic activities lower the water binding capacity in dough and thus decrease viscosity in dough and bread crumb. Enzyme activity in the wheat kernel depended on enzyme concentration, temperature and duration of heat treatment.

Sound cereals have low levels of α-amylase. However, upon germination, the level of α-amylase is increased by many times. This makes α-amylase activity a sensitive measure to detect sprouting of cereal grains. Because of its rapid effect on viscosity of flour-water suspension, measuring of relative viscosity has been widely used to control enzyme activity (Falling Number method). The samples had a low α-amylase

activity (by all samples the Falling Number was higher than 300 s). Investigation results showed that all untreated samples had a low value of α-amylase (Fig. 5). α-Amylase activity decreased at temperatures above 70 °C. WEIPERT (1970) reported that the α-amylase activity and maltose content decreased at temperature above 60 °C for various rye samples. Changes of Falling Number at higher drying temperature of wheat kernel reflected changes of physico-chemical characteristics of starch components (KULP & LORENZ 1981). Viscosity of gelatinized starch increased at the drying temperature up to 70 °C in all samples.

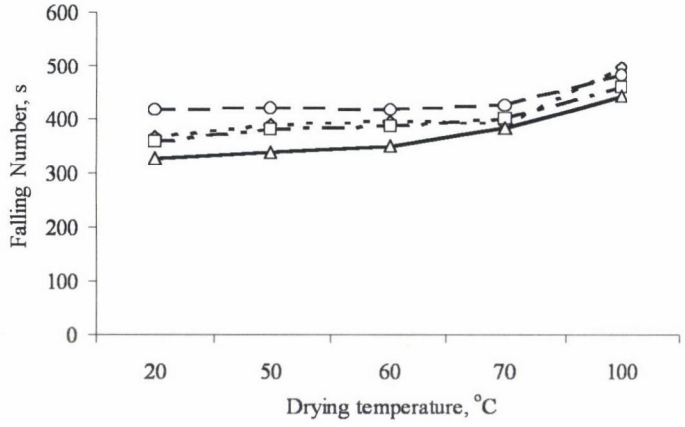

Fig. 5. Changes of Falling Number with drying temperatures. ◊: Sample 1; □: sample 2; Δ: sample 3; O: sample 4

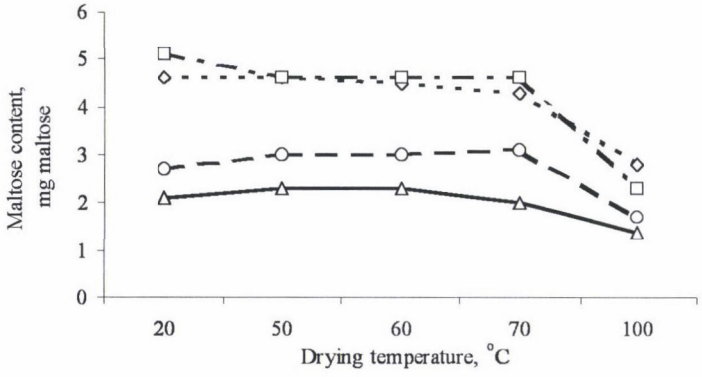

Fig. 6. Changes of maltose content with drying temperatures. ◊: Sample 1; □: sample 2; Δ: sample 3; O: sample 4

The β-amylase activity, as expressed by the maltose quantity, is shown in the Fig. 6. It was found that the β-amylase activity was reduced after drying at 70 °C.

The optimal temperature for the proteolytic enzyme activity in wheat is 50 °C. At temperatures higher than 50 °C enzyme activity decreased, while at 100 °C the activity completely stopped (Fig. 7).

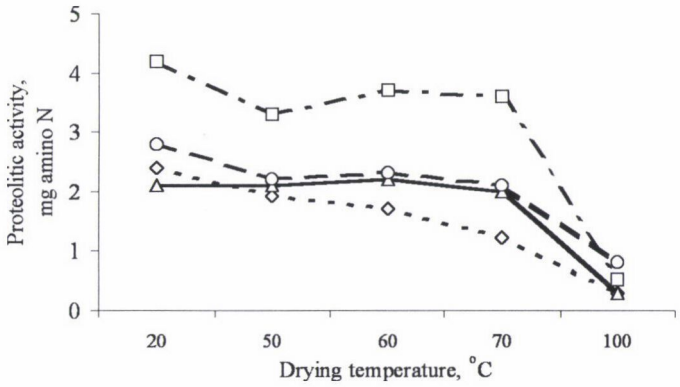

Fig. 7. Changes of proteolitic enzyme activity with drying temperatures. ◊: Sample 1; □: sample 2; Δ: sample 3; O: sample 4

3. Conclusions

To preserve biologically undamaged grain, the drying temperature of wheat should not exceed 50 °C.

The highest differences caused by the thermal wheat treatment were obtained for the sedimentation value and the starch damage. The changes of starch damage percentage are reflected in the higher starch susceptibility to enzymatic disintegration.

The wheat grain drying process at a temperature of 70 °C improves wheat quality with regard to the increased capacity of gluten swelling.

Proteolytic and amylolytic enzyme activities of the wheat grain decrease at a drying temperature higher than 70 °C.

More significant deterioration of wheat quality appeared at a drying temperature higher than 70 °C.

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Acta Alimentaria, Vol. 30 (2), pp. 159–171 (2001)

EFFECT OF TITANIUM ASCORBATE TREATMENT ON RED AND YELLOW PIGMENT COMPOSITION OF PAPRIKA CULTIVARS

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(Received: 7 February 2000; accepted: 30 September 2000)

Paprika and paprika extracts are commercially used as natural colouring and flavouring agents for foods and are good source of carotenoids. *Capsicum annuum* plants, cv. Albar, Negral, KM-622, Mihályteleki, Buketen-50 and Gorogled-6, were treated with Ti^{4+}-ascorbate, a biological activity inductor, to study its effect on the fresh pericarp and spice red pepper pigment compositions. The influence of drying and grinding operations on the pigment composition was also studied. The Ti^{4+} treatment improved the photosynthetic activity of the plants, increasing the concentrations of both red and yellow pigments in fresh and dry-ground pericarp samples, however, the increase of the yellow pigments was higher than that of the red ones. The drying (55 °C for 48 h) and milling of fresh pericarp from control plants (without Ti^{4+} treatment) resulted in a significant decrease of total carotenoids in the cultivars: Albar, Negral, and KM-622, however, Mihályteleki and, especially, Gorogled-6 plants showed a significant increase. On the other hand, all samples from Ti^{4+}-treated plants showed a significant reduction in their total red and yellow pigments content after these conditioning operations. In both fresh and dry-ground pericarps from over-ripe peppers, capsanthin and zeaxanthin and their fatty acid esters were the major red and yellow pigments, respectively. Considering the red/yellow pigment ratio as the main quality parameter, the Ti^{4+} treatment, which was able to improve the biological activity of this plant species, did not lead to an improvement of fruit quality because the increase of the yellow pigments was higher than that of the red ones.

Keywords: biological activity inductor, *Capsicum annuum*, food antioxidant, red pepper

The popularity of red pepper for food uses stems from the combination of colour, taste, and pungency. Increased consumption of peppers in foods has created a need for

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0139-3006/2001/$ 5.00

compositional and nutritional information. Paprika is a good source of vitamins C and A and carotenoids (HOWARD et al., 1994; GREGORY et al., 1987). Carotenoids, vitamin C, and vitamin E are major antioxidants found in the human diet and, therefore, much research has focused on food antioxidants for their cancer protective activity (HOWARD et al., 1994; LEE et al., 1995).

Carotenoids are fat-soluble antioxidants found in many fruits and vegetables, including peppers and they are required for human epithelial cellular differentiation (HOWARD et al., 1994). Levels of these compounds may be affected by maturity, genotype, processing (LEE et al., 1995), and picking time (LEASE & LEASE, 1956; REVERTE et al., 2000).

A large number of carotenoids are biosynthesised during ripening of the *Capsicum annuum* L. peppers. The different pathways of biochemical synthesis always end in capsanthin and capsorubin, and in every variety, the same carotenoids are present, however, they differ in the total quantity of the pigments and in the red/yellow carotenoid ratio. These pigments constitute approximately 1–3% of the total fresh weight of mature pepper fruits.

Titanium exercises some beneficial effects on the biological activity of several crop species. On *Capsicum annuum* L. plants, it has some promotive effects on biomass production, nutrient absorption, several enzymatic system activities (catalase, peroxidase, lipoxygenase and nitrate reductase), malic acid, leaf chlorophylls, fruit carotenoids and ascorbic acid contents, and it decreases plant starch concentration (CARVAJAL & ALCARAZ, 1998).

The main objective of this study was to determine how the application of a biological activity inductor, titanium ascorbate, and the pepper variety (2 Spanish, 2 Hungarian, and 2 Bulgarian, which are the main European paprika-producing countries) affect the concentrations of the red and yellow pigments as well as the red/yellow ratio.

1. Materials and methods

1.1. Plants

Seeds of *C. annuum* L. had the following origin:

The Spanish varieties Albar and Negral were obtained from CEBAS-CSIC (Murcia) and from Ramiro Arnedo, S.A., respectively; the Hungarian KM-622 and Mihályteleki, from the Research Station for Paprika Development (Kalocsa) and from the Food Processing Enterprise (Szeged), respectively; both seeds were granted by the Central Food Research Institute (Budapest). The Bulgarian Buketen-50 and Gorogled-6 were given by the "Maritsa" Vegetable Crop Research Institute (Plovdiv).

1.2. Experimental growing conditions

Plants were grown in a greenhouse equipped with an automatic system to control temperature and humidity. Plants were disposed in polyethylene containers (25 l capacity) filled with a semi-inert substrate [a mixture of peat, murc and pine bark (1:1:1, v:v:v)]. Each container constituted an experimental plot in which four plants were grown. Six experimental blocks (ten containers block^{-1}) were randomly disposed in the greenhouse. Half of these experimental blocks were leaf-sprayed twice with a 42 mmol l^{-1} Ti^{4+}-ascorbate solution, pH 6 [the first Ti application was carried out before flowering (35 ml plant^{-1}), and the second one (35 ml plant^{-1}) 24 days later]. The other half of the plants were treated with identical volumes of 42 mmol l^{-1} ascorbic acid, pH 6, and were used as controls. The fertirrigation of the plants was carried out by an automatically controlled drip system, so that all the plants received identical doses of water, nutrients, phyto-sanitary treatments, etc. Over-ripe fruits were collected 4 months after the first Ti application. In the laboratory, fruits were divided into peduncles, seeds, and pericarps, then washed with 10 g l^{-1} BRIJ 35 solution (non-ionic detergent) and then rinsed three times with deionized water. Half of each sample was frozen with liquid nitrogen and stored at –40 °C until analysis. The other half was dried in darkness, in an air-forced oven, at 55 °C for 2 days. In the industry, when peppers are dried in an oven, the temperature normally used is 65 °C. In our experiments, the temperature was decreased to 55 °C for reducing the pigment oxidation and degradation. The dried pericarps were ground, simulating paprika manufacture and stored at –20 °C in darkness until analysis.

1.3. Analytical procedure

The solvents used for spectral and HPLC analyses were of HPLC grade; all other solvents were of analytical (ACS) grade.

1.3.1. Extraction. Carotenoids from fresh and ground paprika samples were extracted according to a combination of previously described methods (BIACS et al., 1989; DAOOD et al., 1996). Red bell peppers without seeds were cut into small pieces and 2.5 g (random sample) of paprika fresh pericarp was taken and disintegrated with quartz sand in a mortar with pestle. The sample was then mechanically shaken for 15 min with a mixture consisting of 20 ml of dichloromethane, 70 ml of methanol, and 15 ml of distilled water. This mixture was transferred to a separation funnel and the organic layer was dried on Na_2SO_4. The homogenate was evaporated to dryness under vacuum at a maximum temperature of 35 °C. The residues were dissolved in 10 ml of a mixture consisting of 1:1 (v:v) acetone-methanol. A 5 ml aliquot from this mixture was taken for saponification.

Pigments of paprika powders were extracted by shaking 0.25 g of powder with 50 ml of a mixture consisting of 2:1:1 dichloromethane-acetone-methanol for 15 min. The extract was dried on Na_2SO_4 and the solvent was evaporated to dryness under vacuum at T<35 °C. Finally, the residue was dissolved in 5 ml of a mixture consisting of 1:1 acetone-methanol. A 5 ml aliquot from this mixture was taken for saponification.

1.3.2. Saponification procedure. The method described by MÍNGUEZ-MOSQUERA and co-workers (1993) and MÍNGUEZ-MOSQUERA and HORNERO-MÉNDEZ (1993) was slightly modified as follows. A 5 ml aliquot from the extraction process was evaporated to dryness and the residue was redissolved in 40 ml of diethyl ether. This solution was mechanically shaken for 1 h at room temperature after addition of 9 ml of KOH 30% in methanol under nitrogen atmosphere, avoiding light incidence. After this time, the solution was transferred to a separation funnel and washed twice with 40 ml of NaCl (10%) and afterwards with 40 ml of distillated-deionized water until pH 7. The organic phase was filtered and evaporated to dryness under vacuum at T<35 °C. Finally, the residue was dissolved in 10 ml of acetone using an ultrasound bath.

1.3.3. Identification and quantification procedures. A Hewlett Packard (HP) liquid chromatograph (HPLC) equipped with a quaternary pump (HP Model 1050), a variable-wavelength UV detector (HP Model 1050), and a Chem-station HP software were used.

The pigments were separated on a 250×4 mm (i.d.) column packed with Spherisorb ODS 2 C_{18} 5-μm reversed phase and a 20×4 mm (i.d.) precolumn C_{18} ODS-Hypersil 5-μm from Hewlett-Packard. An acetone and water gradient solvent system was used at a flow rate of 1.2 ml min^{-1}, and detection was carried out at 450 nm (MÍNGUEZ-MOSQUERA & HORNERO-MÉNDEZ, 1993; MÍNGUEZ-MOSQUERA & HORNERO-MÉNDEZ, 1994).

2. Results and discussion

In the present experiment, only over-ripe capsicum fruits were used and, therefore, total capsanthin was the major red carotenoid in all six varieties studied (Table 1) and accounted for 75–93% of the total red carotenoids pigments and for 49–68% of the total red + yellow carotenoids pigments. That is why that in industry total red pigments are usually quantified and/or expressed as capsanthin. Capsanthin and capsorubin contents of paprika fruit increase proportionally to the advanced ripeness (BIACS et al., 1989). The relative order of the three red pigments was: trans-capsanthin >> cis-capsanthin ≅ capsorubin. These results agree with those previously reported by BIACS and co-workers (1985; 1989) and PHILIP and FRANCIS (1971), although the latter authors reported that capsanthin accounted only for 35% of the total carotenoids in red peppers.

The mature red fruits from all six cultivars studied contained trans-capsanthin, cis-capsanthin, and capsorubin. Their relative proportions were, however, cultivar-specific and depended on the titanium ascorbate treatment (Table 1). The total amount of red carotenoid pigments is one of the most important pepper fruit quality parameters because the decision on which cultivar should be chosen from the industrial and commercial points of view is usually based on this characteristic. For instance, the Gorogled-6 fruits had the lowest total red pigment content of all studied plants independently from titanium-spray. On the other hand, the Spanish variety Negral presented the highest concentrations of these three red carotenoids pigments in control plants, however, in the Ti^{4+}-treated plants, the levels of both trans- and cis-capsanthin were higher in the Hungarian variety, Mihályteleki.

Table 1

Concentrations of red pigments in fresh pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars as affected by titanium ascorbate treatment*

Cultivar	Trans-capsanthin	Cis-capsanthin	Capsorubin	Total red pigments
		mg kg^{-1} f.w.		
		Control plants		
Albar	2300 b	203 c	326 c	2829 b
Negral	3278 c	322 e	489 d	4089 c
KM-622	3267 c	268 de	240 b	3775 c
Mihályteleki	1527 a	252 d	231 b	2010 a
Buketen-50	2802 bc	130 b	90 a	3022 b
Gorogled-6	1588 a	94 ab	80 a	1762 a
		Leaf-Ti sprayed plants		
Albar	4034 b	73 a	253 b	4360 b
Negral	3526 ab	274 c	602 c	4402 b
KM-622	3238 a	341 d	246 b	3825 ab
Mihályteleki	4323 b	424 d	239 b	4986 b
Buketen-50	4107 b	191 bc	117 a	4415 b
Gorogled-6	3057 a	350 d	230 b	3637 a

Data are the average values from three individual samples. Values followed by the same letter, within the same column and type of plants (Ti-treated or non treated), are not significantly different at a $P = 0.05$ level (unpaired t-test)

The experimental results of the varietal influence on the yellow pigments composition are summarised in Table 2. Data on this table show that the most abundant pigment was zeaxanthin but not β-carotene, which is usually the pigment selected in the industry to express the total concentration of yellow pigments. The relative order of the five yellow pigments was: zeaxanthin >> β-carotene > β-cryptoxanthin > violaxanthin ≅ antheraxanthin.

When the plants were leaf-sprayed with Ti^{4+} during the growing cycle (Table 1), all cultivars, except the variety KM-622, increased their fruit pericarp red carotenoid pigments, with the most positive effect being for the Mihályteleki and Gorogled-6 cultivars. A similar pattern was observed for the yellow pigments (Table 2).

Table 2

Concentrations of yellow pigments in fresh pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars as affected by titanium ascorbate treatment*

Cultivar	Zeax.	β-Carotene	Violax.	β-Cryptox.	Antherax.	Tot. yellow pig.
	mg kg^{-1} f.w.					
Control plants						
Albar	348 a	139 a	96 bc	86 a	88 b	757 a
Negral	600 b	278 cd	109 bc	168 b	110 c	1265 c
KM-622	637 b	155 a	119 c	106 a	107 c	1124 b
Mihályteleki	564 b	199 b	87 b	152 b	71 b	1073 b
Buketen-50	674 b	325 d	89 b	226 c	82 b	1396 c
Gorogled-6	348 a	111 a	25 a	125 ab	21 a	630 a
Leaf-Ti sprayed plants						
Albar	1021 c	479 c	98 a	359 b	44 a	2001 cd
Negral	868 b	531 c	276 c	356 b	203 d	2234 d
KM-622	654 a	227 a	110 ab	131 a	93 b	1215 a
Mihályteleki	750 ab	210 a	150 b	179 a	119 bc	1408 ab
Buketen-50	1124 c	587 c	143 b	389 b	109 bc	2352 e
Gorogled-6	825 ab	330 b	139 b	357 b	121 c	1772 bc

Data are the average values from three individual samples. Values followed by the same letter, within the same column and type of plants (Ti-treated or non treated), are not significantly different at a P = 0.05 level (unpaired t-test)

Titanium spray treatment increased the total concentration of yellow pigments in all six studied cultivars. Because of the non-uniform increase of the red and yellow pigment contents, the red/yellow pigment ratio changed as summarised in Table 3. This ratio remained constant for KM-622, Buketen-50 and Gorogled-6. The ratios of Albar and Negral varieties decreased, due to the higher increase of the yellow pigments compared to the red ones. Finally, the only cultivar in which the ratio increased significantly was Mihályteleki, due to the high increase of the red pigments, especially of trans-capsanthin. Thus, the effect of titanium spray could be summarised as follows: it improved the photosynthetic activity of the plants, increasing the concentration of both red and yellow pigments, however, the increase of the yellow pigments was higher, in general, than that of the red ones. Therefore, the red/yellow pigment ratio decreased, which meant that the quality of the red pepper pericarp did not increase as a result of this treatment. Impact of Ti-treatment on the red/yellow ratio seemed to be related to a variety-dependent factor.

Table 3

Concentrations of total carotenoids, water content and red/yellow pigment ratio in fresh pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars as affected by titanium ascorbate treatment*

Cultivar	Total carotenoids (mg kg^{-1} f.w.)	Water content (%)	Red/yellow pigments ratio
		Control plants	
Albar	3586 b	76.2 b	3.74 d
Negral	5354 d	76.7 b	3.23 d
KM-622	4899 c	81.2 c	3.36 d
Mihályteleki	3083 ab	75.7 b	1.87 a
Buketen-50	4418 c	80.3 c	2.16 b
Gorogled-6	2392 a	61.6 a	2.80 c
		Leaf-Ti sprayed plants	
Albar	6361 b	75.8 b	2.18 b
Negral	6636 b	74.3 b	1.97 a
KM-622	5040 a	74.5 b	3.15 c
Mihályteleki	6394 b	74.1 b	3.54 c
Buketen-50	6767 b	79.9 c	1.88 a
Gorogled-6	5409 a	61.3 a	2.05 b

Data are the average values from three individual samples. Values followed by the same letter, within the same column and type of plants (Ti-treated or non treated), are not significantly different at a $P = 0.05$ level (unpaired *t*-test)

During paprika production, there are some steps that induce a decrease of pigments and bioantioxidants, causing an important reduction of quality. The stability of the paprika pigments has been attributed to a number of factors, including cultivar (ALCARAZ et al., 1991; MARTÍNEZ-SÁNCHEZ et al., 1991), plant nutrition (MARTÍNEZ-SÁNCHEZ et al., 1993), moisture content, stage of ripeness at harvest (KANNER et al., 1979), and antioxidant content (BIACS et al., 1992).

Spice paprika is usually ground to a fine powder of about 0.5 mm particles. This process increases the surface area and thus allows higher lipid oxidation (CARVAJAL et al., 1998). In our study, paprika peppers were processed in a similar way like in paprika powder production (drying in an air-forced oven at 55 °C for 48 h and grinding using a coffee mill) to study how the drying process influences the quality attributes of paprika. Usually seeds are removed from the pericarp before the drying and grinding operations, because the addition of seeds decreases the total pigment content of the product and damages the most important pigments (BIACS et al., 1989). Evidence has been presented for the presence of lipoxygenase in the seeds of paprika (DAOOD & BIACS, 1986), and this enzyme has carotenoid bleaching activity through both aerobic and anaerobic pathways (ESKIN et al., 1977).

The drying process significantly affected the composition of both red and yellow pigments and the order of the six varieties according to their relative pigment composition as described in Tables 4, 5, and 6. There is a varietal incidence on the effects of drying and grinding operation, the concentrations of red and yellow pigments and their ratio. Trans-capsanthin and zeaxanthin were, however, still the most abundant red and yellow pigments, respectively, similarly to the pattern observed in the case of fresh pericarp.

The Spanish varieties exhibited a high loss of total red (Table 4) and yellow (Table 5) pigments after this conditioning of the samples, which agreed with previous results by REVERTE and co-workers (2000). Data on water content in fresh pericarp is included in Table 3, so readers can easily calculate the concentrations of total carotenoids in fresh pericarp on a dry matter basis, and in this way compare results with those of dry-ground paprika samples. There are some interesting results showing that the Bulgarian varieties and the Hungarian Mihályteleki increased their total red pigments by as much as 54% (Gorogled-6) after the drying and grinding processes (Table 4).

Table 4

Concentrations of red pigments in dry and ground pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars as affected by titanium ascorbate treatment*

Cultivar	Trans-capsanthin	Cis-capsanthin	Capsorubin	Total red pigments
	mg kg^{-1} d.w.			
Control plants				
Albar	6450 a	538 a	668 b	7655 a
Negral	8245 b	845 b	1000 c	10090 b
KM-622	10000 c	1681 c	1367 c	13048 c
Mihályteleki	9202 b	535 a	222 a	9959 b
Buketen-50	13421 c	1228 c	1036 c	15685 c
Gorogled-6	5674 a	872 b	534 b	7081 a
Leaf-Ti sprayed plants				
Albar	12467 c	963 a	1517 c	14946 c
Negral	6856 a	992 a	930 b	8778 a
KM-622	8196 b	1416 b	580 a	10192 b
Mihályteleki	8780 b	1444 b	1077 b	11301 b
Buketen-50	12055 c	1522 b	1294 bc	14871 c
Gorogled-6	7121 a	912 a	742 ab	8775 a

Data are the average values from three individual samples. Values followed by the same letter, within the same column and type of plants (Ti-treated or non treated), are not significantly different at a $P = 0.05$ level (unpaired *t*-test)

A similar situation was found for the Gorogled-6 when studying the yellow pigments (Table 5). The increase of the pigment concentrations could be related to the water content in the fruits of these varieties (REVERTE et al., 2000). In fact, Gorogled-6 was the variety that showed the lowest decrease of the total extractable colour during a thermo-stability experiment carried out by REVERTE and co-workers (2000). Paprika contains 10–12% triglycerides, containing high amounts of unsaturated fatty acids and capsanthin esterified as dilaurate (PHILIP & FRANCIS, 1971). The presence of less stable carotenoids as well as the presence of highly autoxidizable fatty acids in paprika may explain the stability of capsanthin (the most abundant red pigment) against oxidation and the low decrease of total red pigments after the drying and grinding operations for most of the studied varieties. Our results agree with those reported by BIACS and co-workers (1985), who stated that the results of enzymatic oxidation indicate that the capsanthin compound-group is more stable in the presence of lipoxygenase than capsorubin and its fatty acid esters.

Table 5

Concentrations of yellow pigments in dry and ground pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars as affected by titanium ascorbate treatment*

Cultivar	Zeax.	β-Carotene	Violax.	β-Cryptox.	Antherax.	Tot. yellow pig.
	mg kg^{-1} d.w.					
Control plants						
Albar	811 a	176 a	55 a	239 a	76 a	1357 a
Negral	1176 ab	541 b	116 a	408 ab	227 b	2468 b
KM-622	1559 b	734 b	574 b	649 b	596 c	4112 c
Mihályteleki	2037 c	621 b	362 b	535 b	309 b	3864 c
Buketen-50	2310 c	1239 c	376 b	807 b	401 bc	5132 d
Gorogled-6	1315 b	591 b	195 a	568 b	195 b	2865 b
Leaf-Ti sprayed plants						
Albar	2421 c	930 bc	384 b	707 b	488 b	4930 d
Negral	895 a	198 a	74 a	323 a	101 a	1591 a
KM-622	1514 b	706 b	129 a	349 a	149 a	2847 b
Mihályteleki	1811 b	595 b	398 b	583 ab	371 ab	3757 c
Buketen-50	1632 b	1154 c	517 b	846 b	517 b	4667 d
Gorogled-6	1744 b	755 b	300 b	724 b	248 a	3770 c

Data are the average values from three individual samples. Values followed by the same letter, within the same column and type of plants (Ti-treated or non treated), are not significantly different at a $P = 0.05$ level (unpaired t-test)

Data on the red/yellow pigment ratio could be divided into two groups according to their thermo-stability (drying operation): a) Albar, Negral, Buketen-50 and Mihályteleki, and b) KM-622 and Gorogled-6, the ratios of the first group increased and those of the second one decreased, respectively. Consequently, the red pigments of the plants belonging to the first group varieties were more thermo-stable than the yellow pigments.

All plants treated with Ti^{4+} showed a significant reduction in their total red and yellow pigment content after the industrial operations. However, and in a general way, total red and yellow pigments of dry and ground pericarp were higher in the Ti^{4+}-treated plants than in the non-treated paprika, showing a positive effect of this treatment, especially in Albar and Gorogled-6 cultivars.

Table 6

Concentrations of total carotenoids and red/yellow pigment ratio in dry and ground pericarp from several Spanish, Hungarian and Bulgarian red pepper (paprika, Capsicum annuum *L.) cultivars as affected by titanium ascorbate treatment*

Cultivar	Total carotenoids(mg kg^{-1} d.w.)	Red/yellow pigments ratio
	Control plants	
Albar	9013 a	5.64 d
Negral	12558 b	4.09 c
KM-622	17160 c	3.17 b
Mihályteleki	13823 b	2.58 a
Buketen-50	20817 c	3.06 b
Gorogled-6	9945 a	2.47 a
	Leaf-Ti sprayed plants	
Albar	19876 c	3.03 b
Negral	10370 a	5.52 c
KM-622	13039 b	3.58 b
Mihályteleki	15058 b	3.01 b
Buketen-50	19537 c	3.19 b
Gorogled-6	12545 ab	2.33 a

Data are the average values from three individual samples. Values followed by the same letter, within the same column and type of plants (Ti-treated or non treated), are not significantly different at a $P = 0.05$ level (unpaired *t*-test).

Although Ti^{4+} treatment increased both red and yellow pigment contents, their increase was not proportional, as previously described for fresh pericarp data. Therefore, the red/yellow pigment ratio remained statistically constant for the Hungarian and Bulgarian varieties. This ratio increased for the Negral and decreased for the Albar species, respectively. Therefore, it must be stressed again, considering the ratio of red/yellow pigments as the main quality parameter, that a beneficial treatment, assumed to improve the biological activity of a crop species, was not useful from an industrial point of view.

3. Conclusion

The low levels of the precursor of various xantophylls, β-carotene, were likely due to the advanced stage of hydroxylation (yielding zeaxanthin), epoxydation (yielding anteraxanthin and violaxanthin) and rearrangement (yielding capsanthin and capsorubin, end of the biosynthesis reactions) reactions of the over-ripe fruits selected for these

experiments. The concentration of capsanthin in both fresh and dry/ground pericarp samples was always higher than that of capsorubin. The most abundant yellow pigments found in peppers were zeaxanthin and β-carotene. The Spanish cultivar Albar showed the highest red/yellow pigment ratio of all studied varieties in control materials (fresh pericarps and spice red pepper), however, in Ti^{4+}-treated plants the Hungarian KM-622 had the highest values of this same ratio. Peppers from Ti^{4+}-ascorbate treated plants had higher levels of both red and yellow pigments, however, this increase of the biological activity of peppers did not lead to a better quality of spice red pepper as indicated by a general decrease in the red/yellow pigments ratio.

*

This work was supported by the EU COPERNICUS program CIPTA-CT94-0222. The authors gratefully thank Prof. I. PAIS (University of Horticulture and Food Industry, Budapest) for providing Titavit® used in the experiments.

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Acta Alimentaria, Vol. 30 (2), pp. 173–187 (2001)

EXAMINATION OF THE PROTEIN–EMULSIFIER–CARBOHYDRATE INTERACTIONS IN AMARANTH BASED PASTA PRODUCTS

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(Received 23 February 2000; accepted: 14 November 2000)

Different pasta products were prepared using amaranth species with the addition of emulsifiers: mono- and diacyl-glyceride, lecithin, lecithin and lysolecithin and diacetyl-tartaric ester of monoglyceride in an amount of 1.2 g/g flour. For the characterisation of interaction between the emulsifiers and the components of raw material properties the cooking values – water uptake and cooking loss –, sensory assessment of the pasta systems, the amount of albumin and globulin as well as "glutelin" fractions, the molecular weight distribution of the soluble protein with SDS-PAGE and complexing rate were examined. It was found that amaranth flour is suitable for pasta products with excellent cooking and sensory properties. However, without emulsifier the production of pasta products was not possible. The amount of soluble fraction was reduced by the addition of emulsifiers, this increasing was significant at the "glutelin" fraction. Emulsifiers to a different degree, depending on the emulsifiers and variety of amaranth, affected the molecular weight distribution of albumin, globulin and "glutelin". In the structure an emulsifier-protein-carbohydrate-lipid complex can be expected. In the presence of diacetyl-tartaric ester of monoglyceride, lysolecithin and mono- and diacylglyceride as well as stearoyl lactylate we could detect the protein – emulsifier and emulsifier–carbohydrate interaction – with high complexing rate, 50–90%. In the presence of lecithin the texture was less good and probably only protein–emulsifier interactions occurred. The complexing rate was as low as 5%. The best pasta product was produced from *Amaranthus cruentus* with diacetyl-tartaric ester of monoglyceride (1.2%).

Keywords: emulsifier, pasta products, interaction and structure, cooking quality, amaranth

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Grain amaranthus was consumed by the Aztecs till the 16th century and amaranthus species even now are cultivated as minor crops in the Central and South America and in limited regions of Asia and Africa (GARCIA et al., 1987; BRESSANI, 1988; KAUFMANN, 1992). The seeds of some species of grain amaranthus are mainly popped to make a confection with sugar syrup or milled for baking application. Nowadays amaranth has the potential to become an improved "new crop" and to be successfully integrated into both modern and traditional cropping systems in several areas of the world. In the last years amaranth was grown in Europe: Austria, Poland and Hungary.

Functional food products in modern eating habits have been gaining ground these days. Components advantageous for health were rendered into 12 categories by the Japanese Ministry of Health (GOLDBERG, 1994). The amaranth seeds have those components. Reports have shown that the seeds contain about 13–18% crude protein with more lysine and a more favourable amino acid composition than any other cereal (BRESSANI et al., 1987; GUPTA et al., 1989; GUPTA & SEGHAL, 1992). Amaranth grain has a particularly high nutritive value with high digestibility and an amino acid composition close to optimal for human requirements (MENDOZA & BRESSANI, 1987; PEDERSEN et al., 1987; PRAKASH & PAL, 1991; LEHMANN, 1992; BERGHOFER et al., 1997). The nutritional value of amaranth seeds has been reported as comparable to that of casein. The protein fractions are albumin and globulin 66%, prolamin 0.7% and gluten 28.5%. Starch content varies from 50 to 70% of which amylopectin can be almost 80–100%. Its oil and fat content is approximately 8% and is rich in vitamin E. Its Ca, K, P as well as Mg and Fe content is relatively high.

Amaranth seeds fulfil the requirements of functional foods in many ways. The prolamin type protein content of amaranth is low, therefore these seeds are suitable for the diet of people suffering from celiac disease and emulsifiers are required to form a structure of the products. In the presence of emulsifier an emulsifier–carbohydrate–protein–lipid complex can be expected. The rate of the individual interactions depends on the components of the given sample. In the literature there are details about the electrophoretic characterisation of the protein fractions of amaranthus, but no details about the change in the fractions to influence the process or use of emulsifiers can be found (GORINSTEIN et al., 1991; BARBA DE LA ROSA et al., 1992).

The aim of this study was to investigate the interactions between the emulsifiers and different amaranth species in model systems as well as to examine how these interactions influence the structure of pasta products, which were made from amaranth flour with different type of emulsifiers.

1. Materials and methods

Different varieties of amaranthus were used: *Amaranthus cruentus* from Austria and *A. molerose, A. mantegazzianus, A. cruentus(I), A. caudatus, A. hypochondriacus* as well as *A. cruentus(II)* from the Cereal Research Non-Profit Company, Szeged, Hungary. The seeds were milled to a size of 250–500 μm. Mono- and diacyl-glyceride, lecithin, lecithin and lysolecithin, sodium-stearoyl lactylate and diacetyl-tartaric ester of monoglyceride were used in the production. The composition of the examined amaranth samples can be seen in Table 1 as well as the applied emulsifiers in Table 2.

Table 1

Composition of the examined amaranth varieties

Sample	Dry material content (%)	Protein (% d.m.)	Total Carbohydrate (% d.m.)	Fat (% d.m.)
A. cruentus (Austria)	90.58	15.97	44.47	7.22
A. molerose (Hungary)	89.41	16.09	51.53	8.22
A. mantegazzianus (Hungary)	90.02	16.41	48.70	7.08
A. cruentus(I) (Hungary)	90.52	16.02	54.11	9.01
A. caudatus (Hungary)	90.18	16.50	54.56	8.22
A. hypochondriacus (Hungary)	88.96	17.52	56.47	9.11
A. cruentus(II) (Hungary)	90.12	16.03	54.64	6.92

Table 2

Type of applied emulsifiers

Name	Company	Type of emulsifiers
Dimodan PM (D)	Grindsted, Denmark	Mono-and diacyl-glyceride
Epikuron 130 P (E)	Lucas Meyer, Germany	Lecithin
VP-618-10 (V)	Lucas Meyer, Germany	Lecithin and Lysolecithin
Multec SSL (S)	Beldem Food Ingredients, Belgium	Sodium-stearoyl lactylate
Multec Data (B)	Beldem Food Ingredients, Belgium	Diacetyl-tartaric ester of monoglyceride

1.1. Preparation of pasta samples

The amount of flour and water accounted for 40% moisture content in model systems with the addition of 1.2% emulsifiers (concerning the mass of flour). Suspension was made from the emulsifier and water. The temperature of suspension was raised to 97 °C. It was stirred for 15 min in a mixer. Fine and small pieces of pasta were prepared by a dough processing machine through Teflon matrix. The product was dried at 39 °C and 87% relative humidity for 24 h.

1.2. Cooking test and sensory assessment

Cooking value: water uptake and cooking loss as well as sensory assessment were determined by cooking test.

1.2.1. Method of cooking test according to KARÁCSONYI (1970). Two hundred and fifty cm^3 tap water (pH=6.80) were boiled in a high, 600 cm^3 beaker on an electric pasta cooker (1200 W output). Twenty five g intact pasta, free of debris were weighed on a counter-balance and then cautiously poured into the water, whereby the water was kept boiling moderately during 10 min. In order to prevent catching, the contents of the beaker were stirred at the beginning of cooking, several times with a glass rod.

In order to determine the wet weight of the cooked pasta, it was rinsed on a Buchner funnel connected to a vacuum pump. After cooling the pasta was weighed in a vessel of known weight. Weighing of the wet mass was followed by sensory assessment.

Water uptake (V) in % is calculated as follows:

$$V = \frac{(a - b)}{a} \cdot 100$$

where, b: mass of wet pasta (g), a: pasta taken for cooking (g).

The cooking and rinsing water obtained in the cooking test was concentrated by evaporation, then dried to constant mass at 102±1 °C. For 25 g pasta, cooking loss (*F*) in % is calculated as follows:

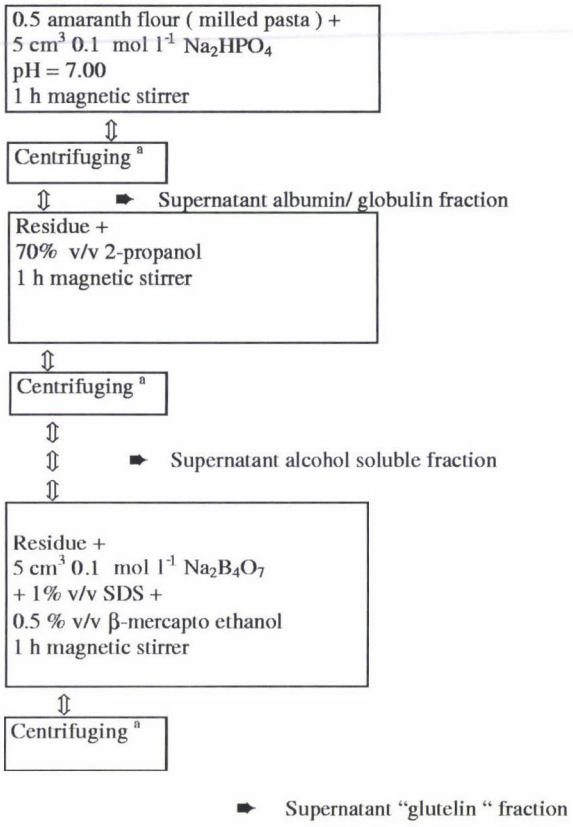
$$F = a \cdot 4$$

where, a: mass of cooking and rinsing water after drying (g).

1.2.2. Sensory assessment according to the HUNGARIAN STANDARD (1986). The pasta product was assessed first in raw state (uncooked) then in the cooked state whereby cooking was performed according to the cooking test. Sensory assessment included four groups of properties: outer appearance, aroma, taste and consistency. Sensory evaluation was performed by a jury of 3 members. The maximum score for the individual groups of properties was 5. Within the individual groups of properties weighted mean values were calculated. Calculation of the weighted mean was performed as follows: appearance × 1.1 + aroma × 0.7 + taste × 0.9 + consistency × 1.3.

1.3. Solubility fractionation

The pasta samples were ground to flour size particles in a Lab Mill 1QC-114 type grinder. Protein extraction of milled samples were carried out by methods of BARBA DE LA ROSA and co-workers (1992). The scheme is shown in Fig. 1. The amount of albumin and globulin as well as "glutelin" fraction was determined by micro-Kjeldahl method.



[a] Janetzki 3 30 centrifuge, min 4000 r.p.m.

Fig. 1. Protein extraction

1.4. SDS–PAGE electrophoretic investigations

Electrophoretic investigations were carried out according to KOVÁCS (1992). Proteins were separated in a 12.5% acrylamide gel. The fractions were diluted (1:4) and 5 µl of the fractions were used for the examinations. Mini-gels were used (vertical electrophoretic apparatus P 2000, Belgium). The size of the gel plates was 100×100×1 mm, the duration 110 min at 140 V (32 mA). The gels were fixed and stained in one step, in a mixture of 12.5% trichloroacetic acid and 0.25% Coomassie Brillant Blue R-250 (19:1). The gels were stained for lipids, too, according to

NEDELKOVICS and TELEKY-VÁMOSY (1974). The molecular weight distribution of the protein fraction in the gel was estimated by densitometer (Biomed Instruments).

1.5. Iodine binding capacity according to CONDE-PETIT (1992)

The iodine binding capacity (IBC) of starch was determined by amperometric titration with the use 140 mV voltage. Twenty cm^3 of pasta suspension portion (1 g milled pasta samples in 100 cm^3 solution) was used for the titration. The titration was carried out with 0.005 mol l^{-1} iodine solution and with a titration rate of 1 cm^3 min^{-1}. The amount of bound iodine (*Ib*) was determined graphically, and the iodine binding capacity (*IBC* %) and the complexing rate (*CR* %) were calculated as follows:

$$IBC = \frac{IB}{S} \cdot 100$$

$$CR = \frac{IBC\ (\text{without}) - IBC\ (\text{with emulsifier}) \cdot 100}{IBC\ (\text{without})}$$

where, *Ib*: amount of bound iodine (mg), *S*: amount of starch in the system (mg), *IBC*: iodine binding capacity.

All investigations were carried out in triplicate.

2. Results and discussion

2.1. Influence of the variety

Pasta samples made from different varieties with diacetyl-tartaric ester of monoglyceride resulted in pasta products with different cooking value and sensory assessment (Table 3). In general the amaranth pasta products need shorter cooking time than the wheat products. For the cooking of amaranth pasta 5 min is enough, and 10 min cooking time is suitable for the characterisation of overcooked condition.

Amaranthus mantegazzianus variety resulted in the best quality of pasta, which can be characterised with excellent sensory assessment and small cooking loss. The emulsifier–amylose complex is considered to be important in the structure formation in the model systems, because the complexing rate is 66%. *Amaranthus mantegazzianus* and *A. hypochondriacus* varieties showed products with good cooking values – high water uptake and low cooking loss – but the sensory assessment presented lower quality in outer appearance. In these systems an emulsifier–amylose complex can be expected, the complexing rate was found to be 60–75%. The *Amaranthus caudatus* and *A. cruentus(II)* varieties were not suitable for pasta production. In all systems the protein-emulsifier interactions are important, too.

Table 3

Properties of amaranth pasta products from different varieties without and with addition of diacetyl-tartaric ester of monoglycerides 1.2% (B)

Variety (Hungary)	Sensory assessment					Cooking value		Complexing rate (%)	Soluble protein	
	Appearance	Aroma	Taste	Consistency	Total Σ	Water uptake (%)	Cooking loss (%)		Albumin+ Globulin (%)[a]	"Glutelin" (%)[a]
A. *mantegazzianus*									46.62	37.39
+B	4	5	5	5	18.9	148.68*	15.35	75.03	45.58	28.30*
A. *cruentus(I)*									51.31	38.78
+B	5	5	5	5	20	125.35	15.53	66.66	57.35	26.00*
A. *caudatus*									53.21	34.66
+B	3	5	5	3	15.2	127.67	26.85*	90.90	49.01	25.59*
A. *hypochondriacus*									42.73	35.22
+B	4	5	5	5	18.9	110.46	24.83*	60.52	42.29	21.70*
A. *cruentus(II)*									48.78	40.83
+B	3	5	5	3	15.2	163.89*	32.53*	75.00	40.02	19.92*

* significant (the values were assessed by ANOVA at P = 5% level)

[a] related to total protein

Table 4

Properties of pasta products from Amaranthus molerose *(Hungary) with different type of emulsifiers 1.2%*

Emulsifier[b]	Cooking time (%)	Sensory assessment					Cooking value		Complexing rate (%)	Soluble protein	
		Appearance	Aroma	Taste	Consistency	Total Σ	Water uptake (%)	Cooking loss (%)		Albumin+ Globulin (%)[a]	"Glutelin" (%)[a]
Raw material										62.18	30.47
E	5	4	5	5	4	17.6	142.94	24.50			
E	10	5	5	5	3	15.2	178.16*	36.80*	5.87	56.21	12.43*
V	5	4	5	5	4	12.8	124.02	31.30			
V	10	2	5	5	2	17.6	135.29	49.46*	60.58	59.7	24.25
B	5	5	5	5	5	20.0	123.04	24.82			
B	10	3	5	5	3	15.2	161.27*	34.02	87.82	58.45	15.54*
S	5	5	5	5	5	20.0	134.35	24.63			
S	10	3	5	5	3	15.2	146.97	41.21*	70.58	62.18	14.92*
D	5	4	5	5	4	17.6	114.02	19.40			
D	10	2	5	5	3	14.1	134.01	41.16*	74.71	52.53	21.79*

* significant (the values were assesed by ANOVA at P = 5% level)

[a] related to total protein

[b] for emulsifier codes see Table 2

Table 5

Properties of amaranth pasta products from Amaranthus cruentus *(Austria) with addition of different emulsifiers 1.2%*

Emulsifier[b]	Cooking time (min)	Sensory assessment					Cooking value		Complexing rate (%)	Soluble protein	
		Appearance ×1.1	Aroma ×0.7	Taste ×0.9	Consistency ×1.3	Total Σ	Water uptake (%)	Cooking loss (%)		Albumin+ Globulin (%)[a]	"Glutelin" (%)[a]
Raw material	–	–	–	–	–	–	–	–		53.6	24.3
E	5	4	5	5	5	18.9	139.5	19.7			
E	10	3	5	5	3	15.2	125.0	23.6	6.72	48.1	7.80*
V	5	4	5	5	5	18.9	129.8	16.3			
V	10	3	5	5	3	15.2	168.7	34.5	56.66	50.9	15.3*
B	5	5	5	5	5	20	141.9	13.1			
B	10	5	5	5	5	20	150.5	22.4	89.99	50.0	10.3*
S	5	5	5	5	5	20	142.9	15.7			
S	10	3	5	5	3	15.2	149.8	26.3	93.20	55.6	22.1
D	5	5	5	5	5	20	163.7	16.0			
D	10	4	5	5	4	17.6	159.2	22.5	86.6	47.3*	17.4

* significant (the values were assessed by ANOVA at P = 5% level)

[a] related to total protein

[b] for emulsifier codes see Table 2

Generally, (Tables 3, 4 and 5) emulsifiers are required for developing the structure. The ethanol soluble fraction was smaller than 1%, so it is the reason why we examined only the albumin and globulin as well as "glutelin" fractions of the samples.

The quality of the developed structure can be characterised by the amount of soluble protein fractions and the molecular weight distribution of the fractions.

In the amount of the protein fractions (Table 3) of albumin and globulin we could not find significant differences between with and without emulsifier produced fractions, but the amount of "glutelin" fractions were reduced significantly by the emulsifiers.

On the basis of electrophoretic pattern, it can be stated clearly that the varieties did not differ from each other in the molecular weight distribution (Figs 2 and 3). We could detect small differences between the varieties. Our results are comparable with results of reference (GORINSTEIN & MOSHE, 1991; BARBA DE LA ROSA et al., 1992).

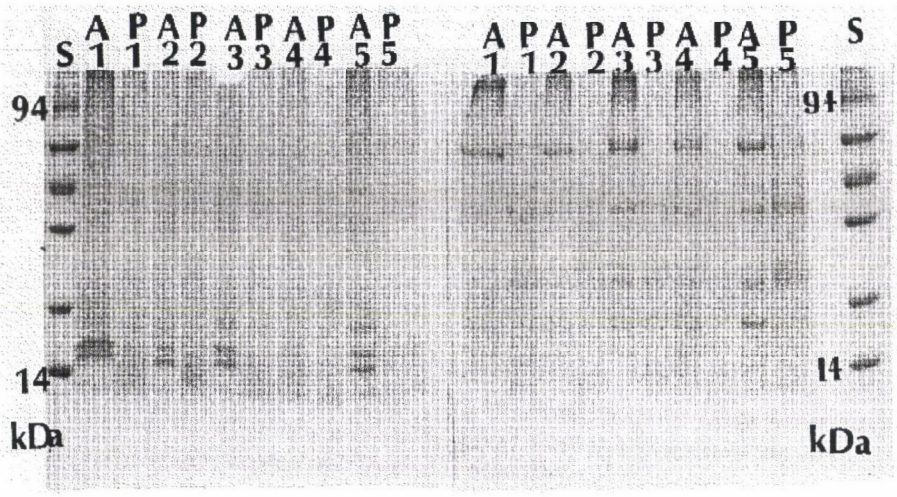


Fig. 2. Electropherograms of the albumin, globulin and the "glutelin" fractions of different varieties of amaranth pasta products made with the addition of diacetyl-tartaric ester of monoglycerides. Vertical electrophoresis apparatus PS 2000, 12.5% acrylamide concentration, 1 mm gel plate, 110 V, 110 min; staining with Coomassie Brillant R 250; S = Standard protein (14 kDa α-Lactalbumin, 20 kDa Trypsin inhibitor, 30 kDa Carbonic Anhydrase, 43 kDa Ovalbumin, 67 kDa Albumin and 94 kDa Phosphorylase b, Pharmacia, Sweden) A: Amaranth varieties; P: pasta products with B emulsifier (1.2%); 1: *A. mantegazzianus*; 2: *A. cruentus(I)*; 3: *A. caudatus*; 4: *A. hypochondriacus*; 5: *A. cruentus (II)*

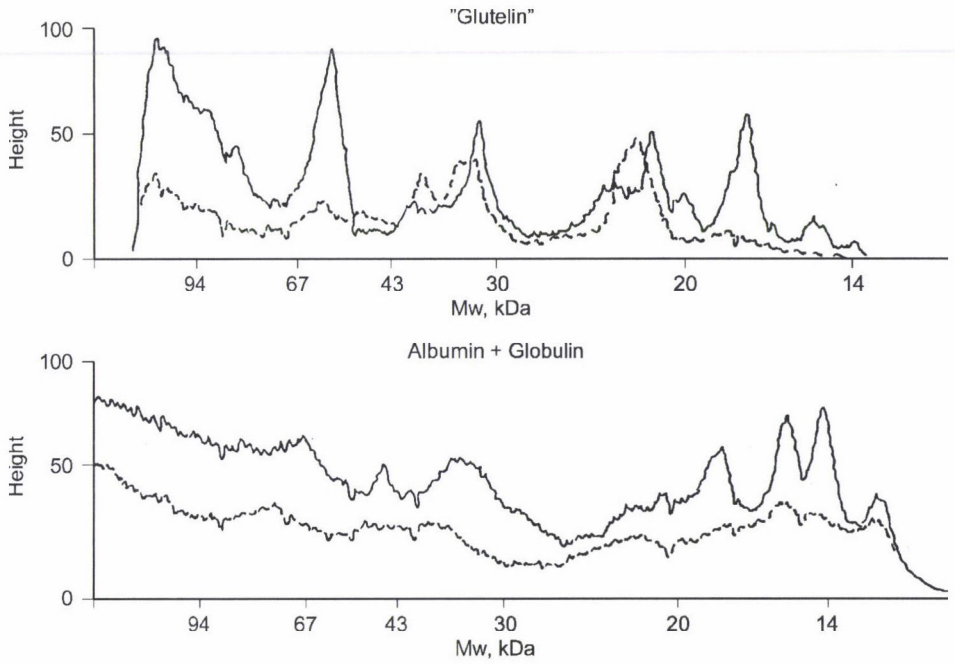


Fig. 3. Molecular weight distribution of the albumin and globulin as well as the "glutelin" fractions in the presence of B emulsifier (1.2%) in the *A. cruentus (II)* (Video Densitometer, Biomed).
—— : Without emulsifier, - - - - -: with emulsifier

In the albumin and globulin fraction of *Amaranthus cruentus(II), A. caudatus, A. cruentus(I)* and *A. mantegazzianus* variety we managed to demonstrate subunits of 12, 14, 16, 18, 35, 43 and 67 kDa region (Figs 2 and 3). In *Amaranthus hypochondriacus* variety we could not find fraction of 43 kDa and the amounts of 12, 14 and 16 kDa subunits were similar. By the addition of diacetyl-tartaric ester of monoglicerid the amount of albumin and globulin fractions were reduced. But aggregation occurred by the emulsifiers in the molecular weight distribution of these fractions, the amount of 12 kDa and 14–20 kDa increased, while that of 38 kDa diminished. In the *Amaranthus cruentus* (Austria) we found subunits of 23 kDa as a characteristic, new fraction.

We could find only a smaller difference in the amount of "glutelin" fraction in the different amaranth varieties, but in all cases it was reduced by the applied emulsifiers (Fig. 2). In the "glutelin" fractions we could observe similar subunits of 16, 18, 23–24, 36–38 and 68 kDa in the *Amaranthus mantegazzianus, cruentus(I)* and *caudatus* varieties and we could detect subunits of 58 kDa instead of 68 kDa fraction in the varieties *Amaranthus hypochondriacus* and *A. cruentus(II)*. The application of diacetyl-tartaric ester of monoglyceride resulted in a similar aggregation in the "glutelin"

fraction: two main fractions were 24 and 38 kDa, and we could detect fractions only in the molecular weight region in a 20–60 kDa. On the basis of lipid staining we could only find lipid fractions both in the albumin and globulin of the amaranth flour and amaranth pasta samples, in the 14–20 kDa molecular weight region.

2.2. Influence of emulsifier

Amaranth pasta products from *Amaranthus cruentus* (Austria) and *A. molerose* (Hungary) were prepared in model systems with addition of different type emulsifier (Tables 4 and 5). The test cooking was made with 5 and 10 min cooking time. On the basis of experiments (Tables 4 and 5) we could state that the emulsifier after 5 min cooking time resulted in a similar structure of pasta with similar cooking value in the two amaranth varieties. The best cooking values were obtained by the use of diacetyl-tartaric ester of monoglyceride 1.2%: high water uptake with small cooking loss. In the structure the emulsifier-protein interaction is important, but we have to count for the emulsifier-carbohydrate complex, too. The values of complexing rate were similar, 87–89%. The emulsifiers sodium-stearoyl lactylate and lysolecithin resulted in similar pasta quality and complexing rate, but the cooking loss was lower in *Amaranthus cruentus* than in *Amaranthus molerose*. The pasta products made with lecithin resulted in no good structure, the products had a high water uptake, and the structure was not as massive as with other emulsifiers. The values of sensory assessment were very poor after 10 min of cooking. In the structure we expected only protein-emulsifier-complex, the complexing rate was only 5–6%.

The amount of soluble fractions in both albumin, globulin and "glutelin" was reduced by applying emulsifiers, and the increase in "glutelin" fractions was significant in all samples.

On the basis of electrophoretic patterns (Figs 4 and 5), it can be stated that the emulsifiers effected the change of molecular weight distribution, which depends on the type of emulsifiers. In the presence of diacetyl-tartaric ester of monoglyceride, stearoyl-lactylate, lecithin and lysolecthin the dominant fractions were 12, 43, 38 and 68 kDa, and at the presence of monoglyceride only 43 and 68 kDa subunits. In the molecular weight distribution of "glutelin" fraction the aggregation was affected by emulsifiers. After adding emulsifiers without monoglyceride we managed to demonstrate an increase in the fraction of 43 and 67 kDa fractions and decrease in 12 kDa fraction, but in the presence of monoglyceride the amount of 33, 43 and 67 kDa subunits were lower.

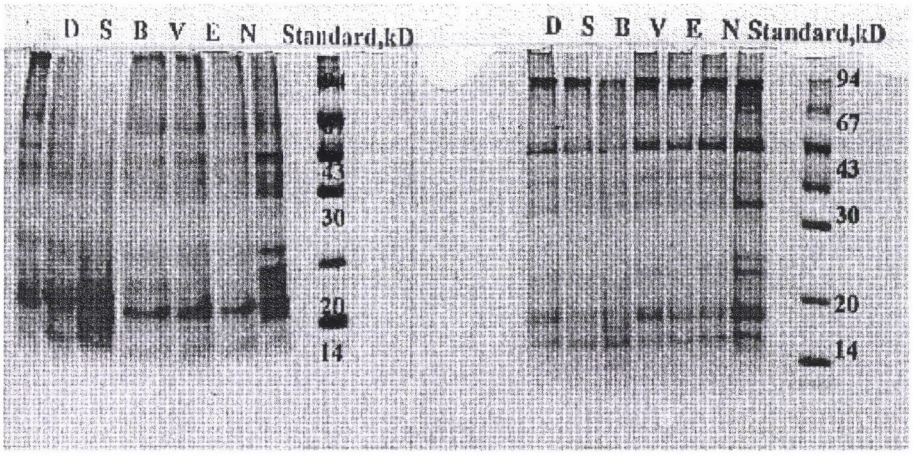


Fig. 4. Electropherograms of the albumin and globulin as well as the "glutelin" fractions of *A. molerose*. M: *A molerose* flour; D: pasta with D; V: pasta with V; B: pasta with B; S: pasta with S; E: pasta with E (Standard can be seen in Fig. 2)

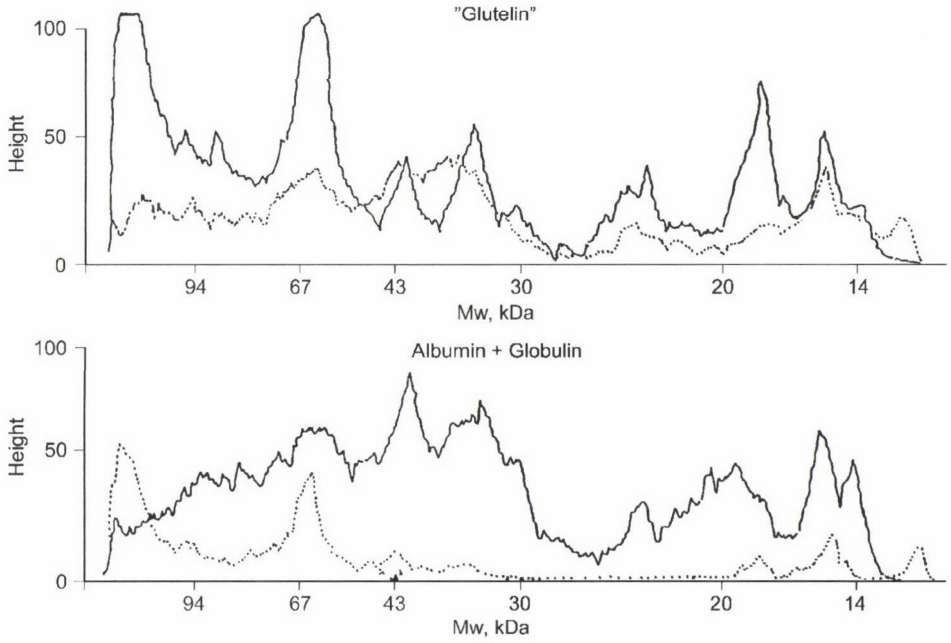


Fig. 5. Molecular weight distribution of albumin and globulin as well as "glutelin" fractions in the presence of D emulsifier in *A. molerose* variety (Video Densitometer, Biomed).
—— : Without emulsifier, - - - - -: with emulsifier

3. Conclusions

On the basis of examined amaranth varieties and applied emulsifiers – mono- and diglyceride, lecithin and lysolecithin, stearoyl-lactylate and diacetyl-tartaric ester of monoglyceride we were able to produce pasta products with different cooking quality in model systems. In the structure of the amaranth pasta systems an emulsifier–protein–carbohydrate–lipid complex can be expected. The amount and molecular weight distribution of albumin, globulin and glutelin fractions were affected by emulsifiers to different degrees depending on the emulsifiers and the variety of amaranth. The emulsifiers had higher influence on the structure than the varieties. Amaranth pasta with high cooking quality was produced from *Amaranthus cruentus* with diacetyl-tartaric ester of monoglycerid 1.2%.

In the future we would like to examine the ratio of the low and high molecular weight fractions in the albumin and globulin as well as in the "glutelin" fraction. Probably this ratio is responsible for the cooking values of the products. The emulsifier alters the ratio of these subunits, so they can help in developing the structure.

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We gratefully acknowledge the financial support from Hungarian Academy of Sciences (OTKA No. T O26447/1998).

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Acta Alimentaria, Vol. 30 (2), pp. 189–203 (2001)

INFLUENCE OF EXTRUSION CONDITIONS ON CASSAVA STARCH AND SOYBEAN PROTEIN CONCENTRATE BLENDS

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(Received: 11 April 2000; accepted: 14 November 2000)

The effects of extrusion parameters on soy protein concentrate (SPC) and cassava starch blends were studied. Extruded samples were prepared by using a Werner and Pfleiderer twin-screw extruder. The study was carried out by using Response Surface Methodology. The highest values for axial expansion were found at the maximum SPC level in the blend and at the lowest feed moisture. Higher levels of SPC, greater than 25%, reduced radial expansion. The specific volume increased as the SPC level in the blend increased and feed moisture decreased. The highest water absorption index (WAI) was found at the highest feed moisture and with the maximum SPC level in the blend, while decreasing the SPC level increased the water solubility index (WSI) of the extrudates.

Keywords: cassava starch, extrusion cooking, snacks, soy protein concentrate

Soy protein products are mainly used as ingredients in formulated foods and are seldom seen by the consumers. Most soy proteins are derived from "white flakes," made by dehulling, flaking and defatting soybeans by hexane extraction. These may then be milled into defatted flours or grits containing ~50–54% protein; extracted with ethanol or acidic water to remove flavour compounds and flatulence sugars, producing soy protein concentrates containing 65–70% protein; the fiber removed by centrifugation and reprecipitate and dry the protein. Mixtures of soy proteins with cereals, dried milk or egg fractions, gelatin, stabilizers and emulsifiers are offered for specific baking, whipping, breading and batter applications. Also, soy protein ingredients are used in compound foods for their functional properties, and to increase the total protein content and improve the essential amino acid profile (LUSAS & RIAZ, 1995).

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0139-3006/2001/$ 5.00

With a model system of soybean protein isolate (SPI) and potato starch YURYEV and co-workers (1995) showed that their blending markedly improved the texture and functional properties of the expanded extrudates. Problems confronted in the marketing of soy-based products are perceived inferior taste quality, cost, inaccessibility in supermarkets and improper shelf placement (SOYFOODS ASSOCIATION OF AMERICA, 1997). There is a potential for extruded soy-snacks to meet consumer expectations. Moisture content and extrusion conditions, as well as soy protein type are directly related to the sensory and physical characteristics (FALLER et al., 1999). Thus, currently there are no commercial soy-based snack products on the U.S. market. Our objective was to develop an extruded snack product containing soy protein concentrate blended with cassava starch, evaluating the influence of the soy protein level, feed moisture and barrel temperature on the extrusion conditions and physical characteristics of the product.

1. Material and methods

1.1. Blend preparation

Cassava starch (CS) and SPC (Proteimax 70) were obtained from Fleischmann Royal (Conchal – SP) and Ceval Alimentos S. A. (Esteio – RS), respectively. CS and SPC powders were mixed at various weight ratios, and the initial moisture contents of the blends were adjusted with distilled water fed into the extruder, to provide the desired values (Table 1).

1.2. Extrusion

Extrusion of the blends of CS and SPC was carried out using a Werner and Pfleiderer twin-screw extruder Model ZSK 30 (Ramsey, NJ) which had two co-rotating and intermeshing screws. The extruder specifications were as follows: barrel bore diameter, 30.9 mm; screw length, 878 mm; screw diameter, 30.7 mm; a die head with circular dual-orifice holes of 3.0 mm diameter. The screw configuration is given in Table 2, and the screw speed was held at 250 r.p.m. The extruder barrel was electrically heated and contained four independently controlled heating zones. Zone 1 was located about 5 mm past the feed entrance, and the zones progressed to the fourth zone, which was located immediately before the die plate. Extrusion temperatures were varied by setting the third and fourth zone simultaneously to the desired temperatures for the experiment (Table 2), while the temperatures for the first and second zones were maintained constant at 60 and 100 °C, respectively. The blends were fed to the extruder with a K-Tron Soder Model K2V-T20 twin-screw volumetric feeding system (K-Tron Corp., Pitman, NJ) with the feed rate held constant at 10 kg h^{-1}. Water was fed into the

Table 1

Independent variables and experimental design levels expressed in coded and natural units for extrusion

Code units[a]			Independent variables		
x_1	x_2	x_3	SPC (%)	Moisture (%)	Temperature (°C)
–1	–1	–1	10.14	20.00	120
1	–1	–1	39.86	20.00	120
–1	1	–1	10.14	26.00	120
1	1	–1	39.86	26.00	120
–1	–1	1	10.14	20.00	160
1	–1	1	39.86	20.00	160
–1	1	1	10.14	26.00	160
1	1	1	39.86	26.00	160
0	0	0	25.00	23.00	140
0	0	0	25.00	23.00	140
0	0	0	25.00	23.00	140
0	0	0	25.00	23.00	140
0	0	0	25.00	23.00	140
0	0	0	25.00	23.00	140
–1.682	0	0	0.00	23.00	140
1.682	0	0	50.00	23.00	140
0	–1.682	0	25.00	17.96	140
0	1.682	0	25.00	28.04	140
0	0	–1.682	25.00	23.00	106.4
0	0	1.682	25.00	23.00	173.6

x_1: soy protein concentrate content (SPC); x_2: moisture content (%); x_3: barrel temperature (°C) 3rd and 4th zone.

[a] $x_i = (Xi–Y)/\Delta Xi$, where:

x_i: coded value of variable Xi; Xi: real value of variable; Y: real value of variable on central point; ΔX: difference module between two consecutive values

first extruder section by a metering pump (model 504S/RL, Watson-Marlow Limited, Falmouth, England). The total moisture was varied (Table 1) by changing the amount of water delivered by the metering pump while maintaining a constant blend feed. The cutter speed was held at 29 r.p.m. and samples were collected during the steady-state operation. Steady-state conditions were assumed when there were no visible drifts in product temperature at different zones and percent torque were steady for at least 5 min.

The extrudates were dried to about 5% moisture in a forced-air oven at 60 °C for 18 h and stored in polyethylene bags until the analyses were carried out. After drying, part of the extrudates were ground in a Brabender Quadrumat Senior Mill (Duisburg, Germany) to produce products with a particle-size <250 μm (60 mesh) to help in further analyses.

Table 2

Screw configuration

Element type	No. of elements
PKR/10[a]	1
20/10[b]	1
60/60SK[c]	2
42/21SK-N	1
60/60	2
42/21	1
42/42	1
28/28	1
20/20	4
KB45°/5/14[d]	1
28/14	1
20/20	2
KB45°/5/14	1
20/20	2
KB45°/5/20	1
20/20	2
KB45°/5/20	1
20/20	1
KB90°/5/28	1
20/10LH[e]	1
42/21	1
28/28	1
20/20	6

[a] Starting or initial element
[b] Screw elements: pitch (mm)/length (mm)
[c] Undercut screw element
[d] Kneading blocks (KB): stagger °/# disks/length (mm)
[e] LH: left-handed

1.3. Experimental design and data analysis

Response Surface Methodology (RSM) was chosen to build up some mathematical models, making it possible to quantitatively interpret and describe the relationships between the selected dependent extrusion variables and the extrusion parameters. The experiments were designed according to the theory and rules of BOX and WILSON (1951). The outline of the experimental design and its independent variables and variation levels are presented in Table 1.

The data obtained were analyzed using the Statistic 6.0 for Windows program (STATSOFT, 1995) to investigate the trend of the radial expansion ratio, specific volume, water absorption and solubility indices, and hardness. It was assumed that one mathematical function is presented for each response variable in terms of two independent processing factors. The effects of extruded blend formulations of cassava starch and soy protein concentrate on selected physico-chemical characteristics were investigated. Responses of the independent variables on the radial expansion ratio (RER), specific volume (SV), water absorption index (WAI), water solubility index (WSI), and hardness (HAR) were monitored, as shown in Table 3. Estimated regression coefficients for each dependent variable were obtained from these responses by multiple linear regression (Table 4).

Table 3

Response of the dependent variables to the extrusion conditions with respect to physico-chemical characteristics

Independent variables				Dependent variables					
Trial	x_1	x_2	x_3	RER	AE	SV	WAI	WSI	HAR
1	−1	−1	−1	3.28	1.62	2.70	1.59	80.8	35.48
2	1	−1	−1	3.23	2.43	5.10	2.13	57.2	25.92
3	−1	1	−1	2.82	1.36	1.89	0.81	86.4	47.76
4	1	1	−1	2.49	2.04	2.53	2.05	58.6	34.73
5	−1	−1	1	3.25	2.79	4.58	0.89	89.5	21.07
6	1	−1	1	3.29	3.86	7.81	3.58	51.6	15.56
7	−1	1	1	2.53	2.19	2.28	3.19	71.0	36.93
8	1	1	1	2.77	3.13	5.53	4.17	46.5	21.76
9	0	0	0	2.92	2.42	3.35	1.53	72.9	28.59
10	0	0	0	2.88	2.52	3.50	1.52	71.9	29.20
11	0	0	0	2.91	2.52	3.73	1.61	69.7	24.42
12	0	0	0	3.10	2.90	4.42	1.76	67.5	26.56
13	0	0	0	3.17	2.70	4.24	1.61	70.3	24.80
14	0	0	0	3.16	3.08	4.15	1.63	71.1	28.13
15	$-\alpha$	0	0	2.66	1.98	1.48	0.60	86.9	59.65
16	α	0	0	2.70	3.78	6.33	2.75	45.2	17.63
17	0	$-\alpha$	0	3.55	3.55	5.91	1.90	68.9	16.07
18	0	α	0	2.44	2.69	2.02	2.51	64.0	40.66
19	0	0	$-\alpha$	2.93	2.81	2.37	1.91	66.0	33.42
20	0	0	α	2.79	3.95	5.25	4.04	52.7	17.15

x_1: soy protein concentrate content (SPC, %); x_2: moisture content (%); x_3: barrel temperature (°C) 3rd and 4th zone; RER: Radial Expansion Ratio; AE: Axial Expansion (cm); SV: Specific Volume (ml g^{-1}); WAI: Water Absorption Index; WSI: Water Solubility Index (%); HAR: Hardness (N); α: axial value of experimental design (1.682)

Table 4

Values of coefficients estimated by multiple linear regression for the physico-chemical characteristics

Coefficients	RER	AE	SV	WAI	WSI	HA
β_0	3.02	2.71	3.89	1.61	70.43	26.98
β_1	0.00	0.48*	1.29*	0.66*	–13.47*	–8.34*
β_2	–0.31*	–0.25*	–1.06*	0.22*	–1.81*	6.19*
β_3	–0.02	0.47*	0.94*	0.65*	–3.43*	–5.56*
β_{11}	–0.09*	–0.08	0.04	0.02	–0.73	3.90*
β_{22}	0.02	0.00	0.06	0.20*	–0.57	0.27
β_{33}	–0.03	0.09	0.00	0.48*	–3.08*	–0.82
β_{12}	–0.01	–0.03	–0.22	–0.13*	1.15	–1.64
β_{13}	0.08	0.06	0.43*	0.24*	–1.38	0.24
β_{23}	0.00	–0.08	–0.15	0.47*	–3.83*	0.12
r^2	0.8979	0.7692	0.9672	0.9588	0.9589	0.9104
P	0.00070	0.02671	0.000003	0.00001	0.00001	0.00038
s	0.0544	0.1917	0.1721	0.1146	1.4541	1.8795
$F_{(9,10)}$	9.7682	3.7024	32.7210	25.8969	25.9370	11.2910

* Significant at P<0.05 level

The model adequacy for each equation was tested by the coefficient of determination (r^2), lack of fit and F tests. The general equation is as follows:

$$Y=\beta_o+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{11}x_1^2+\beta_{22}x_2^2+\beta_{33}x_3^2+\beta_{12}x_1x_2+\beta_{13}x_1x_3+\beta_{23}x_2x_3$$

1.4. Radial expansion

The radial expansion ratio was computed as: $E=(D-D_0)/D_0$, where D is the extrudate diameter dried at equilibrium after leaving the nozzle and D_0 is the nozzle die diameter (3 mm). Five measurements were made on 10 randomly chosen pieces of extrudate from each experiment.

1.5. Axial expansion

The lengths of 15 pieces of extrudate taken at random were measured, and the average was calculated.

1.6. Specific volume

This value was determined using the water displacement method and was recorded as ml g^{-1}. Ten samples of 5 cm were randomly chosen and weighed.

The segments were covered with a thin layer of paraffin and the water volume displacement was determined.

1.7. Water absorption and solubility indices

These were determined in triplicate as described by ANDERSON and co-workers (1969).

1.8. Hardness

Hardness is a textural parameter, which was evaluated from a Texture Profile Analysis using the apparatus "TA-XT2 Texture Analyser" from Texture Technologies Corp., Scarsdale, NY, equipped with the Stable Micro Systems from Haslemere-Surrey, UK. The load cell was of 25 kg capacity and 1.0 g sensitivity. For each trial, ten samples of 5 cm in length were sheared using the "Warner Bratzler" blade probe with a speed of 2.0 mm s^{-1} in order to evaluate the breaking strength.

2. Results and discussion

2.1. Radial expansion ratio

The responses of the dependent variables to the radial expansion ratio (RER) were monitored as shown in Table 3. The estimated regression coefficients for each dependent variable were obtained from these responses by multiple linear regression (Table 4), and the model adequacy for each equation was tested by the coefficient of determination (r^2), lack of fit and F test.

The best explanatory model equation for RER showing a relationship between the independent variables such as SPC content (x_1), moisture content (x_2), barrel temperature (x_3) and dependent variable (RER) is as follows:

$$RER = 3.02–0.31x_2–0.02x_3–0.09x_1^2+0.02x_2^2–0.03x_3^2–0.01x_1x_2+0.08x_1x_3$$

The coefficient of determination (r^2) and level of significance for this model equation were 0.8979 and 0.0007, respectively. Accordingly, this assumed model was significant. From the regression equation it was observed that the SPC content had a quadratic effect and feed moisture had a linear effect at the 5% level. The largest value estimated for the regression coefficient was for moisture content (β_2=–0.31), indicating that this was the most important linear variable influencing RER.

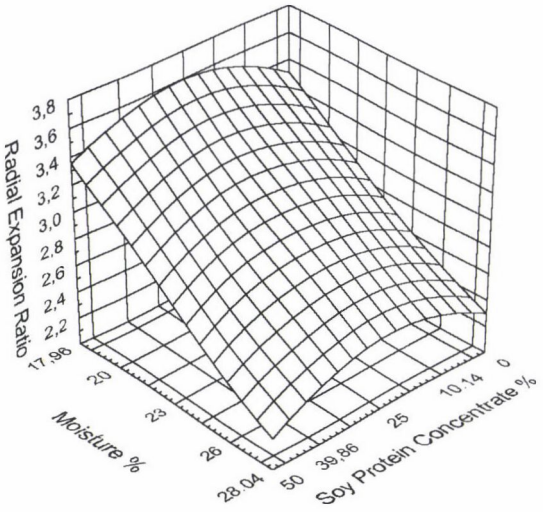

Fig. 1. The influence of moisture and SPC content on the radial expansion ratio of products extruded at 140 °C barrel temperature

The RER ranged from 2.44 to 3.55 for all treatment combinations (Table 3). For practical considerations, maximum expansion is desirable for expanded snacks, so the feed moisture has to be low (Fig. 1). The RER increased as a result of increasing SPC content in the blend up to 25% and then decreased with higher levels of SPC. The RER of the extrudates was higher at medium barrel temperatures (140 °C).

Retention times of 5–15 s, temperatures of 100 to 200 °C and moisture levels of 15 to 30% all influenced the protein dough quality just behind the die and the final product expansion (SMITH, 1975). The extrusion temperature of soybean products must be above the vaporization temperature of water (100 °C) to permit expansion and flash evaporation. Temperatures of 120 to 150 °C are recommended because extrudates processed at lower temperatures may become crumbly broken and disintegrate in boiling water (CUMMINGS et al., 1972).

2.2. Axial expansion

In the case of axial expansion (AE), the best explanatory model equation is as follows and the coefficient of determination (r^2) and level of significance were 0.7692 and 0.02671, respectively. This is the lowest value in accuracy for each surface response model equation. However the model was considered adequate with satisfactory r^2 value

and significant F value. From the regression equations it was observed that the three independent variables had a linear effect at the 5% level. Feed moisture had a negative effect (Table 4).

$$AE = 2.71+0.48x_1-0.25x_2+0.47x_3-0.08x_1^2+0.09x_3^2-0.03x_1x_2+0.06x_1x_3-0.08x_2x_3$$

The axial expansion ranged from 1.36 to 3.95 cm for all treatment combinations and the highest values were found at the maximum SPC level in the blend and at the lowest feed moistures (Fig. 2).

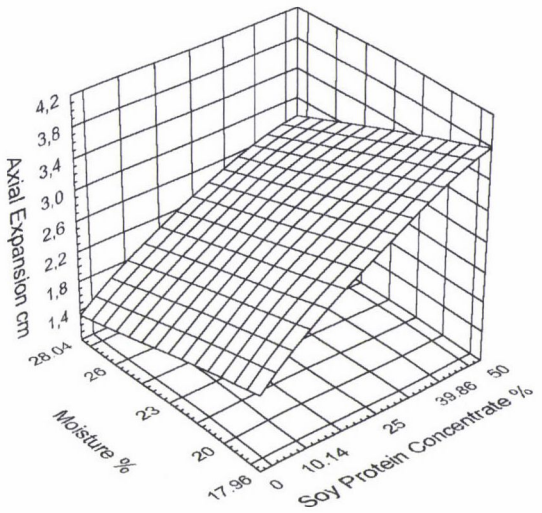

Fig. 2. The influence of moisture and SPC content on the axial expansion of products extruded at 140 °C barrel temperature

CONWAY and ANDERSON (1973) cited that soy protein processed at 130 °C resulted in expansion ratios that were unaffected by feed moisture. The degree of expansion of the extruded product affects product density and friability. Also the starch source is important in expansion (YURYEV et al., 1995).

2.3. Specific volume

The best explanatory model equation for specific volume (SV) is as follows:

$$SV=3.89+1.29x_1-1.06x_2+0.94x_3+0.04x_1^2+0.06x_2^2-0.22x_1x_2+0.43x_1x_3-0.15x_2x_3$$

The coefficient of determination for the specific volume was 0.9672 and this is the highest r^2 value indicating that the model was adequate, and the lack of fit was

significant (level of significance was 0.000003). SPC content was the most important linear variable affecting SV and had the highest regression coefficient ($\beta_1 = 1.29$), but the moisture content ($\beta_2 = -1.06$) and barrel temperature ($\beta_3 = 0.94$) were also important linear variables. Interaction of SPC content and barrel temperature showed a significant effect on the specific volume (Table 4).

The viscous and elastic properties of melted dough are basically the properties which influence the expansion phenomena and volume (LAUNAY & LISCH, 1983). The increased SV with higher SPC (Fig. 3) could be explained by the greater expansion in both axial and radial directions as shown. This was mainly attributed to the decreased feed moisture and increased SPC level in the blend (Fig. 3), which increased the dough visco-elasticity and caused the dough to expand more readily as moisture flashed off at the die (HARMANN & HARPER, 1973).

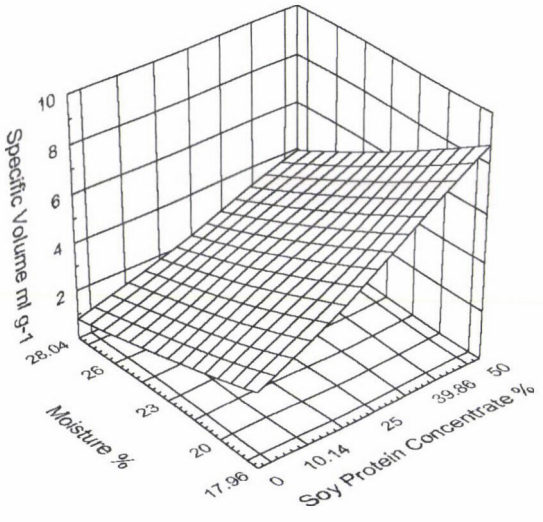

Fig. 3. The influence of moisture and SPC content on the specific volume of products extruded at 140 °C barrel temperature

HARRIS and co-workers (1988) reported that the bulk density values for maize gluten meal–defatted soy flour coextrudates averaged 564 g l^{-1} and these values were inversely related to the initial moisture level of the blend. Maize gluten meal–defatted soy flour blend extruded with relatively low initial moisture levels (20%) exhibited significantly ($P<0.01$) greater bulk density values than those maize gluten meal–defatted soy flour blends extruded with relatively high initial moisture levels (40%) (HARRIS et al., 1988).

MERCIER and FEILLET (1975) also observed that rehydration of an extrudate is closely related to its density. There was no significant difference ($P<0.05$) between the water holding capacity and shear resistance values obtained for the maize gluten meal–defatted soy flour coextrudates processed with initial blend moistures of 20 and 40%. The high (47%) moisture coextrudate exhibited slightly lower water holding capacity and a much greater shear resistance than the maize gluten meal–defatted soy flour coextrudates processed at a lower initial moisture level (30%). KOEPPE and co-workers (1986) observed a similar relationship between feed moisture levels, water holding capacity and shear resistance values with extrudate blends of maize gluten meal and *Amaranthus hypochondriacus* flour. CONWAY (1971) reported that if initial blend moisture levels are too high, insufficient vaporization occurs as the product emerges from the extruder. Therefore extrudates processed at high moisture levels emerge soft and moist and upon drying become hard and tough with possible cracking.

2.4. Water absorption index

The best explanatory model equation for WAI is as follows:

$$WAI=1.61+0.66x_1+0.22x_2+0.65x_3+0.02x_1^2+$$
$$+0.20x_2^2+0.48x_3^2-0.13x_1x_2+0.24x_1x_3+0.47x_2x_3.$$

The coefficient of determination was 0.9588, and the lack of fit was significant (level of significance was 0.00001). The SPC level ($\beta_1=0.66$) and barrel temperature ($\beta_3=0.65$) were the most important variables. From the regression equation it was observed that only SPC content had no quadratic effect on WAI. The blends processed showed higher values for water absorption index (WAI) than starch only. The highest WAI were obtained at the highest feed moistures and with the maximum SPC level in the blend (Fig. 4).

ANDERSON and co-workers (1969) reported that the (WAI) of extruded cereal products was higher for higher moisture extrudates. The ability of maize gluten meal–defatted soy flour coextrudates to hold water was relatively low (2.5) (HARRIS et al., 1988). A lower water holding capacity would be desirable in an extruded product that is to be used in a canned pet food where structural integrity and thermostability of the product during retorting would be important. The optimized maize gluten meal–defatted soy flour coextrudate was of intermediate strength because of its relatively high moisture content, but the slightly acid pH tempered this effect, and the product remained soft, pliable, and chewy upon drying (HARRIS et al., 1988).

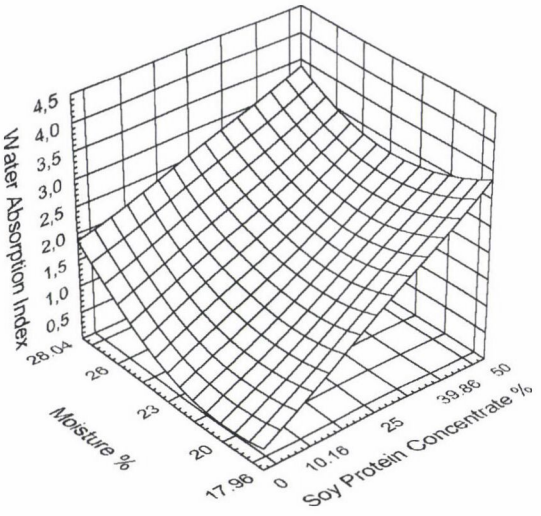

Fig. 4. The influence of moisture and SPC content on the water absorption indices of products extruded at 140 °C barrel temperature

2.5. *Water solubility index*

The best explanatory model equation for WSI is as follows:

$$WSI = 70.43 - 13.47x_1 - 1.81x_2 - 3.43x_3 - 0.73x_1^2 - 0.57x_2^2 - 3.08x_3^2 + 1.15x_1x_2 - 1.38x_1x_3 - 3.83x_2x_3.$$

The coefficient of determination was 0.9589 and the lack of fit was significant (level of significance was 0.00001). The SPC level (β_1=–13.47) was the most important variable.

From the regression equation it was observed that SPC content, feed moisture and barrel temperature had a linear effect and that the barrel temperature also had a quadratic effect. Interaction between feed moisture and barrel temperature showed a significant effect on the WSI at the 5% level.

Decreasing the SPC content increased the WSI of the extrudates (Fig. 5). This result may be related to the degree of starch dextrinization or starch breakdown.

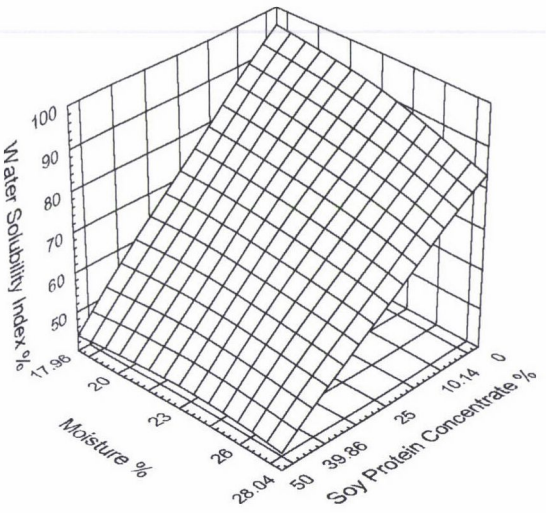

Fig. 5. The influence of moisture and SPC content on the water solubility indices of products extruded at 140 °C barrel temperature

2.6. Hardness

Extrudate hardness was defined in this study as the force to bite through with the incisors, and the best explanatory model equation for hardness is as follows:

$$HAR = 26.98\text{-}8.34x_1+6.19x_2-5.56x_3+3.90x_1^2+$$
$$+0.27x_2^2-0.82x_3^2-1.64x_1x_2+0.24x_1x_3+0.12x_2x_3.$$

The hardness varied from 15.56 to 59.65 N for all treatment combinations (Table 3). The coefficient of determination for hardness was 0.9104, and the lack of fit was significant (level of significance was 0.00038). From the regression equation it was observed that the SPC level had a linear and quadratic effect, whereas feed moisture and barrel temperature had a linear effect at the 5% level. The largest value for the estimated regression coefficient was for the SPC level ($\beta_1 = -8.34$) indicating that this was the most important linear variable negatively influencing HAR, followed by feed moisture and barrel temperature (Table 4). When protein was added to the starch, the texture and sensorial characteristics of the extruded products were improved.

High feed moisture and low SPC content gave high hardness scores (Fig. 6). As the moisture content decreased and SPC content increased hardness decreased, resulting in an expanded and soft product that could easily be broken. The ultrastructure of the

soy protein concentrate appeared to be more flaky and compact than that of the sponge like textured soy flour (TARANTO et al., 1978). BREEN and co-workers (1977) reported that blends of up to 19% soy protein with wheat millfeeds, wheat starch and yellow corn meal were reported to yield good expansion ratios, texture and taste. CUMMINGS and co-workers (1972) observed two structural changes in soybean products as extrusion temperatures increased: an increase in aligned fibers with enhanced cohesiveness and the development of a spongy texture with increased porosity. CUMMINGS and co-workers (1972) reported that as the temperature of extrusion increased, the extruded product became more resistant to shear and compression but decreased in density, also indicating a greater cohesiveness and increased degree of structural integrity under optimum extrusion conditions (pH 6.25, initial moisture 32% and barrel temperature 153 °C). These authors observed that the interior of the maize gluten meal–defatted soy flour coextrudate took on a multilayered appearance, the individual layers appearing "sponge like" with the presence of minute pores on the layers.

CUMMINGS and co-workers (1972) suggested that during extrusion processing the protein bodies are reformed into continuous fiber-like structures. In a histological study, SMITH (1974, 1975) explained that the cellulose capsules surrounding individual protein bodies were ruptured and denatured by extrusion-cooking, allowing the protein bodies to run together in rivulets.

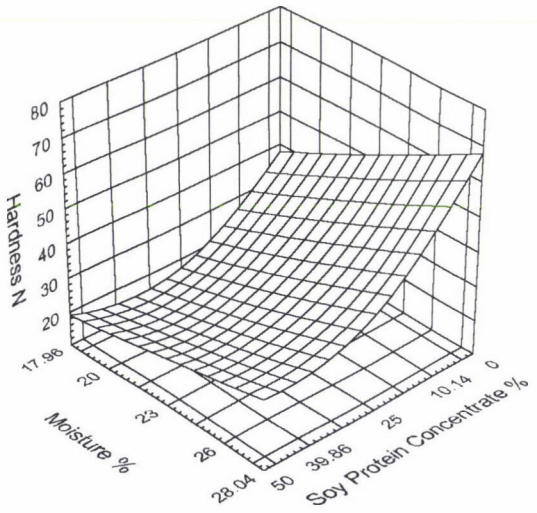

Fig. 6. The influence of moisture and SPC content on the hardness of products extruded at 140 °C barrel temperature

3. Conclusions

Moisture content was the most important linear variable influencing radial expansion. The highest values of axial expansion were found at the maximum SPC level in the blend and at the lowest feed moisture. SPC content was the most important linear variable affecting specific volume. The highest WAI were found at the highest feed moistures and with the maximum SPC levels in the blend, while decreasing SPC level increased the WSI of the extrudates. The functional characteristics of the extruded blends could have diverse applications and biodegradable industrial uses.

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Acta Alimentaria, Vol. 30 (2), pp. 205–218 (2001)

FLAVOUR SUBSTANCES OF CARROT CULTIVARS

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(Received: 24 May 2000; accepted: 19 December 2000)

Different carrot cultivars were examined for glucose, fructose, saccharose and volatile substances. Altogether 95 samples were analysed for sugars in three consecutive years. The effect of storage, irrigation and Mg^{2+} treatment was also studied. Saccharose was the dominant sugar, while glucose to fructose ratio was over one. Irrigation and magnesium foliage spray had almost no influence on sugar content in the tested cultivars according to PCA. The cultivar and crop year was found to be affecting factors. As measured by gas chromatography-mass spectrometry, the major volatile compounds were α-pinene, β-myrcene, α-terpinolene, trans-β-caryophyllene and trans-γ-bisabolene with sesquiterpenes dominating over terpenes in all the cultivars tested in this work (Nantes, Nantes Forto, Fertődi Red, Red Giant, and Danvers).

Keywords: carrot, sugar content, volatile substances

The taste of carrot (*Daucus carota* L.) is mainly determined by sugars (glucose, fructose and saccharose). The glucose to fructose ratio is generally above one. The total sugar content varies with genotype and environment (SIMON et al., 1982). Year and location have a considerable effect on both overall quality and sugar content of carrots, while there are contradictory results concerning the effect of fertilisation (EVERS, 1989). Data found in the literature for whole carrots and juice are shown in Table 1 and 2 respectively.

The sugar components are not evenly distributed in carrots. According to HABEGGER and SCHNITZLER (1997) the vertical and horizontal distribution of flavour substances in carrot root is uneven. The phloem contains more sugars and volatile substances than the xylem (the difference for the individual sugars is between 30 and 98%). The difference between crown, midsection and tip is less (between 10 and 40% for sugars).

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Table 1

Glucose, fructose and saccharose content of fresh carrots (g/100 g fresh weight)

Literature source/number of samples/origin	Glucose mean (range)	Fructose mean (range)	Saccharose mean (range)
SOUCI et al. (1989)	1.40 (0.84–1.71)	1.29 (0.82–1.96)	1.90 (1.55–4.17)
SOMOGYI & TRAUTNER (1974) (30 samples, Swiss)	1.66 ± 0.38[a]	1.47 ± 0.29[a]	1.55 ± 0.71[a]
HAILA et al. (1992) (18 samples, Finnish)	1.41 (0.71–2.27)	1.36 (0.69–2.22)	2.56 (1.32–4.53)
HOFSOMMER & GHERARDI (1985) (40 samples, Italian-German)	1.238 (0.56–1.77)	1.047 (0.39–1.47)	4.33 (2.58–6.56)
EVERS (1989) (Finnish)	1.75 (1.5–2)	1.55 (1.2–1.8)	4.0 (3.6–4.3)

[a] mean ± standard deviation

Table 2

Glucose, fructose and saccharose content of carrot juice (g/100 g fresh weight)

Literature source/number of samples	Glucose mean (range)	Fructose mean (range)	Saccharose mean (range)
OTTENEDER (1982) 29	0.727 (0.53–1.12)	0.711 (0.49–1.02)	2.42 (1.55–3.93)
HOFSOMMER & GHERARDI (1985) 23	1.06 (0.62–1.44)	0.894 (0.53–1.24)	3.13 (1.85–4.86)

The volatile components of carrots have been more extensively studied since the sixties. Already in the early stage of aroma research, BUTTERY and co-workers (1968) has separated more than 100 compounds. Most of them (ca 80%) were identified as terpene hydrocarbons, mainly terpinolene, γ-terpinene, limonene, caryophyllene, sabinene, β- and γ-bisabolene. Components under 1% were α- and β-pinene, camphene, myrcene, α-terpinene and p-cymene, terpinene-4-ol, α-terpineol, bornyl acetate, myristicin and carotol. The total amount extracted with simultaneous steam distillation – pentane extraction was about 40 ppm. HEATHERBELL and co-workers (1971) identified nearly the same substances. SEIFERT and BUTTERY (1978) discovered further sesquiterpenes (α-humulene, β-farnesene, α-bergamotene and γ-muurolene) in carrots, while BUTTERY and co-workers (1979) reported, among others, oxygen-containing terpenes (geranyl esters, geranyl acetone). ALABRAN and co-workers (1975) identified altogether 28 components and studied their odour characteristics. They published quantitative data on essential oil components in fresh carrot, of which some are doubtful, e.g. linalool and the high amounts of aldehydes of longer carbon chain.

SIMON and his group (1980) used polymer trapping for analysis of carrot headspace and compared it to continuous steam distillation – extraction using n-pentane. They measured 32.5 ppm total volatiles for Nantes cultivar. These authors found differences in the vertical and horizontal distribution of terpenes.

SHAMAILA and co-workers (1996) studied the effect of water blanching on headspace volatiles and sensory attributes, while HOWARD and his group (1995) developed regression models relating sensory attributes and composition of fresh and processed carrots. According to these authors, fresh carrot flavour is affected by genetic variations. Cooked flavour and cooked aftertaste were associated with high terpinolene content. Terpinolene, caryophyllene and γ-terpinene were major terpenoids in strained carrots.

Detailed information on carrot components, including sugars and odour components have been reviewed by HERRMANN (1995).

HABEGGER and co-workers (1996) studied the volatile components of Red Giant, Cubic and Nanco cultivars in two consecutive years. They reported 23.6 to 42 ppm volatile oil calculated to fresh weight. For Red Giant, only the total quantity of volatile substances has been given, while for Cubic and Nanco the individual components, too. Some compounds (e.g. β-myrcene as well as β-caryophyllene) showed a great variation between crop years.

It is generally accepted that carrot flavour results probably from several compounds, and terpinolene is thought to be responsible (HOWARD et al., 1995) for cooked carrot aftertaste.

In the literature no data are available on sugar and volatile compounds of Hungarian carrots except for few published in the local journals (TAKÁCSNÉ HÁJOS, 1999). This paper reports the results of investigations of sugars and volatile compounds in different cultivars. The effect of storage, irrigation and Mg treatment was also studied.

1. Materials and methods

1.1. Plants

Carrot cultivars were grown in the Agropark of Research Institute for Irrigation, Szarvas. Cropping and treatments were performed by co-workers of Sámuel Tessedik College, Faculty of Agricultural Water- and Environment Management.

Cultivars studied in 1997: Nantes, Nantes Forto, Fertődi Red, Red Giant (the latter only for volatile substances) and Danvers.

Irrigation studies in 1998 were performed with seven cultivars (Nantes, Record, Fertődi Red, Danvers 126, Red Giant, Flakker, and Chantenay). During cultivation season, 30–40 mm irrigating water was applied three times, and 5 to 10 mm water for sprouting.

As the year 1999 was unusually rainy, there was no need of irrigation, the addition of Mg^{2+} was investigated in triplicates, with cultivars Fertődi Red, Record, Flakker, Supra, K. Gold. The treatment included foliage fertilisation with 2% solution of $MgSO_4$, performed three times during the cultivation season.

1.2. Chemicals

Diethyl ether (Reanal p.a., freshly cleaned by fractional distillation), tridecane (Fluka purum), β-pinene (Fluka puriss), myrcene, d-limonene (Fluka puriss), caryophyllene (Carl Roth GC), terpinolene (Carl Roth), α-humulene (Fluka purum), sucrose-D-glucose-D-fructose enzymatic test kit (Boehringer-Mannheim).

1.3. Sample preparation for sugar analysis

Three replicates of each sample were prepared by removing the crown of the root, washing, grating of two whole carrots. After a thorough mixing, 100 g of the sample was blended in Ultra-Turrax with distilled water until pureed (cca 3 min). The puree was held in a 60 °C water bath for 15 min; filled up to 500 ml with distilled water and filtered. Sugars were determined with a Boehringer-Mannheim enzymatic test at 340 nm.

1.4. Sample preparation for volatiles

Cultivars studied were the following: Nantes, Nantes Forto, Fertődi Red, Red Giant, Danvers. Carrots were washed, ends removed, the remaining root was homogenised with the addition of distilled water. The resulting pulp was treated with a continuous steam distillation-diethyl ether extraction in a Likens-Nickerson apparatus on atmospheric pressure for two hours. For the quantitative determination, n-tridecane as an internal standard was added. In the case of Nantes, Nantes Forto, Fertődi Red four parallel distillations, while with other two cultivars, 3 parallel distillations were performed.

1.5. Separation and identification

Separation and identification of extracted concentrate of volatile substances was performed on a HP 5890 Series II gas chromatograph coupled to HP 5971 mass selective detector. Wiley 275 library was used for library search. Column applied: HP 5, length 25 m, i.d. 0.2 mm, film thickness 0.33 μm. Carrier gas: helium, injector temperature: 250 °C, column temperature program. 60 °C 2 min, rate 6 °C min^{-1}, final temperature 190 °C, final time 15 min. Detector temperature 260 °C, split mode.

1.6. Statistical analysis

Paired comparison of samples was performed using Student's *t*-test. Significant differences were determined at $P \leq 0.05$ level. Results were also assessed by regression analysis, analysis of variance and PCA.

2. Results and discussion

2.1. Sugar composition

Table 3 shows the results obtained from the analysis of sugars in different carrots cultivated in 1997. The measurements were performed at the end of storage time, from healthy carrots.

The lowest values with regard to saccharose and total sugar were estimated with Nantes Forto while the other carrots showed higher saccharose values ranging between 4.87–5.37 g/100 g fresh weight. The highest concentrations of monosaccharides were found in Danvers. According to analysis of variance, there is no difference in sum of glucose and fructose as well as saccharose between cultivars among stored samples. Investigating the averages of total sugar by ANOVA, cultivars could be divided into two groups: Nantes Forto (5.44) is separated from the others. Analysis of variance of the second principal components separated three groups: Nantes Forto is in the first while Danvers is in the third one.

The irrigation experiment data of 1998 are shown in Table 4.

Table 3

Sugar composition of carrot cultivars
(1997 crop, at the end of storage time, 01. 1998, in g/100 g fresh weight)

Cultivar	Glucose $\bar{x}\pm s$	Fructose $\bar{x}\pm s$	Saccharose $\bar{x}\pm s$	Gl:fr $\bar{x}\pm s$	Total sugar $\bar{x}\pm s$
Nantes	0.57±0.34	0.48±0.33	5.37±1.02	1.24±0.14	6.41±0.45[a]
Nantes Forto	0.61±0.37	0.50±0.26	4.32±0.75	1.23±0.21	5.44±0.39[b]
Fertődi Red	0.71±0.29	0.56±0.18	4.92±0.62	1.23±0.16	6.18±0.81[a]
Danvers	1.07±0.67	0.88±0.41	4.87±1.28	1.15±0.25	6.82±0.75[a]

$\bar{x}\pm s$: mean ± standard deviation of at least 3 replicates.
Values having different supercripts are significantly different (ANOVA)

Table 4

The effect of irrigation on sugar composition of carrots (crop year 1998, in g/100 g fresh weight)

Cultivar	Glucose $\bar{x}\pm s$	Fructose $\bar{x}\pm s$	Saccharose $\bar{x}\pm s$	Gl:fr $\bar{x}\pm s$	Total sugar $\bar{x}\pm s$
			Irrigated		
Nantes	0.84±0.61	0.72±0.38	2.37±1.69	1.08±0.23	3.92±0.96
Record	1.07±0.29	1.08±0.29	2.43±0.41	0.98±0.05	4.58±0.78
Fertődi Red	0.27±0.24	0.17±0.13	4.94±0.65	1.49±0.22	5.38±0.73
Danvers126	0.47±0.33	0.41±0.24	4.52±1.70	1.14±0.15	5.40±1.14
Red Giant	0.78±0.40*	0.75±0.29**	2.63±1.50	1.01±0.16	4.16±1.00
Flakker	0.23±0.27	0.21±0.25	4.45±0.30	1.26±0.21	4.89±0.51
Chantenay	1.34±0.22	1.15±0.33	3.40±0.70	1.20±0.18	5.89±0.27
			Without irrigation		
Nantes	0.78±0.18	0.75±0.14	3.37±0.71	1.03±0.05	4.90±1.01
Record	0.32±0.05	0.34±0.02	3.99±1.22	0.92±0.12	4.65±1.21
Fertődi Red	0.41±0.41	0.34±0.36	4.15±1.16	1.25±0.09	4.89±1.07
Danvers126	0.95±0.41	0.63±0.27	3.57±1.71	1.54±0.23	5.15±1.07
Red Giant	1.56±0.39	1.38±0.33	2.35±0.93	1.13±0.07	5.30±0.69
Flakker	0.54±0.72	0.51±0.66	4.51±1.38	1.08±0.25	5.56±0.55
Chantenay	1.49±0.64	1.32±0.64	3.00±1.66	1.17±0.34	5.81±0.79

$\bar{x}\pm s$: mean ± standard deviation of at least 3 replicates

* Significant difference at $P<0.05$;** at $P<0.01$ (t-test)

Irrigation had almost no effect on sugars as shown by t-test, with the exception of Red Giant, where the control samples had significantly higher glucose and fructose content. Red Giant control sample differed also from the other six cultivars studied in its higher monosaccharide concentration. As a rule, irrigation lowered the average total sugar but to a very low extent in four cultivars of the seven tested. Based on sum of glucose and fructose, cultivars could be separated with ANOVA into 3 main groups. Record and Flakker is in the first group, Danvers 126, Fertődi Red and Nantes in the second, while Red Giant and Chantenay in the third. For saccharose two groups can be distinguished by ANOVA: Red Giant, Nantes, Chantenay, Fertődi Red is in the first one while Danvers 126, Flakker and Record in the other. A similar separation was achieved by analysis of variance of the first principal component. Cultivars can be separated into 4 subgroups, Red Giant (–1.92) and Chantenay (–1.52) is in the first, Nantes (–0.75 and Fertődi Red (–0.38) is in the second, Danvers 126 (0.23) in third while Flakker (0.93) and Record (1.23) are in last one. The analysis of variance of the 2nd principal

component has shown a slight effect of irrigation (treated samples –0.37, control –0.03). Clustering of cultivars according to 2nd PC is as follows: Chantenay and Danvers are in separate groups, and all the others are in the third cluster.

The sugars measured with and without Mg^{2+} treatment can be seen in Table 5. Five varieties were tested. Magnesium foliage fertilisation influenced only cultivar Flakker according to *t*-test where treated samples had more saccharose and total sugar present, whereas monosaccharides decreased. The lowest monosaccharide and at the same time the highest saccharose content was found in the control sample Fertődi Red. According to analysis of variance, there is no difference in sum of glucose and fructose as well as in saccharose between cultivars among Mg^{2+} treated samples. According to PCA and analysis of variance of first and second principal component, Mg^{2+} treatment has no influence on sugar composition.

Studying the effect of storage with respect to Fertődi Red, Nantes, and Danvers by *t*-test, the total sugar content was significantly higher in stored samples, which is due to loss of water.

Comparing the sugar composition of the same cultivars (Fertődi Red, Record and Flakker) in the two consecutive years 98/99, when samples were analysed immediately after harvest, no significant differences could be found by *t*-test.

Table 5

The effect of Mg^{2+} treatment on sugar composition of carrot (crop year 1999, in g/100 g fresh weight)

Cultivar	Glucose $\bar{x}\pm s$	Fructose $\bar{x}\pm s$	Saccharose $\bar{x}\pm s$	Gl:fr $\bar{x}\pm s$	Total sugar $\bar{x}\pm s$
			Irrigated		
Fertődi Red	0.68±0.59	0.63±0.52	4.18±1.63	1.06±0.13	5.49±0.52
Record	0.68±0.37	0.55±0.31	4.61±0.69	1.24±0.18	5.84±0.74
Flakker	0.66±0.28	0.51±0.20**	4.67±0.69*	1.30±0.12*	5.84±0.37
Supra	0.82±0.79	0.71±0.72	4.79±1.50	1.20±0.12	6.31±0.26
K. Gold	0.69±0.06	0.63±0.06	3.89±0.43	1.10±0.09	5.21±0.35
			Untreated		
Fertődi Red	0.13±0.04	0.10±0.03	6.73±1.33	1.24±0.15	6.95±1.29
Record	0.71±0.54	0.59±0.35	4.26±2.41	1.14±0.19	5.57±1.53
Flakker	0.95±0.40	0.68±0.19	3.72±0.86	1.37±0.33	5.35±0.43
Supra	0.71±0.38	0.60±0.27	4.71±0.35	1.15±0.30	6.01±0.93
K. Gold	0.90±0.44	0.64±0.29	4.08±1.17	1.38±0.08	5.63±0.50

$\bar{x}\pm s$: mean ± standard deviation of at least 3 replicates.

* Significant difference at $P<0.05$; ** at $P<0.01$ (*t*-test)

Concerning the effect of genotypes, the untreated, freshly harvested samples were compared by *t*-test (in Tables 4 and 5). Generally, there was no significant difference in total sugar content between the cultivars. Fertődi Red differs from others in lower glucose and fructose content. The highest level of glucose and fructose was estimated in Red Giant and Chantenay.

Regression analysis of all the data showed a close correlation of fructose and glucose according to the following equation (rejecting two outliers):

$$\text{Fructose} = 0.81 \times \text{glucose} + 0.029,$$

where $F=1000$ and the standard error is 0.11. Because of this correlation, the sum of glucose and fructose was regarded in further calculations.

The sum of glucose and fructose is closely correlated with saccharose:

$$\text{Glucose+fructose} = -0.45 \times \text{saccharose} + 3.26.$$

F value is 88, standard error is 0.64.

Total sugar is independent of sum of glucose and fructose, and it is in a close correlation with saccharose as described by the next equation:

$$\text{Saccharose} = 1.06 \times \text{total sugar} - 1.7.$$

F value 128, standard error 0.89.

Results of PCA are summarised in Table 6.

Table 6

Results of principal component analysis

Variable	Correlation coefficients		
Glucose	1		
Fructose	0.958	1	
Saccharose	0.674	0.707	1
Principal Components	Eigenvalues	Proportion	Cumulative values
1	2.57	0.86	0.86
2	0.39	0.13	0.99
3	0.04	0.01	1
Variable	Principal component loadings		
Glucose	–0.9559	0.259	–0.1382
Fructose	–0.967	0.2089	0.1455
Saccharose	0.8477	0.5305	0.01017

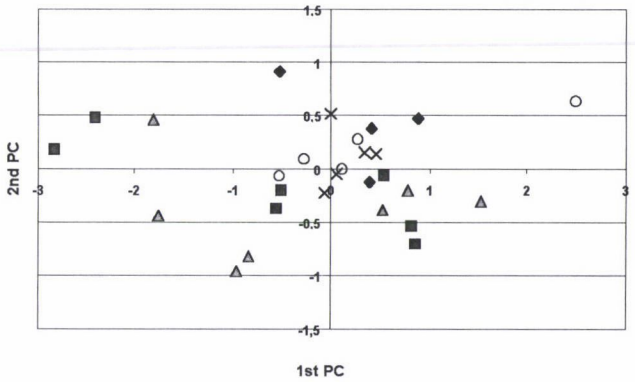

Fig. 1. Principal component score plot. ♦: stored 97, ■ : control 98, Δ: irrigated 98, X: Mg treated 99; O: control 99

Principal component scores plot of cultivars (average) is shown on Fig. 1.

First principal component, accounting for 86% of the total variability, reflects the resolution of change along correlation of glucose plus fructose versus saccharose. Second principal component presents the resolution of deviation from regression. According to the analysis of variance of the first principal component, there is no difference between treatments. Starting from this, the treated and untreated samples were pooled in further calculations.

Crop years can be distinguished by variance analysis of the second principal component (rejecting 11 outliers out of 95, residual error 0.39, F value within a crop year 2.7, among crop years 12.2, which shows a significant difference).

Sugar composition of carrot cultivars studied show a great variation due to year-by-year differences as well as inhomogeneous distribution of sugars within the root. Saccharose is the dominating sugar. In the tested carrots saccharose concentration ranged from 2.37 to 6.73 g/100 g fresh weight, glucose changed between 0.13 and 1.56 g/100 g, while fructose from 0.10 to 1.38 g/100 g. The range of glucose to fructose ratio was 0.92–1.54, with an average of 1.2. A strong correlation was found between glucose and fructose. Our measurements indicated a lower monosaccharide content than most of the sources (see Tables 1 and 2). Monosaccharide to disaccharide ratio found is in accordance with OTTENEDER's (1982), furthermore, with results of HOFSOMMER and GHERARDI (1985). Irrigation and magnesium foliage spraying had almost no influence. The effect of cultivars and crop years was proved by PCA. This result supports SIMON and co-workers' (1982) opinion, that cultivar and environment are the most important factors. The natural occurrence and activity of invertase in carrots (ZAMSKI & BARNEA, 1996) possibly cause the large deviation of monosaccharide and sucrose contents.

2.2. Volatile substances

Experiments were carried out in 1997. The quantity of total volatile substances can be seen in Table 7.

Table 7

Total volatile content (ppm) of carrot cultivars cultivated in 1997

Cultivar studied	Total volatiles ppm $\bar{x}\pm s$
Nantes	68.3 ± 7.2
Nantes Forto	64.7 ± 7.8
Fertődi Red	74.0 ± 4.4
Red Giant	79.2 ± 8.3
Danvers	64.1 ± 9.5

$\bar{x}\pm s$: mean ± standard deviation of at least 3 replicates

The majority of the components identified belong to the terpene and sesquiterpene family. The total amount is in the same order of magnitude as reported by BUTTERY and co-workers (1968), SIMON and co-workers (1980), HABEGGER and co-workers (1996).

Figure 2 shows the relatively rich terpene region of the cultivar "Nantes Forto", while the sesquiterpene composition of Fertődi Red is depicted in Fig. 3.

The main components were α-pinene, β-myrcene, α-terpinolene, trans-β-caryophyllene and trans-γ-bisabolene. In the cultivars examined these substances countered for 64–82% of the total peak area. It was characteristic of each cultivar that sesquiterpenes dominated over terpenes. The main components of the cultivar Nantes were α-terpinolene and trans-γ-bisabolene, countering for approximately 60% of the total volatile oil. Quantity of pinene, myrcene, limonene and terpinolene were in accordance with SIMON and co-workers' data (1980), while the lower level of caryophyllene and bisabolene sesquiterpenes reported by these authors explains the difference found in total volatiles.

Main components of cultivars Nantes Forto, Fertődi Red and Red Giant were trans-β-caryophyllene and trans-γ-bisabolene. Red Giant and Danvers is characterised by a low α-terpinolene level. Principal volatile components of Danvers are trans-γ-bisabolene and trans-β-caryophyllene. Trans-β-caryophyllene is probably not an artefact, as it was shown to impart a fresh carrot, fruity note when sniffing (HABEGGER et al., 1996). Bisabolene has also been reported in carrots by numerous authors (BUTTERY et al., 1968, HEATHERBELL et al., 1971, SEIFERT & BUTTERY, 1978, SIMON et al., 1980).

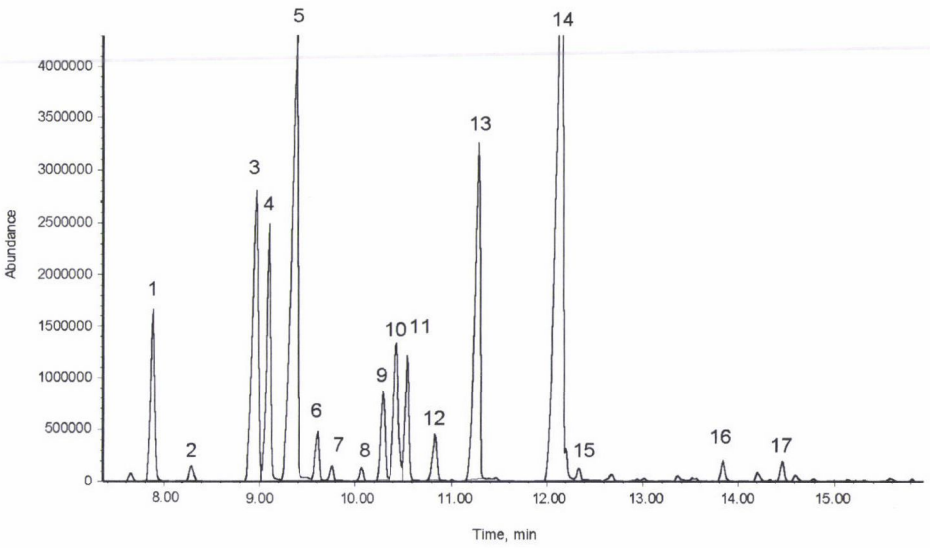

Fig. 2. Terpene region in total ion chromatogram of volatiles from carrot cultivar "Nantes Forto." 1: α-pinene, 2: camphene, 3: sabinene, 4: β-pinene, 5: myrcene, 6: octanal, 7: phellandrene, 8: α-terpinene, 9: p-cymene, 10: limonene, 11: cis-ocimene, 12: trans-ocimene, 13: γ-terpinene, 14: terpinolene, 15: nonanal, 16: 2-nonenal, 17: terpinen-4-ol

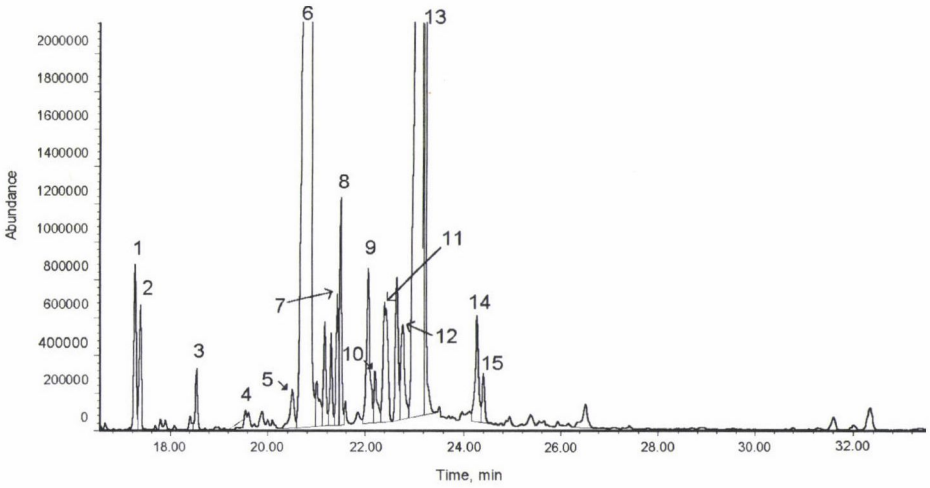

Fig. 3. Sesquiterpene region in total ion chromatogram of volatiles from Fertődi Red carrot cultivar. 1: bornyl acetate; 2: tridecane internal standard; 3: δ-elemene; 4: copaene; 5: bergamotene; 6: trans-caryophyllene; 7: trans-farnesene; 8: α-humulene; 9: cubebene; 10: selinene; 11: β-bisabolene; 12: myristicine; 13: trans-γ-bisabolene; 14: geranyl ester; 15: caryophyllene oxide

Table 8

Average quantity and standard deviation of main volatile components (ppm) from different cultivars of carrot (1997)

Cultivar	β-Pinene $\bar{x}\pm s$	β-Myrcene $\bar{x}\pm s$	Limonene $\bar{x}\pm s$	α-Terpinolene $\bar{x}\pm s$	β-Caryophyllene $\bar{x}\pm s$	γ-Bisabolene $\bar{x}\pm s$
Nantes	0.58±0.02	3.29±0.02	1.07±0.22	19.99±2.78	11.20±3.13	37.56±5.85
Nantes Forto	0.55±0.05	2.32±0.91	0.83±0.05	13.07±0.72	24.10±1.49	21.36±3.93
Fertődi Red	1.22±0.70	6.48±2.81	1.17±0.35	13.00±3.07	17.11±1.80	17.62±0.92
Red Giant	0.49±0.16	5.79±1.00	1.99±0.51	7.17±1.56	25.48±2.24	23.11±1.58
Danvers	1.19±0.23	3.28±0.50	1.67±0.46	8.77±4.24	15.34±2.65	30.14±5.85

$\bar{x}\pm s$: mean ± standard deviation of at least 3 replicates

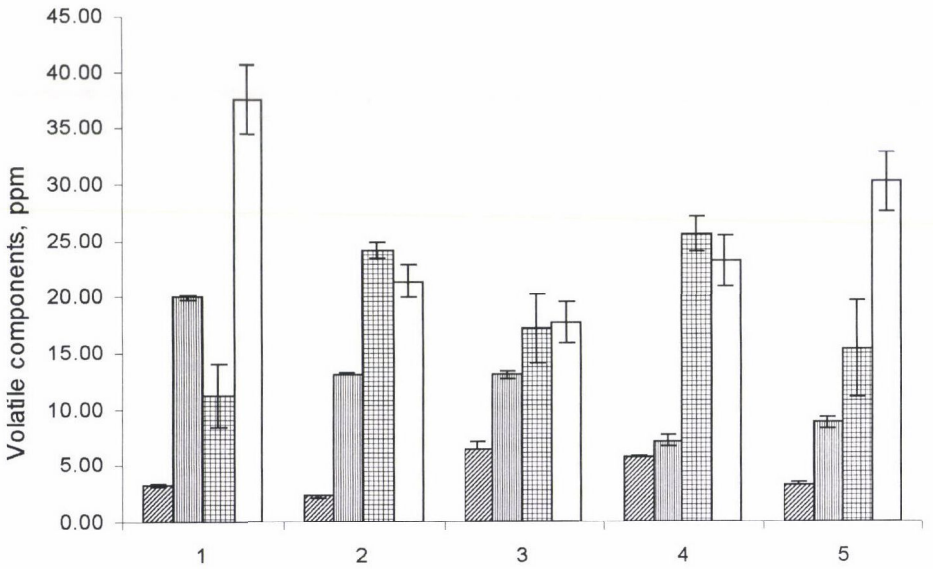

Fig. 4. Distribution of main volatile components in carrot cultivars (ppm). 1: Nantes; 2: Nantes Forto; 3: Fertődi Red; 4: Red Giant; 5: Danvers; ▨: myrcene; ▥: terpinolene; ▦: caryophyllene; □: bisabolene

Oxygen containing compounds with a low odour threshold value are octanal, nonanal, 2-nonenal, 4-terpinenol, bornyl acetate and a geranyl ester, probably 2-methybutyrate. The mean values and standard deviations of main components are tabulated in Table 8.

The terpinolene content of cultivar Nantes is much higher than that of all the other varieties studied. Nantes had significantly higher bisabolene and lower caryophyllene content than that of Nantes Forto, Fertődi Red and Red Giant. Nantes Forto and Red Giant are unlike in terpinolene level. The significant difference was found between Nantes Forto and Danvers in caryophyllene, between Fertődi Red and Red Giant in terpinolene, caryophyllene and bisabolene content (Fig. 4).

Our results are in accordance with the data in the literature, as the composition of volatile substances in Hungarian carrots was similar to that of foreign carrot cultivars. However, their quantitative distribution is slightly different.

3. Conclusion

Our study indicated the dominance of saccharose over glucose and fructose in all the cultivars studied. Glucose and fructose are strongly correlated; average glucose to fructose ratio is 1.2. Saccharose and total sugar significantly increased as a function of storage. The cultivars studied did not show a clear response to magnesium foliage treatment. No substantial effect of irrigation to sugar composition was found. Cultivars and crop years influence the sugar composition as justified by analysis of variance of first two principal components.

Studies of volatile flavour components show large differences between cultivars. Sesquiterpene hydrocarbons, mainly trans-β-caryophyllene and bisabolene, were present in high concentrations.

*

This work was supported by the National Scientific Research Foundation (OTKA T 23841).
We thank E. ZUKÁL for his help in analysis of variance and PCA.

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Acta Alimentaria, Vol. 30 (2), pp. 219–226 (2001)

Preliminary communication

GRAPE SPIRIT OBTAINED FROM TABLE GRAPES: PRELIMINARY RESULTS

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(Received: 27 April 2000; accepted: 14 November 2000)

Fermented table grapes have been used as raw material for grape-spirit production. The free and potentially volatile monoterpene flavourants of fermented table grapes treated with exogenous glycosidases by *A. niger* have been studied. The enzyme preparation added to the must in the presence of solids was found to be less efficient in hydrolysing the glycosides than when added to the fermented juice. A comparison between the volatile composition of grape-spirits obtained from table grapes and from wine grape has been carried out. The content of esters of the medium-long chain fatty acids in table grape spirit was found to be significantly higher than in wine grape. The results of the sensory analysis pointed out that table grapes seem to be not suitable to produce high quality grape spirit.

Keywords: table grapes, volatile compounds, exogenous glycosidases, terpenols, distillation, sensory analysis

The flavour of grape-spirit (PAUNOVIC, 1991) is affected by many variables such as raw material and processing steps which include fermentation, distillation and maturation (DA PORTO, 1998). Actually, a limited selection of wine grape varieties that contain flavour impact compounds such as terpenols in the Muscat varieties and non-muscat aromatic varieties (Prosecco, Riesling, Traminer, etc.) (GUNATA et al., 1985; GUNATA et al., 1992; STRAUSS et al., 1986; NOBLE & SHANNON, 1987; USSEGLIO-TOMASSET, 1987; PARK et al., 1991; VERSINI, 1992; VERSINI et al., 1995) and alkoxypyrazines in the 'herbaceous' cultivars (Sauvignon, Cabernet, etc.) (BAYONOVE et al., 1975; ALLEN et al., 1991; DARRIET & DUBOURDIEU, 1993) have been used to produce grape-spirit.

Since grape-spirit is a fruit-spirit (EC, 1989) not only wine grapes but also table grapes could be used as raw material. In this preliminary research we studied the

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0139-3006/2001/$ 5.00

influence of the addition of exogenous glycosidases on the concentration of some monoterpenes in fermented table grapes to be distilled. A comparison between the volatile composition of table grape spirit and wine grape spirit was carried out. The sensory evaluation of the grape spirit samples is also discussed.

1. Material and methods

1.1. Raw material

White table grapes (ca. 350 kg) harvested at 14 °Brix were stemmed and crushed. Maceration with fermentation was carried out at 20 °C after addition of thiamin, mutè concentrate (up to 20% of sugars), inoculation with pure yeast starter and the addition of pectolytic enzymes (Zimopec P110, Perdomini S.p.A., Verona, Italy). Close to the end of fermentation (ethanol content about 12%) the mass was divided into two portions: the first portion consisted of fermented juice drawn off from solids (J) and the second one must plus solids (M). Commercial glycosidases (6 g hl^{-1}) produced by *Aspergillus niger* (Pectasin FL, Genecor International, Verona, Italy) were added to the must plus solids and to the fermented juice.

The must was left in contact with skins for seven days (20 °C) (M+E), the fermented juice (J+E) was stored for seven days at 18–20 °C. The skins of the fermented juice was stored in inert gas and added to the fermented juice (J+E+S) before distillation. Samples of M, M+E, J and J+E were stored at –20 °C until extraction.

1.2. Experimental distillation plant

After seven days M+E and J+E+S were distilled at atmospheric pressure (100.9 kPa). The distillation equipment used to obtain the samples is shown in Fig. 1. It consists of a copper boiler (A) (capacity ca. 30–40 kg) with a hermetically sealed hatch for loading the fermented grapes and, at the top, a large hemispherical hat. The vapour is injected into a space around the bottom of the boiler. A pipe inserted into the top of the boiler conveys phlegm vapours to column (B). Two condensers (D-E) connected in series condense the vapours and cool the distillate. The distillation pilot plant can work at atmospheric pressure or, by means of the vacuum pump, at reduced pressure (F).

1.3. Wine grape spirit

Since in Italy the bulk of grape spirit production uses Prosecco and Riesling grapes as raw materials, the control spirit chosen to compare table grape spirit compositions was a Prosecco grape spirit. This wine grape spirit was obtained using the

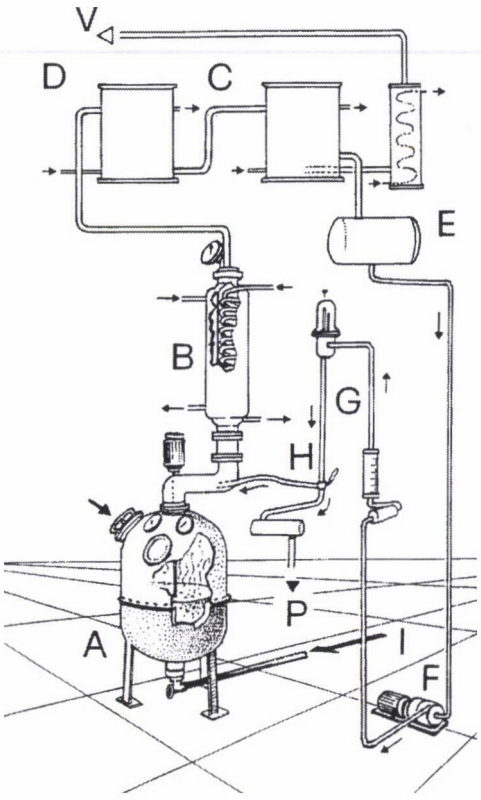

Fig. 1. Pilot distillation equipment. A: copper boiler; B: column; C–D: condensers; F: liquid sealed vacuum pump

same fermentation and distillation conditions used for the experimental table grape spirit M+S.

1.4. Chemical analysis

Extraction and determination of free and bound terpenols in M, M+E, J and J+E samples were carried out using the procedure described by BATTISTUTTA and co-workers (1998). Samples were clarified by centrifugation before analysis. The juices were adsorbed on reverse phase C_{18} Sep-Pak cartridge (Waters Associates Milford, MA) and then eluted with a mixture of n-hexane:chloroform (2:1 v/v).This solvent mixture permits the on-column injection. Free forms were determined directly by gas chromatography, while glycosidically bound forms were first enzymatically hydrolised to release aglycones.

Gas chromatographic analysis of grape spirits M+E and J+E+S were performed directly using a 30 m Supelcowax 10 capillary column (0.32 mm i.d. and 0.5 μm film thickness) (Supelco Inc.). The operating conditions were as follows: injector and detector temperature 250 °C, column temperature was programmed from 50 °C for 5 min, 50–190 °C at 3 °C min^{-1} and 190–220 °C at 10 °C min^{-1}. The carrier gas (helium) flow rate was 2 ml min^{-1} and the split ratio was 1:20 v/v. The repeatability of the HRGC analysis was tested using a grape spirit sample analysed six times. The relative standard deviations were lower than 8%. Quantification was expressed as mg/100 ml absolute ethanol.

1.5. Sensory analysis

A panel of 10 experienced assessors evaluated the table grape spirits and the wine grape spirit ordering the samples for 'preference'. The samples (20 ml) were presented to the panel in standard grape–spirit glasses.

2. Results and discussion

The use of exogenous glycosidases in M+E and J+E resulted in an increase in the concentration some monoterpens when compared to control samples M and J as shown in Table 1. The total free terpenols increased by 10% in M+E and 56% in J+E. The enzyme preparation added to the must in the presence of solids, in spite of the extended maceration time, was found to be less efficient in hydrolysing the glycosides than when added to the juice.

The enzymatically treated must plus solids had a higher aroma potential in the form of bound terpenols than the enzymatically treated juice. Under the acidic conditions prevailing during fermented grape and juice distillation, these compounds should be hydrolysed to form volatile monoterpenes which should distil over in the spirit (STRAUSS & WILLIAMS, 1983).

By comparing the volatile composition of the table grape-spirits with the control wine grape spirit (Table 2), it is interesting to note that the most considerable variation occurred in the ester content. The amount of esters of medium-long chain fatty acids (from C_6 to C_{18}) in table grape-spirits was significantly higher than in wine grape-spirit. From an aromatic point of view these compounds, relatively high-boiling, begin to diverge widely from the pleasant odour of the fruit esters.

Neither the table grape spirits nor the wine grape spirit had any problem with methanol content, being its concentration very low (0.17–0.20 mg% ml absolute ethanol).

Table 1

Changes in free (f) and bound (b) forms of terpenols ($\mu g\ l^{-1}$) in must plus solids without (M) and with (M+E) glycosidases and in fermented juice without (J) and with (J+E) glycosidases

Compounds ($\mu g\ l^{-1}$)		Fermented table grapes treatments			
		M	M+E	J	J+E
Linalool	(f)	146	170	126	172
	(b)	364	300	274	214
α-Terpineol	(f)	5	14	4	22
	(b)	56	49	49	31
β-Citronellol	(f)	32	16	4	5
	(b)	5	2	5	3
Nerol	(f)	49	59	15	41
	(b)	174	147	150	110
Geraniol	(f)	n.d.	n.d.	n.d.	n.d.
	(b)	438	344	350	288
Trans-furanic linalool ox	(f)	n.d.	n.d.	n.d.	n.d.
	(b)	250	207	147	216
Cis-furanic linalool ox	(f)	12	10	10	9
	(b)	7	4	2	11
Total terpenols	(f)	244	269	159	249
	(b)	1294	1053	977	873

n.d.: not detected

Usually, to maintain the level of methanol within its legal limit in fruit spirits (1g% ml absolute ethanol) it is removed by rectification that results in a loss of flavour volatiles (DÜRR & TANNER, 1983). In grape spirit production methanol formation is reduced by short storage time of grapes before winemaking process, without any treatment of fermented grapes, such as acidification, or demethylization of the raw distillate (DA PORTO, 1998). The level of higher alcohols in the different grape spirits varied in relation to the fermentation conditions; foremost in importance was the content of nitrogenous components (BERRY, 1995). The slightly higher amount of terpenols found in table grape-spirits cannot be attributed only to the enzyme hydrolysis of the commercial glycosidases used as chemical hydrolysis of glycosides naturally occurred during distillation.

Table 3 shows the results of the sensory evaluation. The panel preferred the wine grape-spirit. Probably the high content of esters of medium-long chain fatty acids in table grape spirit increased the heavy aroma fraction of the spirit and consequently the panel chose the most balanced and finest grape spirit.

Table 2

Comparison of the composition of some volatile compounds (mg/100 ml absolute ethanol) of grape spirits obtained by table grapes and wine grapes

Compounds	Table grapes		Wine grapes cv Prosecco
	M+E	J+E+S	
Ethanol content (% vol.)	42.0	41.5	41.0
Methanol	0.19	0.17	0.2
1-Propanol	39.00	42.50	39.00
2-Methyl-1-propanol	29.50	30.00	71.00
2-Methyl-1-butanol	26.50	27.00	67.00
3-Methyl-1-butanol	120	129.5	135
2-Phenylethanol	4.65	2.42	0.66
Higher alcohols. sum	219.65	231.42	312.66
1-Hexanol	0.47	0.71	1.34
Trans-3-Hexen-1-ol	0.04	0.01	0.15
Cis-3-Hexen-1-ol	0.06	0.15	0.15
Trans-2-Hexen-1-ol	0.01	0.01	0.07
1-Eptanol	0.02	0.01	0.06
1-Octanol	0.01	0.01	0.04
1-Decanol	0.01	0.01	0.03
Furfurol	0.6	0.31	0.37
Benzyl alcohol	0.02	0.01	0.01
Linalool	0.45	0.14	0.36
α-Terpineol	0.26	0.45	0.23
Geraniol	0.17	0.20	0.11
Terpens. sum	0.88	0.79	0.70
Isoamyl acetate	13.23	4.09	0.80
Hexyl acetate	0.14	0.17	0.03
Butyl acetate	0.01	0.01	0.02
Ethyl lactate	0.27	1.64	13.30
Ethyl acetate	34	10	26
2-Phenyl acetate	0.88	1.39	0.10
Ethyl caproate	1.93	1.04	0.65
Ethyl caprylate	6.75	3.90	3.53
Ethyl caprate	11.57	9.52	7.80
Ethyl laurate+ n.i.	5.74	7.02	4.29
Ethyl myristate	0.70	0.99	0.75
Ethyl palmytate	2.67	4.20	1.59
Ethyl palmytoleate	0.64	0.71	0.07
Ethyl-stearate	0.18	0.27	0.02
Ethyl-oleate	0.15	0.19	0.04
Ethyl linoleate	2.07	2.78	0.40
Ethyl linolenate	0.51	0.35	0.24
Higher esters (C_6-C_{18})	32.91	30.97	19.38
Esters. sum	81.44	48.27	59.63

n.i.: not identified

Table 3

Results of the sensory analysis. The values indicate how many members of the panel gave the sample that rank

Ranks	Table grape spirit		Wine grape spirit cv Prosecco
	M+E	J+E+S	
I	0	0	8
II	3	1	2
III	7	9	0

3. Conclusions

The use of fungal glycosidases during winemaking of table grapes resulted in a little release of flavour compounds such as linalool, geraniol and nerol. An extended maceration time did not improve the free forms of compounds as interesting flavour properties. Distillation of this raw material gave a ratio of volatile compounds which resulted in a non balanced spirit. Results confirm that table grapes are not suitable to produce high quality grape spirit.

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Acta Alimentaria, Vol. 30 (2), pp. 227–230 (2001)

Book reviews

Handbook of cereal science and technology

K. KULP and J. G. PONTE (Eds)

2nd ed., Marcel Dekker, New York, Basel, 2000, ISBN No 0-8247-8294-1, 790 pages

A second edition of a book is always a sign of success. Analyzing the factors taking role in the favourable acceptance of the first edition of this book, it should be primarily mentioned that this book addresses the full spectrum of cereal grain science, employing agronomic, chemical, nutritional and technological aspects and perspectives. Secondly, all economically important grains and a practically full scale of cereal-based products are treated. Finally, the authors of individual chapters are well known specialists of this field, giving an up to date, high level overview about science and practice related to cereals.

In this second edition cereals are discussed, similarly to first edition, from three points of view: agronomic, chemical and technological utilization of each grain. To every chapter a list of references is attached. According to main topics the 27 chapters of book may be divided to three sections.

In the 11 chapters of first section an overview is given about history, production and use, breeding, kernel structure, chemical composition, commercial grading, storage and milling procedure of each cereal (wheat, corn, barley, oats, sorghum, millets, rye, triticale, and wild rice). A new chapter on soybeans and oilseed grains is included into the second edition. Taking in mind some newer tendencies it seems that next a chapter should be devoted also to pseudo cereals such as buckwheat, amaranth etc.

Comparing the extent of chapters it could be stated that chapters dealing with corn, barley and oilseeds are the longest. This is connected with the fact that these chapters include the full spectrum of utilization including the technologies applied. For the reader the most interesting is the part dealing with separation of corn into its component fractions. Dry- and wet milling procedure are discussed and the further possibilities of utilization of products of milling. Among others edible oil recovery from corn germ, the starch production, recovery of zein from corn gluten meal are treated. Special attention is paid to the conversion of raw fractions into value-added ingredients such as modified starches and corn sweeteners. Among the future prospects an overview is given about possibilities of chemical conversion of corn fractions and perspectives of development of production of chemicals based on biochemical conversion of different corn components. The biggest chapter of this section is devoted to oilseeds and oil-bearing materials. Although this chapter contains valuable, up to date informations concerning oilseeds production and processing with very rich material concerning technologies and equipment, it seems that it is somehow oversized.

The second section is devoted to chemistry of main components of the cereals starting with proteins. Taking in mind that basic knowledge concerning the protein of individual cereals was shortly treated in previous chapters, here primarily the isolation and separation techniques and the variety identification are discussed. Although the problems of quality determination and nutritional aspects are included in separate chapters, the reader feels that protein-bread making quality correlations in wheat, some effects of protein on end-use properties of other cereals, the GMO problem and a short review of minor protein components are missing from this chapter.

Akadémiai Kiadó, Budapest

Cereal carbohydrates are the topic of next chapter. An up to date review is given about structure and changes of starch during processing. The main value of this chapter is a detailed treatment of nonstarch polysaccharides, attracting recently the attention of both nutritionists and technologists.

The next chapter gives an overview of the comparative values of cereal lipids – how they differ in quantity and composition in various cereal grains. In more than 60 tables are these data collected and include practically all recent data in this field. What is missing nevertheless from this chapter that's the protein-lipid interaction and its role in processing.

A valuable chapter is dealing with the minor constituents of cereals including vitamins, minerals, enzymes, pigments, phytates, enzyme inhibitors and tannins.

The third section of the handbook is devoted to quality evaluation of cereals and cereal products, to production of different varieties of cereal-based products and to the nutritional quality of cereal-based foods. The chapter dealing with quality evaluation concentrates primarily on instrumental physical dough testing and pasting tests giving an excellent up to date overview, but other methods particularly those of wheat and rye evaluation are treated too.

Among the cereal-based products first the bread and other yeast-leavened products are reviewed. The modern technological processes are described including typical formulas, production equipment, functions of bread ingredients and stability of products. According to my opinion from this chapter a short overview about yeasts and sour dough microorganisms is missing.

Soft wheat products are treated in the next chapter. After an overview of ingredients, the processing of the most typical types of products (crackers, cookies, cakes, donuts etc) is described together with some formulas and quality characteristics.

Ready to eat (RTE) breakfast cereals play an important role in US cereal-based food production and a growing consumption in Europe may be observed. A very good review is given about processing stages (cooking, extrusion and extruders, expansing and puffing, flaking, shredding, coating, packaging), additives, formulation, and finally a list of most typical products is given.

The chapter named "Pasta: Raw materials and processing" provides an overview starting with raw materials (primarily durum wheat and its milling products) till processing and quality evaluation. A short chapter is intended for cereal-based snack foods and also for barley malting and brewing.

The topics "Cereal enrichment and nutrient labeling" and "Nutritional quality of cereal-based foods" are treated in two separate chapters. A specific value of these chapters is the detailed overview of nutritive role of minor cereal components (such as vitamins, microelements, natural antioxidants, antinutrients, etc.) and dietary fiber. Allergy to cereal proteins is also shortly treated. For practice the regulations concerning enrichment (fortification) and labeling may be also useful.

The growing nonfood uses of cereals stimulated probably the editors to include a separate chapter dealing with this topic. As expected, ethanol production from cereal grains is primarily treated both from technical and economical point of view and perspectives. Nonfood uses of starch and cereal proteins are also described.

The topics of the last two chapters are: "Fermentation and microbiological processes in cereal foods" and "Special food ingredients from cereals". A unique list containing more than 100 special cereal-based fermented foods from all continents, together with a list of representative microorganisms isolated from these foods is published. A short review is given about fermentation process in yeast leavened dough and rye sourdough. However, as mentioned before, this topic should be better located in chapter dealing with bread production and in bigger extent.

In the framework of special cereal-based food ingredients an excellent review is given about wheat gluten production including processing equipment and technologies. Cereal starches are shortly treated in this chapter, however it should be noted that this topic was discussed more deeply in a chapter in the first section of the book dealing with corn processing.

Summarizing, it can be stated that this book written and edited by well-known specialists in this field is a valuable source of useful information for all specialists involved in cereal production, processing, utilization and distribution, and to some extent for nutritionists and postgradual students, as well.

R. LÁSZTITY

Food analysis by HPLC

L. M. L. NOLLET (Ed.)

Marcel Dekker, New York, Basel, 2000 ISBN No 0-8247-8460-X, 1049 pages

The growing demands for the nutritional quality and safety of our foods make food analysis develop very fast. The advent of HPLC gave an extremely effective tool into the hands of analysts exactly on those fields, where the capability of classical analytical and gas chromatographic methods ends. Marcel Dekkers' *Food Analysis by HPLC* (ed. Leo M. L. Nollet) is a magic key to this miraculous technique. Several monographs relating to high performance liquid chromatography were published in the last decade, but this book is the most excellent one that can be found in food analysis.

The monograph starts with the discussion of the principals of liquid chromatography not in details but in the depth that is inevitable for a user. Its 24 chapters cover all the topics of great importance in food analysis. Beside the traditional HPLC tasks like carbohydrate or vitamin determinations, new fields of residue analysis, naturally occuring food toxicants and other "favoured" compounds of food additives e.g. artificial sweeteners, or colourants and antioxidants are also discussed. All chapter contains a short historical review of the related field and after a brief theoretical introduction deals with the analytical problem from sample preparation to the conditions of measurements in details, including the troubles caused by the matrix. The greatest desire of all analyst in practice to have literature and references at hand so that new analitical tasks and problems should be solved in a short time. By their very up to date and most recent references all chapters match this demand.

Thanks to the editor the monograph in spite of its many authors prossesses a very uniform and professional style. It makes the book suitable for teaching purposes, that means being used in the education at universities. It is perfectly illustrated with figures and tables, the appearance of the book is really nice. It is a great pleasure to work with it.

I am convinced, that Marcel Dekkers' monograph of Food Analysis by HPLC is the very book that must be present on the book shelves of every chromatographer and expert involved in food analysis.

K. KORÁNY

Modern chromatographic analysis of vitamins

A. P. DE LEENHEET, W. E. LAMBERT and J. F. VAN BOCXLAER (Eds)

3rd ed., Marcel Dekker, New York, Basel, 2000, ISBN No 0-8247-0316-2, 632 pages

The first edition of this important handbook was published in 1985 and the second in 1992. The third edition covered the enormous progress in the chromatographic techniques for the determination of vitamins in different matrices in the last decade.

The work covers both the fat-soluble (Chapters 1–4) and the water-soluble (Chapters 5–13) vitamins, with emphasis on state-of-the-art chromatography, sample preparation, and final measurement. The 30 contributors include many of best known and most knowledgeable workers in the field of vitamin analysis throughout the world and are all recognised experts on their topics under the direction of the 3 editors produced a book, that is a valuable source of information for scientists, with a high degree of expertise in chromatographic analysis of vitamins. However, most chapters contain considerable introductory and background material, making this book also appropriate for the relatively inexperienced researcher in this field.

Contents: Vitamin A and carotenoids; Vitamin Ds: metabolites and analogs; Vitamin E; Vitamin K; Ascorbic acid; Chromatographic determination of folates; Nicotinic acid and nicotinamide; Thiamine; Flavins; Vitamin B6; Biotin; Cobalamins; Pantotenic acid.

All chapters begin with the chemistry, biochemistry of vitamins and continue with the sample extraction, handling and storage, application of standards and internal standards. The methodical part gives information on all possible chromatographic methods including thin-layer chromatography, high performance liquid chromatography, gas chromatography and other possible techniques as well as recent development and future trends. The reader can find all necessary information regarding the methodical details in sample figures and tables or in the plentiful references. The index of book gives good help for quick searching for information.

F. ÖRSI

Typeset by
WEGATREND Kft., Budapest

PRINTED IN HUNGARY
PXP Ltd., Budapest

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ACTA ALIMENTARIA

An International Journal of Food Science

Editor
J. HOLLÓ

Volume 30 **September 2001** **Number 3**

Akadémiai Kiadó
Budapest

ISSN 0139-3006
CODEN ACALDI

ACTA ALIMENTARIA

An International Journal of Food Science

Sponsored by the Joint Complex Committee on Food Science of the Hungarian Academy of Sciences and published by financial support of the Ministry of Agriculture and Regional Development.

Editorial office:

Central Food Research Institute
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Acta Alimentaria is a quarterly journal in English, publishing original papers on food science. The main subjects covered are: physics, physical chemistry, chemistry, analysis, biology, microbiology, enzymology, engineering, instrumentation, automation and economics of foods and food production.

Distributor:

AKADÉMIAI KIADÓ
H-1519 Budapest, P.O. Box 245, Hungary

Fax: (36-1) 464-8221
E-mail: kiss.s@akkrt.hu
http://www.akkrt.hu

Publication programme, 2001: Volume 30 (4 issues)

Subscription price per volume: US$ 236.00, including normal postage, airmail delivery US$ 20.00.

Acta Alimentaria is published 4 times per annum: March, June, September and December

Acta Alimentaria is surveyed by Chemical Abstracts, Current Contents/Agriculture, Biology and Environmental Science, ASCA, BIOSIS, Nutrion Abstracts and Reviews.

AAlim 30 (2001) 3

ACTA ALIMENTARIA

AN INTERNATIONAL JOURNAL OF FOOD SCIENCE

VOLUME 30
2001

AKADÉMIAI KIADÓ
BUDAPEST

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Acta Alimentaria, Vol. 30 (3), pp. 231–232 (2001)

Editorial

FOOD SAFETY AND CONSUMER ACCEPTANCE

Outbreaks of food-borne illnesses have occurred over the past world-wide nevertheless recently have made consumers increasingly aware of the naturally occurring threats and production-induced risks to food safety. We have to be aware that food is not only an agricultural and trade commodity but also an essential emotional, political and public health issue. Consumers – at least those with sufficient buying power – make usually an emotional choice based on their cultural, educational and social background. They have to be assured that the food they consume is safe and wholesome, sometimes they require total safety and zero tolerance, however zero tolerance does not exist in practice.

How can we ensure that consumers for whom food is produced after all have their requirements fulfilled? Could their expectations be met? Can food safety be ensured? Do we need to know what consumers think and require?

Once we are aware of their expectations we have to ensure that food producers have the responsibility for food safety nevertheless in effective and efficient co-ordination of the food safety functions of all governmental and non-governmental agencies will share responsibilities for food. Furthermore food legislation has to assist food producers to comply with the high requirements of the consumers. The harmonised legislation has to supply a ground for safe and high quality food production at all stages of the food chain.

A rapidly growing number of people understand requirements, the significance of proper specifications, which has created a processing culture in which food safety management systems could develop. Quality management principles and preventive, proactive approach were introduced to provide uniform, reliable quality and food safety. The importance of risk analysis should be further emphasised and responsibilities to perform risk assessment, risk management ought to be declared as well. The importance of risk communication need to be understood and consumers should be informed on a regular basis. Risk communication means an information flow amongst all interested parties. Risk perception, consumer perception and education of consumers should be considered as well. All problems arising continuously, sometimes unexpectedly posing risks for all of us, for the society, for consumers all over should be and could be solved by co-operation of those in the food chain "from farm to fork" from the producers, processors, through retailers to the consumer.

0139–3006/2001/$ 5.00

In order to strengthen our efforts food safety policies are prepared or proposed at national, regional and international level. Several member states of the EU are preparing food safety offices, authorities, agencies, institutes and even the European Commission has declared to establish a Food Safety Authority. International bodies prepare strategies, status reports, framework programmes in order to establish, to ensure or to strengthen food safety. In summer 1999 the G-8 Countries requested the OECD to prepare a Report on Food Safety for which purpose an OECD Ad hoc Committee on Food Safety has been created and an extensive report was published afterwards.

International organisations, bodies with historical background have several professionals working on the subject (e.g. Codex Committees) nevertheless emerging issues such as the GMOs urging the international flora to develop strategies based on new considerations. For this reason OECD Task Force for the safety of novel foods and feeds and Codex Alimentarius Task Force for the safety of foods derived from modern biotechnology have been created and the work intensified.

The FAO Regional Conference for Europe in July 2000 at the Ministerial Roundtable on Food Safety and Quality has stated its recommendations by supporting the "from farm to fork" approach to food safety which would require food operators throughout the food chain to be responsible and accountable for the safety of their products. They emphasised the importance of traceability of all food, feed and their ingredients and recommended maximum transparency in the operation of both components of risk analysis. This Conference took note with interest of the proposal to hold a Pan-European Conference on Food Safety. This is a great honour to note that the conference will be held in Hungary, February 2002.

The issue of traceability has been emerged as the processing of foodstuffs and their international trade became more sophisticated sometimes complicated and large-scale allowing the spread of diseases caused by a single mistake. The importance of traceability was further emphasised as novel ingredients and raw materials were prepared making consumers uncertain demanding the trace-back.

To ensure that situations making consumers even more reluctant to food safety and influencing their perception the EU has established the precautionary principle. This would enable decision-makers to base their decisions even more for the sake of consumers. Risk assessment, risk management measures carried out by whatever means would not enable us to complete our tasks in order to protect the consumer and ensure the safety of foodstuffs without listening to the consumer and educating all those – including consumers themselves – in the food chain.

Capacity building and networking would or could support these activities. But let us not forget about the importance of scientific co-operation which supports the legislation giving the framework for the production, processing and marketing and assists decision-makers or risk managers by risk assessment and allow consumers to trust in food, the safety of foods consumed and in the food chain as a whole.

D. BÁNÁTI

Acta Alimentaria, Vol. 30 (3), pp. 233–246 (2001)

THE QUALITY OF SLICED CARROTS AFFECTED BY MODIFIED POLYETHYLENE FOIL AND STORAGE TEMPERATURES

J. POSPIŠIL, N. CIKOVIĆ, V. DRAGOVIĆ-UZELAC, V. LUKIN and D. BRUSIĆ

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(Received: 15 May 2000; revision received: 3 January 2001; accepted: 27 March 2001)

The aim of this study was to examine the influence of different additive contents for oxygen absorption (5, 10 and 15%) in low-density polyethylene (PELD) plastic foils and storage temperatures (4 and 28 °C) on the quality and shelf-life of sliced carrots during storage. Quality and storage-life of packaged carrots slices were determined by observing changes of mass, total carotenoide pigments, microbial counts (mesophilic aerobic bacteria, enterobacteria, sulphite-reducing clostridia, yeast and moulds), sensory quality and texture by the use of penetrometer.

The PELD foils modified with 10 and 15% of oxygen absorber (O_2, CO_2 and N_2 permeability at 4 °C of around 700 ml $m^{-2}d^{-1}atm^{-1}$) were the most suitable for the storage and prevention of deterioration of minimally processed carrots. Findings indicated that in these foils the best quality and shelf-life of carrot were maintained by 6 days of storage at 4 °C, without significant changes in parameters studied.

The absorber for oxygen added to the foil had no influence on the permeability to CO_2, O_2 and N_2. The permeability of foils, which were used for carrot packaging increased by the increase of storage temperature to 28 °C and decreased by decreasing the temperature to 4 °C, and was not significantly affected by the additive content either. In the same time the diffusion constants of unused and used PELD foils for carrot packaging at 4 °C and 28 °C changed according to the change of film permeability during storage at those temperatures.

Keywords: PELD modified with oxygen absorber, permeability, sliced carrot, carotenoids, microbes, sensory

Recent years have seen a rapid expansion in small-batch packaging and sale of minimally ("light") processed vegetables, aimed at individual consumers (home consumption, restaurants, tourist camps), that can be used for salads or main courses.They are washed, peeled, sliced or shredded and, wrapped raw vegetables are stored below 10 °C and sold within 8 to 10 days (NGUYEN-THE & PRIUNER, 1989). The disadvantage of the pre-cut product is that its storage life may be greatly reduced as compared to the intact vegetable. Tissue disruption caused by cutting results in elevated respiration and transpiration, which leads to rapid deterioration and subsequently loss of quality (ROLLE & CHISM, 1987; WATADA et al., 1990). In addition, cutting increases the

0139–3006/2001/$ 5.00

area of injured tissue available for microbial degradation (BREIDT & FLEMING, 1997). Some problems related to cell disruption are leakage of nutrients, enzymatic reactions, mould growth, lactic acid fermentation, loss of texture, development of off-flavors and off-odors and appearance defects (CARLIN et al., 1990). Minimally processed carrot may become slimy, lose firmness and produce off-odors (MCLACHLAN & STARK, 1985; CARLIN et al., 1989; POSPIŠIL et al., 1989).

The most common methods used to improve the storage stability of minimally processed vegetable are low temperature, chemical treatments, modification of pH, modified or controlled atmospheres, active packaging or combination of two or more of these (BRACKETT, 1987; HUXSOLL & BOLIN, 1989; KING & BOLIN, 1989; KADER et al., 1989; LABUZA & BREENE, 1989; BOLIN & HUXSOLL, 1991; IZUMA et al., 1996; VERMEIREN et al., 1999; DEVLIEGHERE et al., 2000). KADER (1986) critically reviewed the responses of fresh fruits and vegetables to modified atmosphere packaging, summarizing the implications, status and future of this technology.

No systematic study has been conducted to establish which commercially available plastic films would be most suitable for modified atmosphere packaging of a particular produce (EXAMA et al., 1993). It is well known that different polymer structures have very different properties, giving to the polyethylene family the opportunity to cover a wide range of applications. The reaction of oxidation in a polyethylene film can be slowed or inhibited by compounds that interrupt the chain reaction at some points.

Our work was undertaken to examine the effect of different additive contents (oxygen scavenger) in the low density polyethylene foil (PELD) on the quality and shelf-life of sliced carrot during storage at 4 and 28 °C. Changes in mass, total carotenoids, microbial count, sensory quality and texture in carrot slices were determined. The gas permeability and diffusivity coefficients of foils before and after being used for carrot packaging and storage at two temperatures, were measured.

1. Materials and methods

Carrots of the Nantes cultivar (Napoli variety) were grown on a local farm in the summer of 1998. Before the beginning of the experiments the carrots were held at 4 °C for two days. After washing and hand-peeling the carrots were cut into 5–7 mm thick slices (using a home food processor) and packed in polyethylene pouches (22×17 cm) made from modified polyethylene foils. Antioxidant was added to the plastic to inhibit its degradation during processing and subsequent usage. Although the exact chemical formula is not provided, the applied antioxidant belongs to the group of organic compounds, possesing double bounds, which act as oxygen scavengers. The antioxidant used is, according to the producer, on the EU list of additives which may be used in the

manufacture of plastic materials and articles. (COUNCIL DIRECTIVE, 1990). Polyethylene foils had different additive contents: 5%, 10% and 15%, with an active component of 0.2%; 0.4% and 0.6%, respectively. As a control, a polyethylene foil containing no additive (0%) was used (Table 1).

Each polyethylene pouch contained 300 g of sliced carrots, stored at 4 and 28 °C and were removed periodically for chemical and sensory analysis (after 3, 6, 9 and 14 days at 4 °C and after 2, 5 and 9 days at 28 °C). The samples were evaluated in duplicate.

Chemical composition of sliced carrots was determined by official methods. Dry matter content was determined by drying at 105 °C to the constant mass (A.O.A.C, 1990). Total carotenoid pigments were determined in carrots by petrolether extract, according to the procedure for the vitamin A analysis in food products (VUILLEUMIER et al., 1967).

The sensory evaluation was carried out by 8 panelists, scoring on a rating scale from 1 to 5 (1 is very poor, 2 to 3 is fair and 5 is excellent). The scoring system included assessment of browning (color), odor (aroma), taste and texture. Fresh carrots, from the batch used for processing were used as the control (5 scores). To determine the shelf-life of sliced carrots, the sensory evaluation for single and total sensory attributes were used (in the first case the score of 3 or below and in the second case the score of 15 or below were taken to indicate the end of shelf-life). Besides, the texture of sliced carrots was determined by using a penetrometer (EFFEGI, Italy) with 8 mm radius cylinder.

Microbiological analyses were done after cutting carrots into slices and storing them for 14 days at 4 °C and for 9 days at 28 °C in modified polyethylene foils. Analysis included the determination of total number of mesophilic aerobic bacteria, enterobacteria, sulfite-reducing clostridia, yeast and moulds (ANON, 1980) and *Listeria* sp. (ISO 11290-1, 1996). Duplicate and control samples were prepared for each sample.

Table 1

Polyethylene foils used for packaging

Polymer	Thickness (μm)	Active component (%)
Polyethylene without additive	36	0
Polyethylene with 5% additive	42	0.2
Polyethylene with 10% additive	44	0.4
Polyethylene with 15% additive	44	0.6

Permeability to CO_2, O_2 and N_2 of modified polyethylene foils was measured using a manometric method on a permeability testing appliance, Type GDP-E (Burger Feinmechanik GmBH) (BECKER, 1982). Monofilm material was analysed using method A, which provides for the determination of permeability and diffusion constants of the gas in the film.

Gas permeability calculations for the samples (monofilms) analysed using method A were determined from the equation (1)

$$q = k_1 \frac{V}{T \times (29 \times N - t_L)} \quad (1)$$

where:

k_1: calibration constant (9.89×10^8)

q: gas permeability ($cm^3\ m^{-2}\ d^{-1}\ atm^{-1}$)

V: measurement volume (ml)

N: slope of the measurement curve (s)

T: temperature (K)

Diffusion constant was calculated according to the following equation (2)

$$D = \frac{l^2}{6 \times t_L} \quad (2)$$

where:

D: diffusion constant ($cm^2\ s^{-1}$)

l: polymer thickness (cm)

t_L: time –lag value

Determinations were made before and after usage of modified polyethylene foils for carrot packaging, at selected temperatures.

Variance analysis was used to determine the influence of various additive contents in the polyethylene foils, on the changes of analysed parameters in carrot during storage (DAVIES, 1961).

2. Results

The permeability of modified polyethylene (PE) foils for O_2, N_2 and CO_2, before and after usage for carrot packaging at 4 °C and 28 °C is represented in Fig. 1. The investigated samples of unused foils (A) show almost identical values for O_2 and CO_2 permeabilities at both tempratures, which is in particular expressed at 4 °C. Slightly higher values were observed in the case of nitrogen permeability for PE foils containing 5% and 10% of additive, as well as without the additive (0%). The foils permeability for all three gases increased by increasing the temperature to 28 °C.

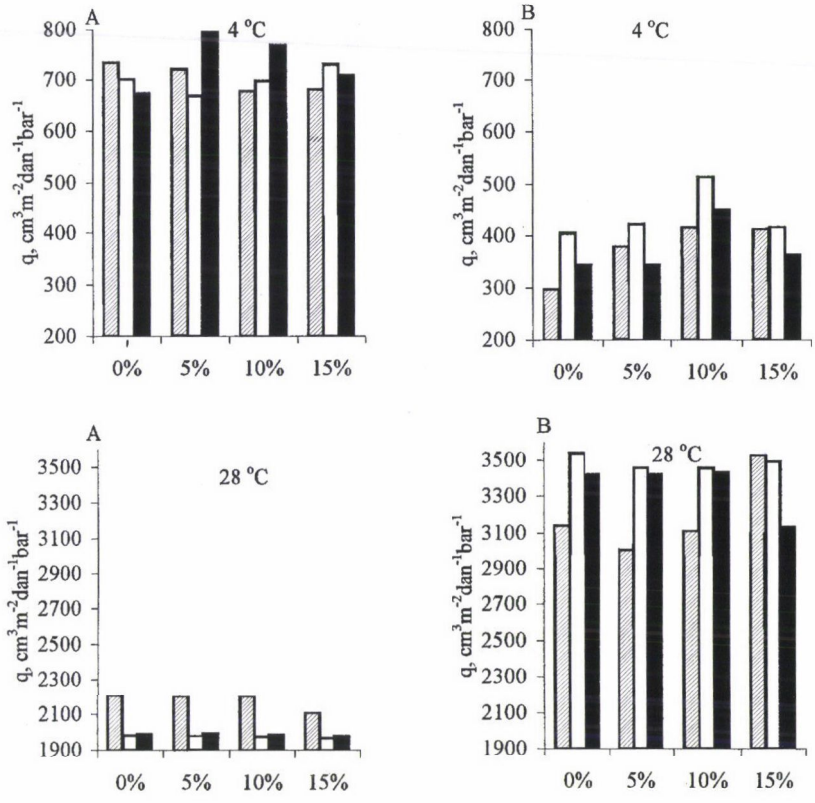

Fig. 1. Permeability values of gases in modified polyethylene foils at 4 and 28 °C, before (A) and after (B) usage for sliced carrot packaging. ▨: N_2; □: O_2; ■: CO_2

It is interesting to note that the permeability of the foils is not affected by the additive content. Such behavior may be attributed to antioxidant presence and an organic compound possessing reactive double bound for oxygen molecular bonding.

The modified PE foils after usage for carrot packaging (B) show decreasing permeability for O_2, N_2 and CO_2 at 4 °C and at 28 °C. This phenomenon is probably due to rising the small polymer crystals and less pronounced diffusion and solubility of gases. The increase of foil permeability at 28 °C is a consequence of foil swelling in the contact with metabolic products of the minimally processed carrots.

The polymer permeability to the gases depends on their diffusion and solubility constants as well. Diffusion coeffiecient presents the measure of polymer capability to differentiate the molecules of different dimensions and shapes (HOEKSTRA et al., 1996).

Diffusion coefficient values for gases in modified PELD foils are presented in Fig. 2. It was interesting that the diffusion constants remained unchanged regardless of the type of gas or percentage of the additive added at 4 °C and 28 °C (Fig. 2A). Because of the more intensive polymer segment movement at higher temperatures, the foils capability in differentiation of the molecules to different dimensions and shapes declined, which results in losing diffusion selectivity. It was probably the reason that the diffusion constants of unused foils at 28 °C were only somewhat higher than those at 4 °C. After the foils were in contact with sliced carrot, their diffusion constants changed in the same direction as their permeability values to the gases at 4 °C and 28 °C (Fig. 2B).

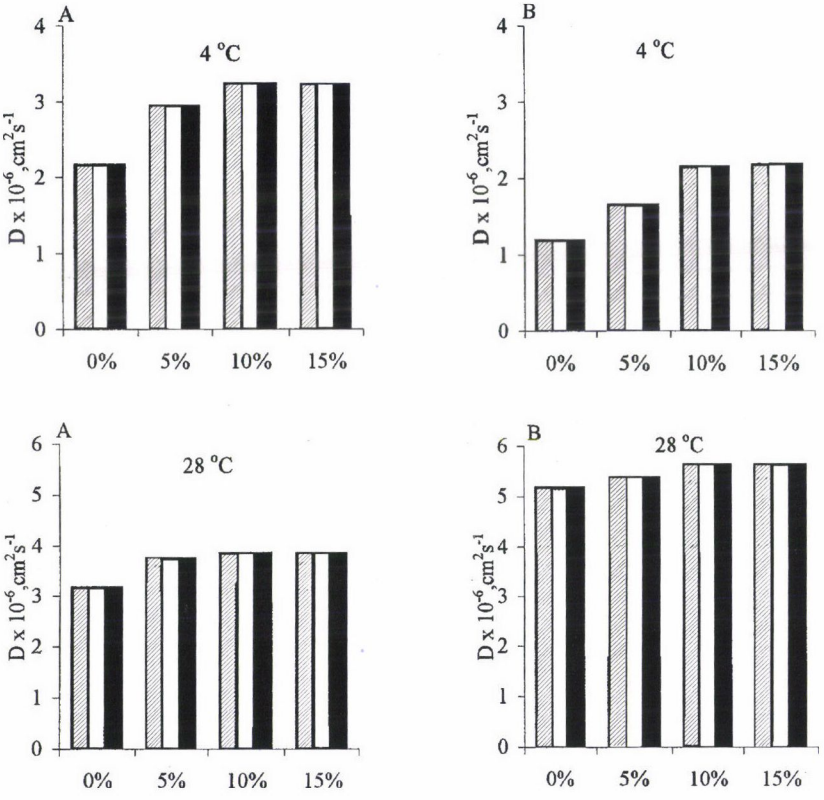

Fig. 2. Diffusion coefficient values for gases in modified polyethylene foils at 4 and 28 °C, before (A) and after (B) usage for sliced carrot packaging. ▨: N_2; □: O_2; ■: CO_2

The results linked to the changes in carrot mass show (Table 2) that storage caused mass losses in all packaged samples are more noticeable at 28 than at 4 °C. In the foil containing additive the mass showed little more losses compared to the foil without additive (0%). Our results showed that the highest losses in the mass content occurred after 6-day storage of samples, at both temperatures (0.57–0.63% at 4 °C and 3.93–4.23% at 28 °C).

The results of determining carotenoid pigments in sliced carrot indicate that storage caused their decomposition in all samples at both temperatures, but significantly more in the PE foil without additive (0%) and at 28 °C (Fig. 3). Namely, at 4 °C the additive for oxygen absorption in 5, 10 and 15% concentrations had a dominant effect on the stability of carotenoid pigments in carrot, whereas at 28 °C the increased temperature had a much greater influence. The foil with 10 and 15% of additives gave higher level of total carotenoid contents at 4 °C than the foil with 5% of additive.

Carotenoid pigments exhibited the highest losses after 6 days of storage (just like mass), when about 20% of the pigment was decomposed in the foils without additive stored at 4 °C, and in the foils with additive only about 5%. In samples stored at 28 °C, more than 50% of pigment was decomposed after 6 days as a consequence of high temperature influence, and greater foil permeability for oxygen at higher temperatures.

Aerobic microbial populations on fresh sliced carrots ranged from 10^4 to 10^7 CFU g^{-1} on 0 day (Table 3 and Fig. 4). Microbiological analyses of the sliced carrots revealed that after 14 days of storage at 4 °C and after 9 days of storage at 28 °C, all samples exhibited a slight decrease in the count of mesophilic aerobic bacteria and enterobacteria, and an increase in the number of yeasts (Table 3 and Fig. 4).

Table 2

Mass loss of sliced carrots in modified polyethylene foils, during storage at 4° and 28 °C

Measurements	Storage temp.	Storage time	Additive (%)			
	(°C)	(days)	0	5	10	15
Mass loss,%	4	3	0.53	0.27	0.37	0.37
		6	0.57	0.63	0.63	0.63
		9	0.73	0.83	0.97	0.97
		14	0.96	1.13	1.06	1.06
	28	3	0.73	0.97	0.83	0.61
		6	3.93	4.23	4.13	4.10
		9	4.01	4.20	4.12	4.07

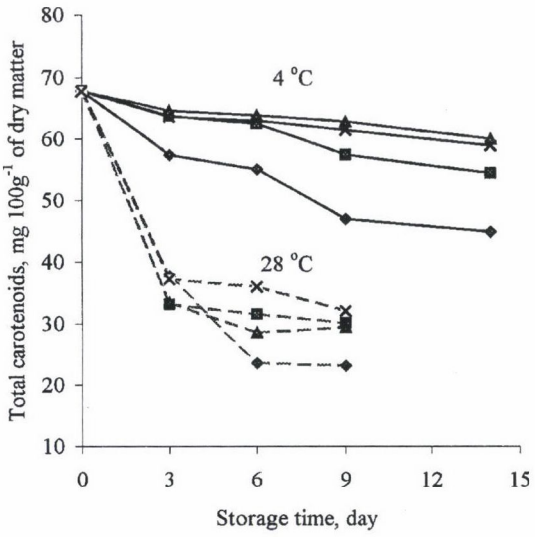

Fig. 3. Changes in total carotenoid pigments of sliced carrot packaged in modified polyethylene foils, during storage at 4 and 28 °C. ◆ = 0%; ■ = 5%; ▲ = 10%; ✕ = 15%

However, the decrease in the total count of mesophilic aerobic bacteria and enterobacteria was more pronounced in the samples stored at 28 °C than in those stored at 4 °C (Fig. 4). The similar trend was observed by MUNSCH and co-workers (1982) working on the effect of various treatments on the microflora of commercially washed carrot stored at 5, 22 and 37 °C. They found that microflora was the most abundant at 5 and 22 °C and significantly lower at 37 °C in all schemes.

Overall growth of mesophilic aerobic bacteria and enterobacteria on sliced carrots, during storage at 4 and 28 °C, were significantly influenced by the additive contents in the PE foils. The lowest number of the total aerobic bacteria was detected in the PE foil with 15% of additive at both temperatures (10^3–10^5 CFU g^{-1}). Storage life of most prepared vegetables is known to be terminated when microbial growth reaches 10^7–10^8 CFU g^{-1} (O'BEIRNE, 1989).

In the samples before storage, only the presence of yeasts (10^4 CFU g^{-1}) but not moulds was detected. During storage the yeast population increased to 10^5 CFU g^{-1} in all samples at both temperatures.Yeasts grow well at neutrality and under alkaline conditions but they do not compete well with bacteria at these pH values (BRACKETT, 1987). The level of additive in the PE foil did not significantly affect the population of moulds and yeasts.

Table 3

Growth of microorganisms in sliced carrot in modified polyethylene foils during storage at 4 and 28 °C[a]

Microorganisms	Storage temp.	Storage time	Additive (%)			
($\log_{10}$ CFU g^{-1})[b]	(°C)	(days)	0	5	10	15
Mesophilic	4	0	7.60	7.60	7.60	7.60
aerobic		14	7.30	7.00	6.84	4.85
bacteria	28	0	7.60	7.60	7.60	7.60
		9	6.85	6.48	6.00	5.00
Enterobacteriaceae	4	0	6.00	6.00	6.00	6.00
		14	4.70	5.00	4.90	4.00
	28	0	6.00	6.00	6.00	6.00
		9	5.00	4.00	3.85	3.30
Yeasts and	4	0	4.00	4.00	4.00	4.00
		14	5.00	5.30	5.30	5.30
moulds	28	0	4.00	4.00	4.00	4.00
		9	5.00	4.00	5.00	5.00

[a] Evolution of sulfite-reducing clostridia or *Listeria* sp. were not detected
[b] CFU means colony forming unit

There was no increase in sulfite-reducing clostridia or *Listeria* sp. in the fresh sample or in samples stored at 4 °C and 28 °C.

In Fig. 5 the scores for sensory quality evaluation of sliced carrots are presented. It is evident that the appearance of sliced carrots is degraded during storage in all foils and depends primarily on the temperature, followed by the time of storage and additive content in the foil.

According to the total score for sensory quality of carrots, the samples in foils with and without additive were of satisfactory sensory quality after 6–9 days of storage at 4 °C (total score for the assessed properties was greater than 15, which is limit value of acceptability). During this storage time the highest scores for the color and aroma attributes of carrot were given in the modified foils containing 10 and 15% of additives (total score for the assessed properties was greater than 3). Samples stored in the foil without additive (0%) received lowest colour and aroma scores. On the other hand, at 28 °C the sensory quality of carrot samples became unsatisfactory as early as 3–4 days into the storage period.

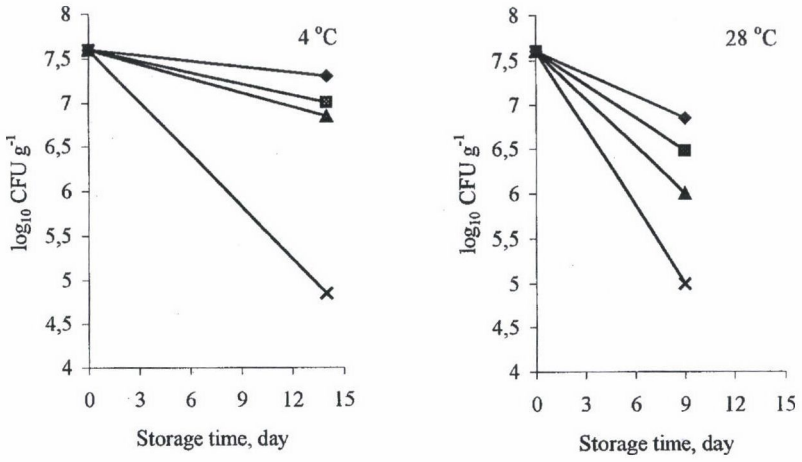

Fig. 4. Changes in number of total aerobic bacteria (CFU) of sliced carrot packaged in modified polyethylene foils, during storage at 4 and 28 °C. ◆ = 0%; ■ = 5%; ▲ = 10%; ✕ = 15%

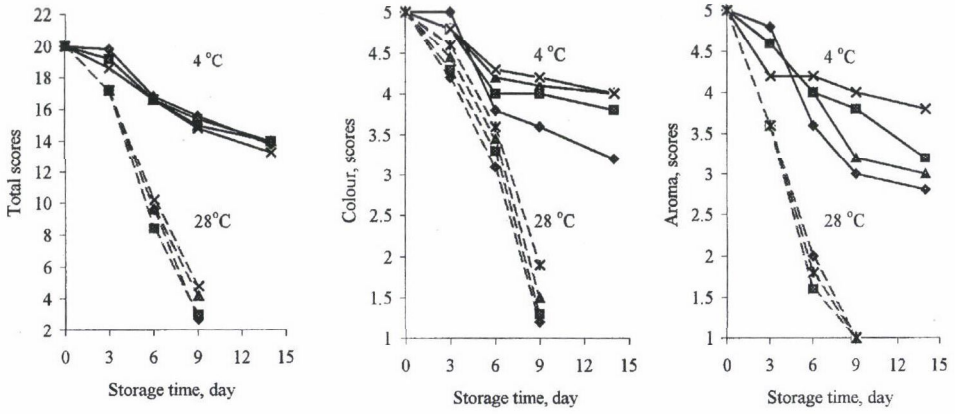

Fig. 5. Changes in total sensory scores and scores for colour and aroma of sliced carrot packaged in modified polyethylene foils, during storage at 4 and 28 °C. ◆ = 0%; ■ = 5%; ▲ = 10%; ✕ = 15%

The scores for colour were supported by the results of determinations of carotenoid pigment contents (Fig. 3). In addition, better colour quality correlated with lower total aerobic bacteria counts in sliced carrots (Fig. 4). Browning of injured vegetables is due to a large extent to oxidized phenols acting as an infection barrier (SKOVGAARD, 1984) and changes in tissue appearance caused by pectinolytic breakdown can be an indicator of microbial growth (WALL & ELLIOT, 1986).

The values for carrot texture measured instrumentally (Fig. 6) were very high in all samples stored for 6–9 days at 4 °C and very low in all samples stored at 28 °C. At 4 °C the significant differences were found in the texture between the foils with and without additive.

In order to examine the influence of various additive contents in the modified PELD foils and storage time at 4 °C and 28 °C on the changes of physico-chemical, microbiological and sensory variables in sliced (F) carrots, analysis of variance was done (Table 4). It was found that the Fisher quotient values for % of additive and storage days as sources of variation, were higher than the limit values ($F_{crit.}$) at P=0.05. It means that added absorber for oxygen to the foils and storage time had statisticaly significant influence on most investigated carrot parameters. The differences between additive fractions in the foils were examined by Duncan's test.

Table 4

Variance analysis of chemical variables in sliced carrots during storage in modified foils at 4 °C

Chemical variables	Source of variation	SS	df	MS	F	$F_{crit.}$
Mass loss	% additive	0.039	3	0.013	4.33	3.49
	Storage days	2.880	4	0.720	240	3.26
	Error	0.036	12	0.003		
	Total	2.955	19			
Carotenoids	% additive	268.1	3	89.64	10.11	3.49
	Storage days	418.72	4	104.68	11.81	3.26
	Error	106.27	12	8.86		
	Total	793.90	19			
Sensory evaluation	% additive	0.770	3	0.257	4.35	3.49
	Storage days	110.38	4	27.595	467.71	3.26
	Error	0.708	12	0.059		
	Total	11.858	19			

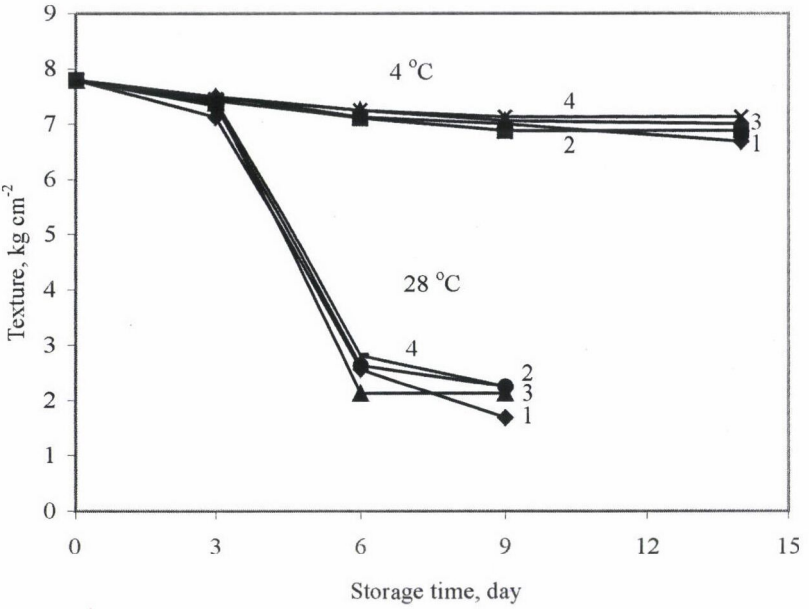

Fig. 6. Changes in texture of sliced carrot packaged in modified polyethylene foils, during storage at 4 and 28 °C. ◆ = 0%; ■ = 5%; ▲ = 10%; X= 15%

3. Conclusions

Based on the obtained results it may be concluded that the additives have no impact on the permeability of polyethylene foils to O_2, CO_2 or N_2. The permeability is significantly decreased in all foils (with or without additive) by storage at 4 °C for 14 days, and increased by storage at 28 °C for 9 days. The similar changes showed the values for the diffusion constants.

The addition of oxygen absorber to the foil and the creation of modified atmosphere in the packages, significantly reduced the mass loss, carotenoid pigments decomposition, microbial growth and changes in sensory properties of sliced carrots (particularly colour), probably by limiting plant and microbial enzyme activity. The best effects on the carrot quality was observed for the foil with 10 and 15% of additive at 4 °C. These conditions may prolong the shelf-life and appearance quality of sliced carrots by 6 days, with no significant changes in the assessed properties as compared with the original material.

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Acta Alimentaria, Vol. 30 (3), pp. 247–265 (2001)

INTERACTION BETWEEN *LACTOCOCCUS LACTIS* STRAINS AND *LISTERIA MONOCYTOGENES* IN MIXED CULTURES

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(Received: 20 July 2000; accepted: 18 December 2000)

Competition between a bacteriocinogenic and a non-bacteriocinogenic *Lactococcus lactis* strain, respectively, and a *Listeria monocytogenes* strain was studied in two semi-synthetic liquid media at various temperatures. The media used for the study were ST I and modified ST I broth (ST I broth + 1 g l^{-1} Tween 80). In both media, at 30 °C, a significant cell count reduction (5 log) of *L. monocytogenes* occurred only when the cell concentration of the bacteriocinogenic competitor reached the level of at least 10^7 CFU ml^{-1} required for the production of sufficient concentration of nisin-like bacteriocin. The same phenomenon was also observed when the initial level of the lactic acid bacteria (LAB) was one log higher or lower than that of the *Listeria*, however, the reduction of *Listeria* cell count occurred earlier with the higher initial concentration of the LAB.

Incubation of the mixed cultures at 20 °C gave similar results but the bacteriocinogenic activity resulted in only a three log decline of the cell count of *L. monocytogenes*.

At 10 °C *Lactococcus lactis* produced much less bacteriocin than at 30 °C, therefore, a drastic decrease of the *Listeria* cell count was not observed. Suppression of the *Listeria* growth was expressed in its decreased maximum population level (i.e. in an earlier appearance of the stationary phase). When the non-bacteriocinogenic *Lac. lactis* and *Listeria* were present at the same initial level (approx. 10^5 CFU ml^{-1}), the *Lactococcus* did not affect the growth of *L. monocytogenes* at 30 °C in modified ST I broth.

Keywords: interaction, *Lactococcus lactis, Listeria monocytogenes*

In recent years, interest in the role of microbial competition in assuring food safety has increased. A number of studies have reported that growth of *Listeria monocytogenes* is suppressed in the presence of high levels of lactic acid bacteria (HARRIS et al., 1989; BUCHANAN & BAGI, 1997; NILSSON et al., 1999). Antagonistic effects of lactic acid bacteria have often been attributed to the production of acids (SCHILLINGER & LÜCKE, 1989) or hydrogen peroxide (TAGG et al., 1976; GILLILAND & SPECK, 1977) although others have shown that the inhibition is due to the production of

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antimicrobial substances called bacteriocins (KLAENHAMMER, 1988). A variety of bacteriocin-producing strains of lactic acid bacteria occurring commonly in foods have been identified and are being evaluated as potential means for controlling foodborne pathogens such as *Listeria* (BUCHANAN & BAGI, 1997). One of them is *Lactococcus lactis,* which produces nisin. Nisin has bactericidal activity against a broad range of Gram-positive microorganisms (SPELHAUG & HARLANDER, 1989; DELVES-BROUGTHON, 1990). Because of their antilisterial activity, nisin producer strains of lactococci may be suitable candidates as protective cultures to control *Listeria* in food systems. For effective commercial application their nisin production must be optimalized. The non-ionic surfactant Tween 80 seems to have an effect on bacteriocin synthesis by *Lactococcus* spp. HUOT and co-workers (1996) suggested that this surfactant is of importance since the bacteriocin activity sharply increased when the culture medium was supplemented with Tween 80. Furthermore, JUNG and co-workers (1992) reported that the addition of Tween 80 has been shown to increase significantly the activity of nisin against *L. monocytogenes* in milk regardless of fat content.

The aim of present work was to study the competition of a bacteriocinogenic and a non-bacteriocinogenic *Lactococcus lactis* strain, respectively, with a *Listeria monocytogenes* strain in two semi-synthetic liquid media at various temperatures. The effect of the non-ionic detergent Tween 80 on bacteriocin activity during the co-culture studies was investigated. The role of inoculum levels of the competing strains in the suppression of growth of *L. monocytogenes* was also studied.

1. Materials and methods

1.1. Microorganisms

Lactococcus lactis BFE 920 (bact+) (Federal Research Centre for Nutrition, Karlsruhe, Germany) a bacteriocinogenic strain, *Lactococcus lactis* DSM 20384 (bact-), a non-bacteriocinogenic strain (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) and *Listeria monocytogenes* SLCC 2540 (serotype 3b) (Federal Centre for Meat Research, Kulmbach, Germany) were used in the experiments. Stock cultures of *Lactococcus* strains and *Listeria monocytogenes* were kept in MRS broth (Merck, Germany) and in ST I broth (Merck, Germany) with 15% glycerol at –18 °C.

1.2. Inoculum

Before the initiation of a co-culture trial, 100 µl of the stock cultures was inoculated into 9 ml of medium. Lactic acid bacteria were grown in MRS broth at 30 °C and *Listeria monocytogenes* in ST-I broth at 37 °C for 24 h. After 24 h 100 µl of these

cultures were inoculated into MRS and ST I broth. Tubes were incubated at the same temperature mentioned before. These 24-h cultures, which composed of stationary phase cells, were used for inoculation.

1.3. Media

The media used for the competitive studies were Standard I broth (Merck, Germany) and modified Standard I broth. The latter was prepared from Standard I broth supplemented with 1 g l^{-1} Tween 80. The broth was divided into 100 ml portions in 250 ml screw capped Duran flasks and sterilised after closure by autoclaving for 20 min at 121 °C. The pH of the broth was not adjusted. It was approx. 7.5.

1.4. Competitive growth studies

The Duran flasks containing 100 ml of ST-I broth or modified ST-I broth were inoculated with 1 ml of appropriate dilutions of the 24-h cultures of *Lac. lactis* and/or *L. monocytogenes* to achieve the following initial viable cell counts:

– *Lac. lactis* (bact+): 10^5 CFU ml^{-1}
– *L. monocytogenes*: 10^5 CFU ml^{-1}
– *Lac. lactis* (bact+): 10^5 CFU ml^{-1} + *L. monocytogenes*: 10^5 CFU ml^{-1}
– *Lac. lactis* (bact+): 10^5 CFU ml^{-1} + *L. monocytogenes*: 10^6 CFU ml^{-1}
– *Lac. lactis* (bact+): 10^6 CFU ml^{-1} + *L. monocytogenes*: 10^5 CFU ml^{-1}
– *Lac. lactis* (bact-): 10^5 CFU ml^{-1}
– *Lac. lactis* (bact-): 10^5 CFU ml^{-1} + *L. monocytogenes*: 10^5 CFU ml^{-1}

The co-cultures and the monocultures were incubated at 30 °C, 20 °C and 10 °C, respectively, for various time periods.

Periodically, 1 ml sample of each co-culture and each monoculture were removed, diluted as needed in sterile 0.1% peptone water. Bacterial counts were determined by selective spread-plating on MRS agar (Merck 10660) for *Lactococcus lactis*, and on Standard I agar (Merck 7881) and Palcam agar (Merck 11755) for *Listeria monocytogenes* and bacteria were incubated at 30 °C and 37 °C for 24–48 h, respectively.

1.5. Determination of spectrum of antagonistic activity

Cell-free neutralised supernatant (CFNS) was used to determine the spectrum of antagonistic activity. For preparation of CFNS , bacteriocinogenic *Lac. lactis* was grown in MRS broth for 24 h at 30 °C. The culture was centrifuged using an Eppendorf-centrifuge (Biofuge 13, Heraeus Sepatech) at 10 000 r.p.m for 10 min, supernatant was adjusted to pH 6.5–7 using 1 M NaOH and heated at 100 °C for 5 min to inactivate remaining cells. The spectrum of antagonistic activity was tested against a wide range of

lactic acid bacteria (LAB) as well as foodborne pathoghens (Table 1) by spotting CFNS onto soft agar beds with indicator microorganism (ca. 10^8 CFU ml^{-1}) according to previously described methods (SCHILLINGER et al., 1993). Antagonistic activity was manifested when a clear inhibition zone was observed after 24 h incubation on the indicator lawn.

1.6. Determination of bacteriocin activity

Bacteriocin activity assays were performed by the critical dilution method as described previously by SCHILLINGER and LÜCKE (1989). Cell-free supernatant of *Lac. lactis* was used to determine the bacteriocin activity against *L. monocytogenes*. Bacteriocinogenic *Lac. lactis* was grown in a pure culture and in a mixed culture with *L. monocytogenes* at different temperatures in two semi-synthetic media (ST-I or modified ST-I). At certain time intervals 1 ml samples were withdrawn, centrifuged at 10 000 r.p.m. for 10 min. The supernatants were transferred to a new tube and heated at 100 °C for 5 min to inactivate remaining cells. Ten µl-volumes of two-fold serial dilutions of the supernatants were spotted onto the surface of a Standard I agar plate overlaid with 7 ml of Standard I soft agar (0.7%) which had been inoculated with 0.2 ml of a 24-h culture of *L. monocytogenes*. The plates were incubated at 30 °C for 24 h. Bacteriocin activity in arbitrary activity units (AU) was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the indicator lawn, and was multiplied by a factor of 100 to obtain the AU ml^{-1} of the original sample. Unless stated otherwise, *Listeria monocytogenes* SLCC 2540 was used as indicator strain.

2. Results and discussion

The bacteriocinogenic *Lactococcus lactis* strain used in this work was isolated from mungbean by FRANZ and co-workers (1997). They suggested that *Lac. lactis* BFE 920 (bact+) produced a nisin-like bacteriocin. According to their finding it was active against a wide range of bacteria, including several lactic acid bacteria and some relevant foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*.

Our preliminary studies gave similar results (Table 1). Furthermore, we realised that from all of the tested *Listeria* spp. *Listeria monocytogenes* SLCC 2540 proved to be the most sensitive strain to the nisin-like bacteriocin in the bacteriocin activity assay (data are not shown). Thus, *Listeria monocytogenes* SLCC 2540 was used during the competitive growth studies.

Table 1

Inhibitory spectrum of cell free neutralised culture supernatants (CFNS) of bacteriocin producing Lactococcus lactis *BFE 920*

Indicator	Sensitivity to CFNS of *L. lactis* BFE 920
Lactobacillus sake DSM 20017	+
Enterococcus faecium DSM 20477	+
Pediococcus acidilactici DSM 20333	+
Streptococcus mutans DSM 6178	–
Salmonella enteritidis 5271	–
Bacillus cereus CCM 2010	(+)
Escherichia coli DSM 116	–
Staphylococcus aureus ST 11	–
Staphylococcus aureus ST 13	+
Staphylococcus aureus ATCC 14458	+
Staphylococcus aureus DSM 1104	–
Listeria monocytogenes SLCC 2540	+
Listeria monocytogenes DSM 20600	+
Listeria monocytogenes WS 2251	+
Listeria monocytogenes Scott A	+
Listeria ivanovii WS 2254	+
Listeria seeligeri WS 2253	+
Listeria welshimeri WS 2254	+

– no sensitivity; + sensitivity; (+) small sensitivity

The results of the study with pure cultures in two semi-synthetic media showed that the growth of the bacteriocinogenic *Lac. lactis* and *L. monocytogenes* in their monocultures did not differ significantly in ST-I and modified ST-I broth at 30 °C. However, when *Lac. lactis* (bact+) was grown in ST-I broth bacteriocin activity was not detectable against *L. monocytogenes* (Figs 1, 2).

The competition studies demonstrated that the growth of *Lac. lactis* (bact+) and *L.monocytogenes* in a co-culture was similar to the aforementioned two growth media, when the initial cell numbers of the two bacteria were about equal (Figs 3a,b-4a,b). In both media, the inactivation of *L. monocytogenes* occurred only when the cell concentration of the bacteriocinogenic competitor reached the level of at least 10^7 CFU ml^{-1}. Although bacteriocin activity was not detectable against *L. monocytogenes* when the co-culture was inoculated into ST I broth, the decrease of the viable cell count of *Listeria* seemed to follow the same manner as in modified ST I broth.

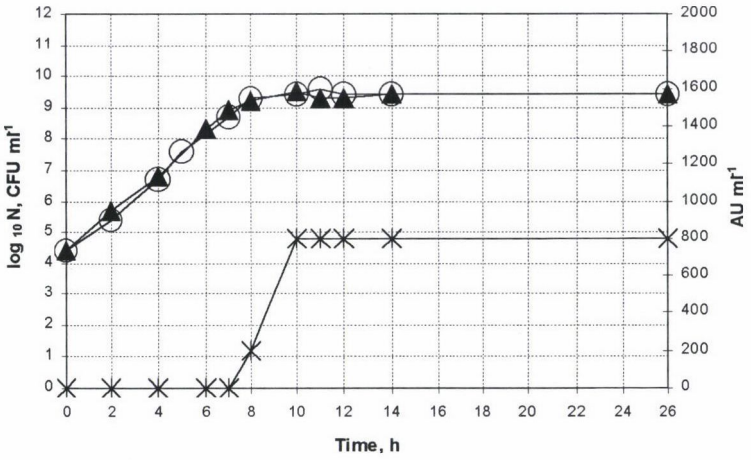

Fig. 1. Growth and bacteriocin production of *Lactococcus lactis* (bact+) in ST-I broth and in modified ST-I broth at 30 °C. s: *Lac. lactis* (bact+) ST-I; ○: *Lac. lactis* (bact+) modified ST-I; s: bacteriocin activity modified ST-I

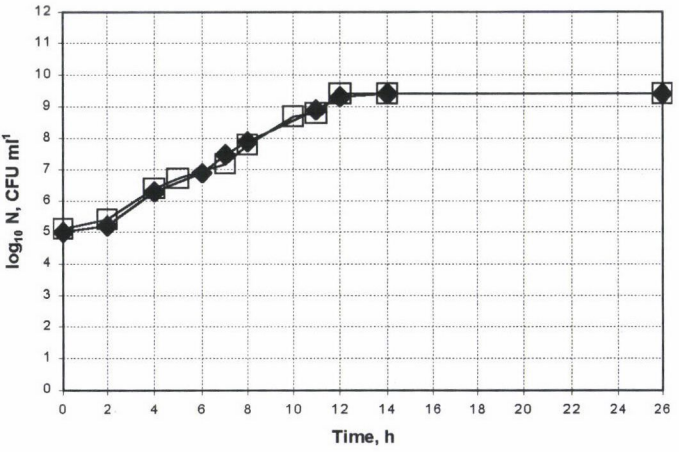

Fig. 2. Growth of *Listeria monocytogenes* in ST-I broth and in modified ST-I broth at 30 °C. u: *L. monocytogenes* ST-I; □: *L. monocytogenes* modified ST-I

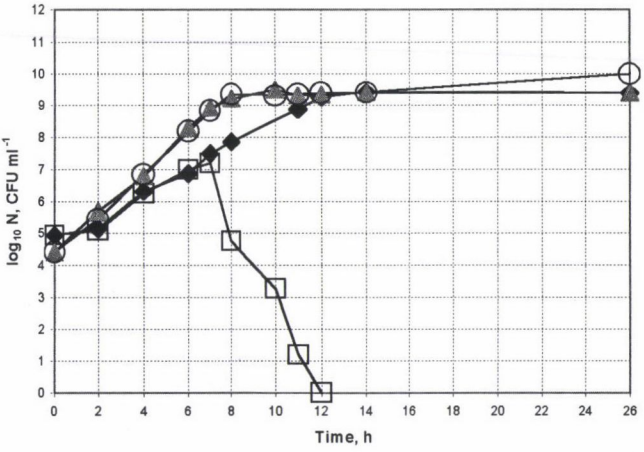

Fig. 3a. Growth of *Listeria monocytogenes* in the presence of the bacteriocinogenic *Lactococcus lactis* (bact+) strain in ST-I broth. Culture conditions, 30 °C, pH 7.5. u: *L. monocytogenes*; □: *L. monocytogenes* (co-culture); s: *Lac. lactis* (bact+); ○: *Lac. lactis* (bact+) (co-culture)

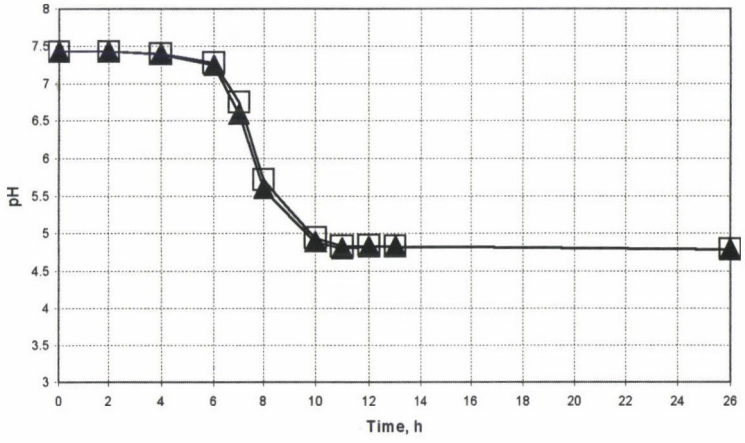

Fig. 3b. pH-drop in the monoculture and in the co-culture. □: *Lac. lactis* (bact+) (pH); s: co-culture (pH)

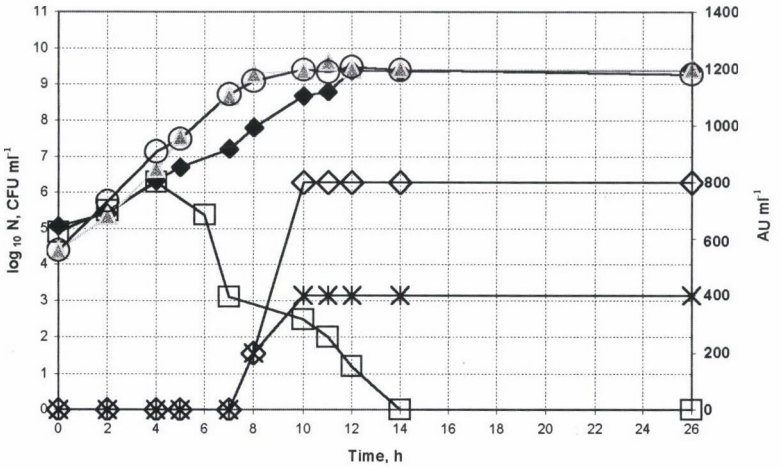

Fig. 4a. Effect of *Lactococcus lactis* (bact+) on the growth of *Listeria monocytogenes* in modified ST-I broth. Bacteriocin production of *Lactococcus lactis* BFE 920. Culture conditions, 30 °C, pH 7.5. u: *L. monocytogenes*; s: *Lac. lactis* (bact+); ◊: bacteriocin activity (monoculture); □: *L. monocytogenes* (co-culture); ○: *Lac. lactis* (bact+) (co-culture); S: bacteriocin activity (co-culture)

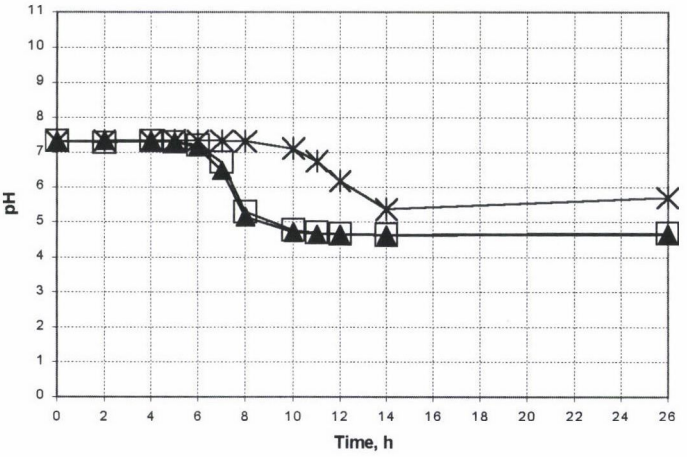

Fig. 4b. pH-drop in the monoculture and in the co-culture. □: *Lac. lactis* (bact+) (pH); s: *Lac. lactis* (bact+) (co-culture) (pH); S: *L. monocytogenes* (pH)

When the non-bacteriocinogenic *Lac. lactis* and *Listeria* as competitors were present in a co-culture in the same initial level (approx. 10^5 CFU ml^{-1}), the *Lactococcus* did not affect the growth of *L. monocytogenes* at 30 °C in modified ST I broth. This is shown in Figs 5a, b.

From the competition studies with different initial cell numbers of the bacteriocinogenic strain and *L. monocytogenes* at 30 °C, 20 °C and 10 °C in the modified medium the following conclusions can be drawn: The two bacteria grew in a mixed culture in the same way as their monocultures at 30 °C, until nisin-like bacteriocin reached the detectable concentration, when a five log decrease of the viable count of *L. monocytogenes* occurred. The same phenomenon was also observed when the initial level of the lactic acid bacteria was one log higher or lower than that of the *Listeria*. Differences were noticed only between the time periods needed for the bacteriocinogenic competitor to reach the cell concentration required for the sufficient bacteriocin production needed for the *Listeria* inactivation. When the *Lac. lactis* initial cell number was higher or equal to its competitor the reduction of *Listeria* cell number was observed earlier than by lower initial LAB concentration (Figs 6a, b, c, d).

Incubation of the mixed cultures at 20 °C gave similar results but the bacteriocinogenic activity resulted in only a three log decline of the cell count of *L. monocytogenes*. Furthermore, the suppression of the pathogen was induced much later at this temperature than at 30 °C (Figs 7a, b, c, d).

At 10 °C *Lactococcus lactis* produced much less bacteriocin than at 30 °C, therefore, a drastic decrease of the *Listeria* cell count was not observed. Suppression of the *Listeria* growth was expressed in its decreased maximum population level (i.e. in an earlier appearance of the stationary phase). The results of the co-culture study at 10 °C are shown in Figs 8a, b, c, d.

No significant difference could be observed at each temperature between the bacteriocin production of *Lactococcus lactis* in co-cultures with *Listeria* as compared to its monoculture. However, at lower temperature, *Lac. lactis* produced much less nisin both in the monoculture and the mixed culture.

The results of this study showed that Tween 80 did not affect the growth of *Lactococcus lactis* and *Listeria monocytogenes* in their monocultures as well as in their co-cultures, however, in the present work it is supposed that the addition of Tween 80 to the culture media influenced the detection of bacteriocin production beneficially.

The role of Tween 80 in bacteriocin production is not totally clear yet. GRAVER and MURIANA (1994) noted that attempts to produce curvaticin FS47 in MRS broth without Tween 80 were unsuccessful, and although *Lactobacillus curvatus* FS47 grew luxuriously, bacteriocin activity was not detectable. HUOT and co-workers (1996) reported that at pH 5.5, and in the presence of 1% (v/v) Tween 80, bacteriocin

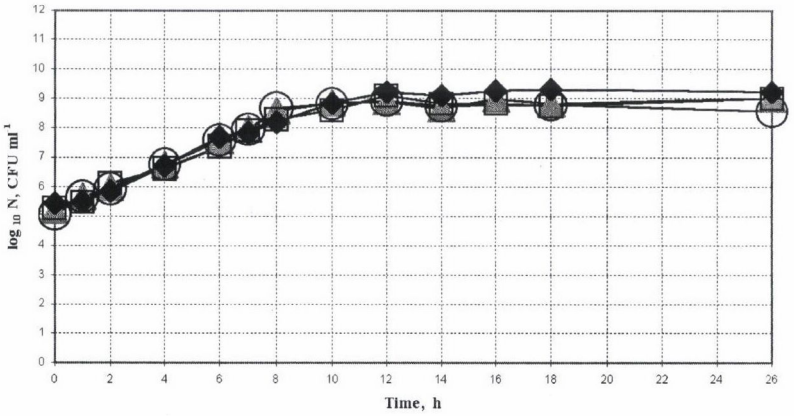

Fig. 5a. Effect of the non-bacteriocinogenic *Lactococcus lactis* (bact-) strain on the growth of *Listeria monocytogenes* in modified ST-I broth. Culture conditions, 30 °C, pH 7.5. s: *Lac. lactis* (bact-); O: *Lac. lactis* (bact-)(co-culture); u: *L. monocytogenes*; □: *L. monocytogenes* (co-culture)

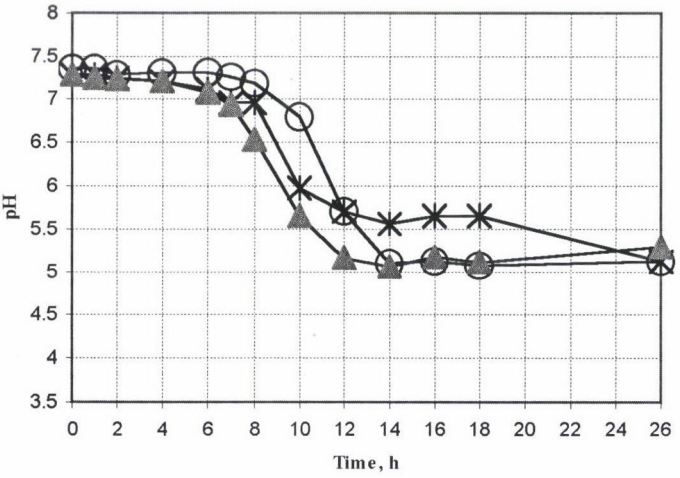

Fig. 5b. pH-drop in the monoculture and in the co-culture. S: *Lac. lactis* (bact-) (pH); O: *L. monocytogenes* (pH); s: co-culture (pH)

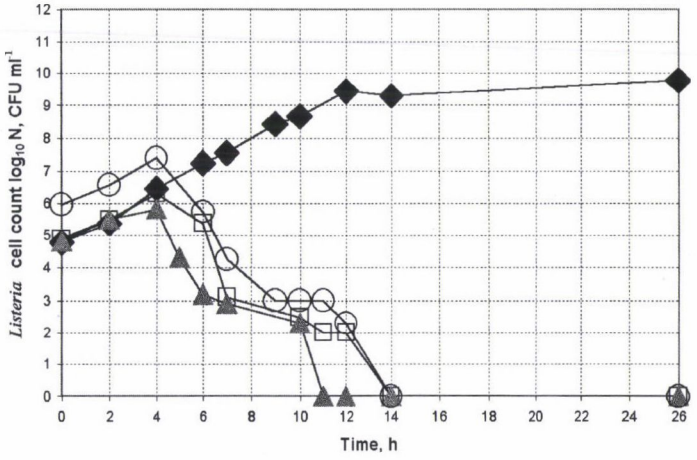

Fig. 6a. Effect of the initial cell number of the competing strains (the bacteriocinogenic *Lac. lactis* (bact+) and *L. monocytogenes*) in the inactivation of *Listeria monocytogenes* in modified ST I broth. Culture conditions, 30 °C, pH 7.5. u: *L. monocytogenes;* □: *L. monocytogenes : Lac. lactis* (bact+) (1:1); s: *L. monocytogenes : Lac. lactis* (bact+) (1:10); ○: *L. monocytogenes : Lac. lactis* (bact+) (10:1)

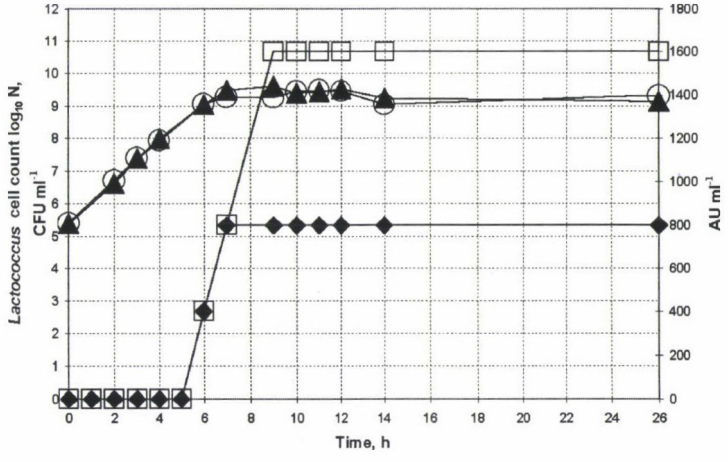

Fig. 6b. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (1:1) in modified ST I broth. Culture conditions, 30 °C, pH 7.5. s: *Lac. lactis* (bact+); ○: *Lac. lactis* (bact+) (1:1); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (1:1)

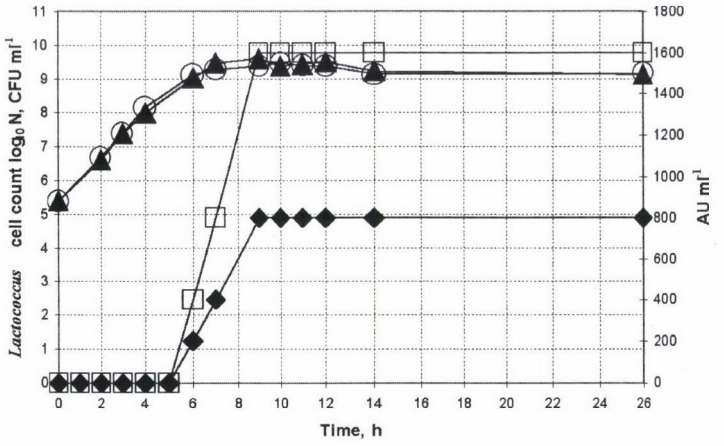

Fig. 6c. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (10:1) in modified ST I broth. Culture conditions, 30 °C, pH 7.5. s: *Lac. lactis* (bact+); O: *Lac. lactis* (bact+) : *L. monocytogenes* (1:10); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (1:10)

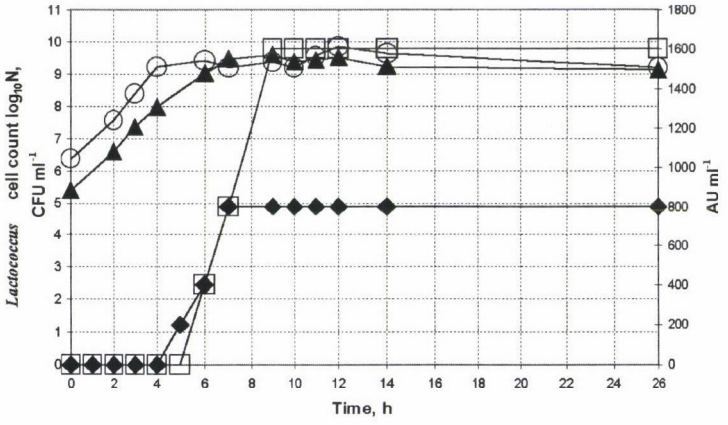

Fig. 6d. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (10:1) in modified ST I broth. Culture conditions, 30 °C, pH 7.5. s: *Lac. lactis* (bact+); O: *Lac. lactis* (bact+): *L. monocytogenes* (10:1); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (10:1)

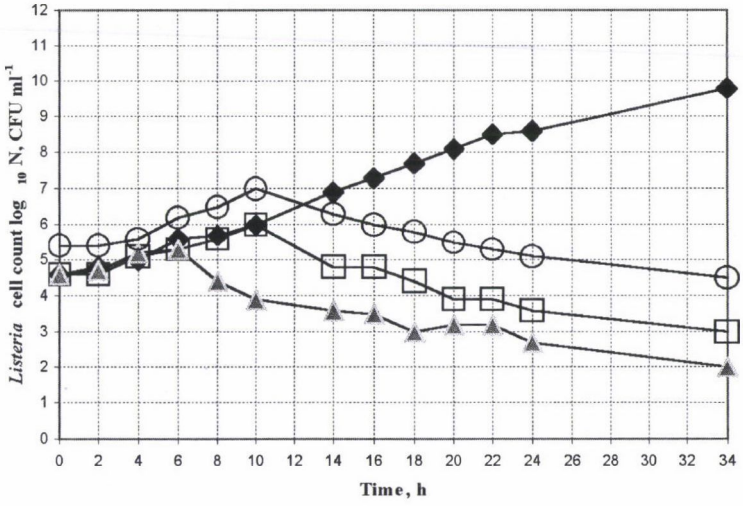

Fig. 7a. Effect of the initial cell number of the competing strains (the bacteriocinogenic *Lac. lactis* (bact+) and *L. monocytogenes*) in the suppression of growth of *Listeria monocytogenes* in modified ST I broth. Culture conditions, 20 °C, pH 7.5. u: *Listeria monocytogenes;* □: *L. monocytogenes : Lac. lactis* (bact+) (1:1); s: *L. monocytogenes : Lac. lactis* (bact+) (1:10); ○: *L. monocytogenes : Lac. lactis* (bact+) (10:1)

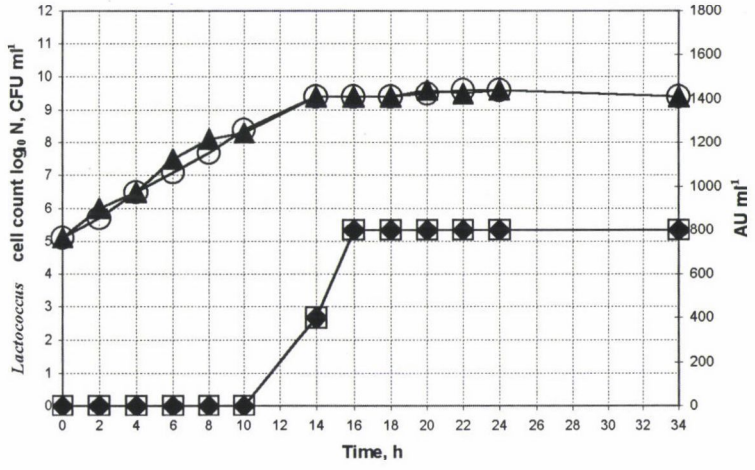

Fig. 7b. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (1:1) in modified ST I broth. Culture conditions, 20 °C, pH 7.5. s: *Lac. lactis* (bact+); ○: *Lac. lactis* (bact+) : *L. monocytogenes* (1:1); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (co-culture) (1:1)

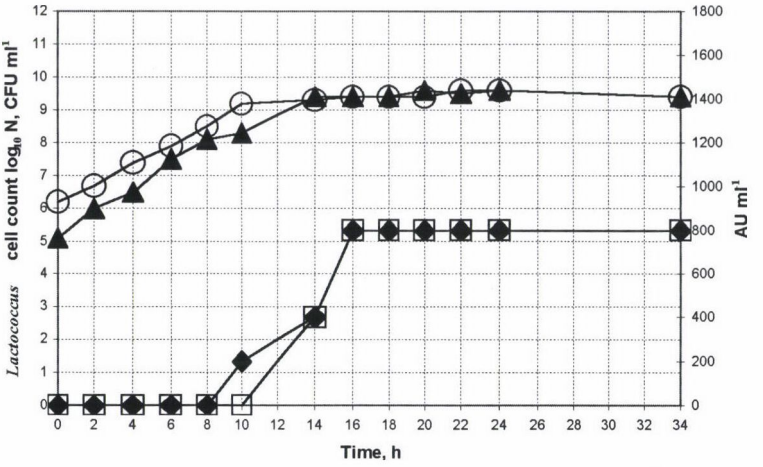

Fig. 7c. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (10:1) in modified ST I broth. Culture conditions, 20 °C, pH 7.5. s: *Lac. lactis* (bact+); O: *Lac. lactis* (bact+) : *L. monocytogenes* (10:1); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (co-culture) (10:1)

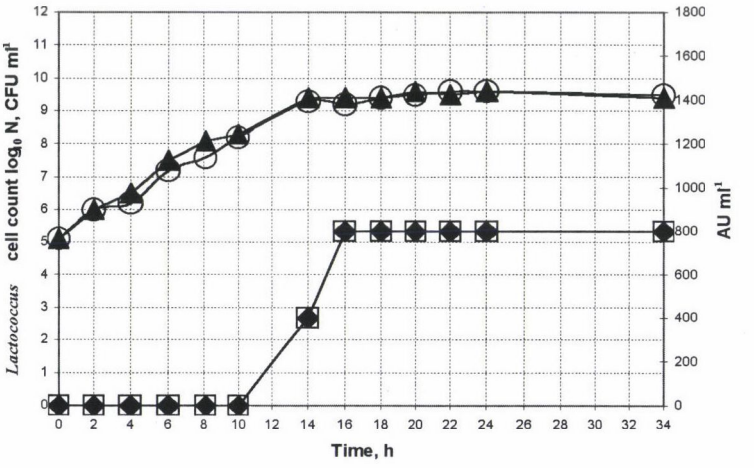

Fig. 7d. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (1:10) in modified ST I broth. Culture conditions, 20 °C, pH 7.5. s: *Lac. lactis* (bact+); O: *Lac. lactis* (bact+) : *L. monocytogenes* (1:10); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (co-culture) (1:10)

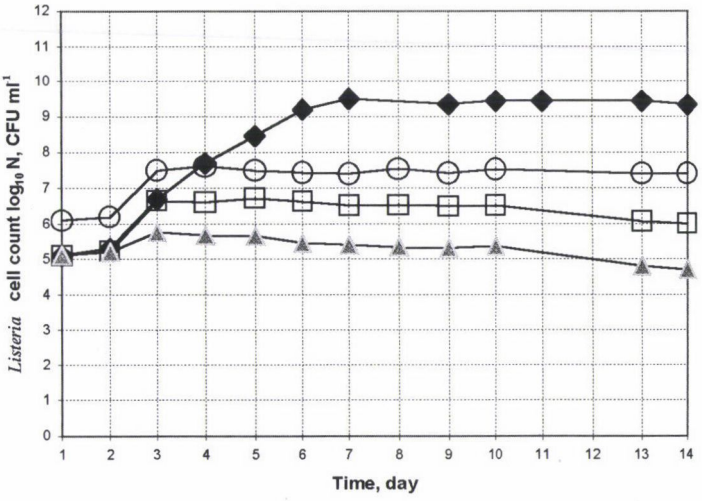

Fig. 8a. Effect of the initial cell number of the competing strains (the bacteriocinogenic *Lac. lactis* (bact+) and *L. monocytogenes*) in the suppression of growth of *Listeria monocytogenes* in modified ST I broth. Culture conditions, 10 °C, pH 7.5. u: *L. monocytogenes;* □: *L. monocytogenes : Lac. lactis* (bact+) (1:1); s: *L. monocytogenes : Lac. lactis* (bact+) (1:10); ○: *L. monocytogenes : Lac. lactis* (bact+) (10:1)

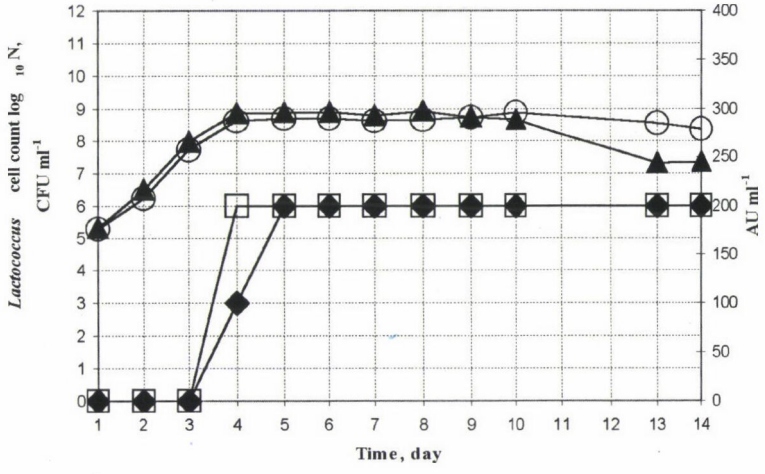

Fig. 8b. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (1:1) in modified ST I broth. Culture conditions, 10 °C, pH 7.5. s: *Lac. lactis* (bact+); ○: *Lac. lactis* (bact+) : *L. monocytogenes* (1:1); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (co-culture) (1:1)

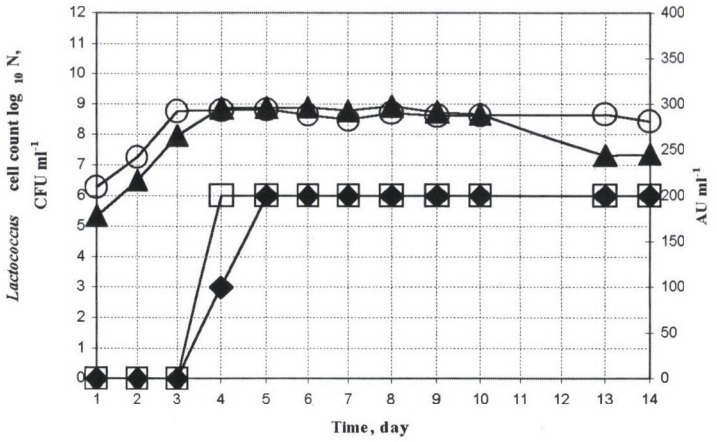

Fig. 8c. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (10:1) in modified ST I broth. Culture conditions, 10 °C, pH 7.5. s: *Lac. lactis* (bact+); O: *Lac. lactis* (bact+) : *L. monoculture* (10:1); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (co-culture) (10:1)

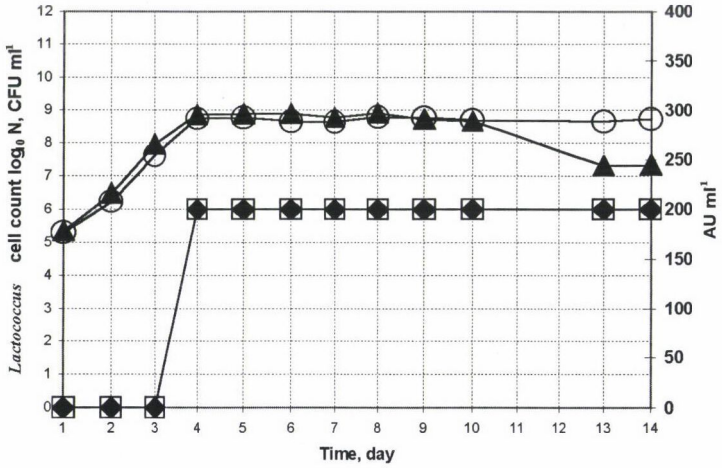

Fig. 8d. Growth and bacteriocin production of *Lactococcus lactis* in its monoculture and in a co-culture with *Listeria monocytogenes* (1:10) in modified ST I broth. Culture conditions, 10 °C, pH 7.5. s: *Lac. lactis* (bact+); O: *Lac. lactis* (bact+) : *L. monocytogenes* (1:10); □: bacteriocin activity of *Lac. lactis* (bact+); u: bacteriocin activity of *Lac. lactis* (bact+) (co-culture) (1:10)

production by *Lac. cremoris* was about fourfold higher than in its absence. However, further increase in Tween 80 concentration did not result in a significant modification of the bacteriocin titre. NIESSEN-MEYER and co-workers (1992) showed that the addition of this surfactant to the microplate wells in the bacteriocin activity tests increased the sensitivity of the assays 2- to 10-fold. The effect of Tween 80 may be due to a reduction of the binding of bacteriocin to the plastic microplate wells. On the other hand, when Tween was added to the full grown *Lactococcus* culture of a 0% Tween broth, no stimulation of the antibacterial activity was observed, whatever the concentration added in range 0–3% (HUOT et al., 1996).

Since the reduction of *Listeria* cell number in a co-culture with *Lac. lactis* (bact+) in ST-I broth and modified ST-I broth succeeded in similar ways at 30 °C, it is supposed that the bacteriocinogenic *Lac. lactis* also produced nisin in ST I broth, although this activity was not detectable by the critical dilution method. This hypothesis seems to confirm the fact that the non-bacteriocinogenic *Lac. lactis* did not suppress the growth of *Listeria* in a co-culture of the two bacteria in modified ST-I broth at 30 °C. Furthermore, one can conclude from this latter observation that the considerable decline of the viable cell counts of *L. monocytogenes* found in the presence of the bacteriocinogenic *Lac. lactis* was not caused alone by lactic acid production by the lactic acid bacteria. Inhibition of the target organism can be explained by (i) nutrient depletion (BUCHANAN & BAGI, 1997), (ii) excretion of antilisterial compounds in laboratory broth at concentrations below the detection level when assayed in an agar diffusion test of antagonism (SCHILLINGER & HOLZAPFEL, 1990) or (iii) excretion of antilisterial compounds, such as bacteriocins (HARRIS et al., 1989). Results of the present study with bacteriocinogenic and non-bacteriocinogenic lactococci indicated that the antilisterial effect was primarily due to production of nisin-like bacteriocin. However, nutrient depletion and production of other antilisterial compounds could also contribute to the *Listeria* inactivation. BUCHANAN and BAGI (1997), who have investigated the interaction between *L. monocytogenes* and antilisterial strains of *C. piscicola*, found that suppression of *Listeria* was not always attributed to the production of antilisterial compounds but could be partially due to nutrient depletion. DEGNAN and co-workers (1992) suggested that the inhibitory effect of *Pediococcus acidilactici* LB42 against *L. monocytogenes* was caused by competitive antagonism.

The results of the competitive studies at different temperatures in the modified medium indicate that the production of the bacteriocin is regulated depending on environmental conditions. GEISEN and co-workers (1993) reported that bacteriocin production of *Leuconostoc carnosum* LA54A showed no obvious correlation between the cell number of the culture and bacteriocin production. Furthermore, cultures with slow growth produced more than one order of magnitude less bacteriocin, than cultures with higher growth rates, despite the fact that maximum cell numbers are reached after

extended incubation. This tendency was also observed in the case of *Lactoccoccus lactis*, however the maximum population density reached by the strain was also a little bit less at 10 °C than at 30 °C (Table 2) In spite of this observation the bacteriocin production of *Lac. lactis* seems to be tightly coupled to the growth rate.

Table 2

Comparison of the determined specific growth rate of Lactococcus lactis *BFE 920 to maximum population density (MPD) attained by its monoculture and to the maximum amount of bacteriocin (MAB) produced at different temperatures in modified ST I broth at a pH level 7.5*

	MPD Log_{10} CFU ml^{-1}	MAB AU ml^{-1}	Growth rate (μ) 1/h
30 °C	9.6	1600	1.61
20 °C	9.6	800	0.63
10 °C	8.92	200	0.12

3. Conclusions

Summarised, the results of the current study suggest that the bacteriocinogenic *Lactococcus lactis* BFE 920 is able to inactivate *Listeria monocytogenes* under environmental conditions that are beneficial for its bacteriocin production. The effect of Tween 80 on the bacteriocin production is not clear yet. It will require additional research, however supplementation of the culture media with this surfactant proved to enhance the detectable bacteriocin activity. At low temperature the bacteriocinogenic strain grew slowly and the low level of the produced nisin-like bacteriocin was not able to inhibit the growth of *Listeria monocytogenes* sufficiently. Since this pathogen microbe is commonly associated with refrigerated food products, it should be considered that *Lactococcus lactis* BFE 920 might not be able to control alone the growth of *Listeria* at low temperature. Thus additional controlling factors have to be used under those conditions.

*

The experimental work was performed in the Federal Research Centre for Nutrition, Karlsruhe, Germany during a Ph.D. internship of the author. The esteemed cooperation of the Host Institution is highly appreciated. The author thanks the professional help during the study to Ms. B. BECKER and Prof. W. H. HOLZAPFEL and the critical review of this paper, to Prof. J. FARKAS.

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Acta Alimentaria, Vol. 30 (3), pp. 267–279 (2001)

FUTURE TRENDS IN FOOD TECHNOLOGY – NOVEL FOOD AND TRANSGENIC FOOD*

A review

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(Received: 6 October 2000; accepted: 26 March 2001)

The paper reviews briefly the present status and future trends of some new, emerging food preservation technologies and anticipates the major lines of developments in relation to functional and transgenic foods. These topics of innovative food technologies and food products are of particular importance in view of the European Union's regulation concerning "novel foods and novel ingredients". The author considers the obstacles and the needs for more research and other actions such as safety assessment and balanced communication to the public.

Keywords: food preservation, functional food, transgenic food

"Securing Food in Global Dimension" was rightly one of the mottos in the German Pavilion of the present EXPO 2000 at a time of explosively growing world population, which demands world-wide efforts to overcome problems related to the food supply. It is a peculiar contradiction that the centre of rapid population increase is in developing countries while food technologies bring forward newer opportunities in developed countries where food is abundant. Most developing countries are not able to undertake effective research and development of food technologies without the scientific and financial support of industrial countries. However, it is not only the bare quantity of food that counts. This is shown also by the first RTD co-operation programme of the EU's 5th Framework Programme, which is devoted to the problems related to "Quality of Life and Management of Living Resources", and the first key action of this programme is "Food, Nutrition and Health". "Health related quality of

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* This paper was presented at the Symposium on "Nutrition and Food Safety", Hannover, 12 August 2000, within the framework of World Congress on Medicine and Health: "Medicine Meets Millennium".

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0139–3006/2001/$ 5.00

life" is the perceived impact of health on a person's life. That concept evolved from the WHO's constitution defining health as a state of complete physical, mental and social well being and not merely the absence of disease. Safe food must be interesting, diversified and nutritionally balanced. Thus, not only is the EU programme in harmony with the agenda of the Symposium on "Nutrition and Food Safety" held in Hannover, 12 August 2000, in the frame of the World Congress on Medicine and Health: "Medicine Meets Millennium", but the organizers of that meeting recognized the significance of the three interrelated main research fields expressed by the above key action as well. These form one of the major areas where implementation of scientific knowledge obtained by RTD co-operation can best serve society's needs and the quality of life of not only the European consumer but of mankind in general. The above needs and the necessity for new and integrated concepts of food product innovation were also justly reflected by convening an international conference held this year in Porto under the title "Nutritionists Meet Food Scientists and Technologists", and have been addressed in the programme of the 5th Karslruhe Nutrition Symposium held October 2000.

As a food technologist, I should try – within the space limits for presentation – to restrict myself to the topics of

- some new food technologies which respond to the needs of our epoch, and
- foods with improved nutritional value.

Table 1

Article 1 of Regulation (EC) No. 258/97 of the EUROPEAN PARLIAMENT and of THE COUNCIL of 27 January 1997 concerning novel foods and novel food ingredients

Art. 1.1. "This Regulation concerns the placing on the market within the Community of novel foods or novel food ingredients.

Art. 1.2. "This Regulation shall apply to the placing on the market within the Community of foods and food ingredients which have not hitherto been used for human consumption to a significant degree with the Community and which fall under the following categories:

(a) foods and food ingredients containing or consisting of genetically modified organisms within the meaning of Directive 90/220/EEC;

(b) foods and food ingredients produced from, but not containing, genetically modified organisms;

(c) foods and food ingredients with a new or intentionally modified primary molecular structure;

(d) foods and food ingredients consisting of or isolated from micro-organisms, fungi and algae;

(e) foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for food and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use;

(f) foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances."

In the latter context, I can't avoid to touch upon questions of the presently highly controversial topic of transgenic foods or food components. These topics of innovative food technologies and food products are of particular importance in view of the European Union's Regulation of 1997 concerning "novel foods and novel ingredients" (EC, 1997). The Regulation covers all foods and food ingredients that "have not hitherto been used for human consumption to a significant degree within the Community". The scope of this Regulation covers biological, chemical and technological innovations as quoted in detail in Table 1.

New, emerging food preservation technologies

Several well-established traditional processing options are available for the preservation of food. The most widely used among them, thermal processing, provides a high degree of microbial safety. It tends, however, to degrade the quality of foods to some extent. Freezing and distribution of frozen foods is an important technology which retains very well the nutritive quality, but changes the physical state and consumes a great amount of energy.

The present is a time of rapid change in the field of food technologies and the pace of change is increasing. Major motivations determining trends of development of new, emerging or future food technologies are those which signify responses of food science and industry to

- demands of consumers due to their changing lifestyles and expectations for
  - fresher, more natural foods, which
    - are less severely processed (less heat- or freeze-damaged),
    - contain less preservatives, or are even free from "artificial" additives;
  - nutritionally more advantageous food (e.g. containing less salt, less sugar or fat);
  - safer food (posing no microbiological or chemical health hazards); and at the same time are
  - foods convenient to handle ([semi]prepared or ready for consumption and with a sufficient shelf-life);
- needs for less energy requirement of processing;
- the necessity for lower impact on the environment.

These – to some extent conflicting – requirements motivated the introduction of less severe or "minimal processing" technologies such as controlled atmosphere storage of fruits and vegetables, and modified atmosphere packaging of foods, or the development of extended shelf-life refrigerated foods such as "sous-vide" cooked products. These requirements have also resulted in growing interest in new, "non-thermal" methods of food preservation such as

- using new "biopreservatives" or new "protective cultures" utilizing their antagonism against pathogenic microorganisms;
  or
- new "physical" technologies:
  - ionising radiation treatment;
  - high hydrostatic pressure treatment;
  - high voltage electric field pulses

to inactivate pathogenic and spoilage microorganisms. (Other new physical methods of antimicrobial treatment such as high intensity light pulses, "manothermosonication" (combination of pressure, heat and ultrasound), or treatment with oscillating magnetic fields (FARKAS, 1997; GOULD, 2000) are either of more limited scope, or they are not yet sufficiently ready scientifically or technically for implementation).

Application of the technologies listed above offers various opportunities for mildly processed products by preserving their sensory quality, nutritional value and appearance. However, the application potential of any new technologies, which are coming from research laboratories and not "sanctioned" by centuries of empirical use, is influenced by many factors:

- technological feasibility;
- technical possibility;
- health impact
  - wholesomeness of the product;
  - occupational safety;
- environmental friendliness;
- economic feasibility (including their energy demand);
- infrastructural conditions/requirements;
- investment need and availability of investment power;
- political attitude;
- social consequences;
- psychological aspects/ risk-benefit perception.

Whereas the first four aspects listed can be scientifically studied, and clear-cut general answers can be given according to the status of science and technology of a given epoch, the other factors are very much interrelated and depend on local conditions.

A brief sketch of the state of the art of the new/emerging technologies can be given as follows:

"New" natural antimicrobial substances, particularly the use of bacteriocins produced by some lactic acid bacteria seem to gain a role in eliminating the risk of specific microorganisms in some foods (ABEE et al., 1995; KNORR, 1998). Some strains of lactic acid bacteria are considered as protective cultures, if they can be inoculated

into certain foods, such as vacuum-packaged processed meats, and assert an inhibitory effect on pathogens during storage at abuse temperature, while having negligible effect on the sensory quality of the products (BREDHOLDT et al., 1999). Because of associated flavours that can alter the taste of food, future uses of *plant-derived antimicrobials* as food preservatives are not likely on their own, but as part of a preservation system (CHERRY, 1999).

Ionising radiation is a versatile form of processing energy used already in a wide range of non-food applications. It offers various technological benefits by reducing food losses and improving food safety (WILKINSON & GOULD, 1996; PATTERSON & LOAHARANU, 2000). Irradiation extends to solid and semi-solid foods like meat, poultry and seafood the same benefits as thermal pasteurization provided for liquids (SATIN, 1996). Radiation treatment at doses of 1.5 to 7 kGy – depending on conditions of irradiation and of the food – can effectively eliminate potentially pathogenic non-sporeforming bacteria from suspected food products without affecting nutritional and technical qualities. These bacteria include both long-time recognized hazards such as *Salmonella* spp. and *Staphylococcus aureus,* as well as emerging or "new" pathogens such as *Campylobacter* spp., *Listeria monocytogenes* or *Escherichia coli* O157:H7 (FARKAS, 1998). In addition to control of the aforementioned bacteriological hazards, "radicidation" of perishable commodities can extend their shelf-life 2- to 3-fold, and inactivate food-borne parasites, the latter being of particular importance for developing countries. After decades of unprecedentedly intense and wide-ranging research efforts, food irradiation is now a well understood and controllable food processing technology supported by all relevant specialized agencies of the United Nations (the World Health Organization, the Food and Agriculture Organization and the International Atomic Energy Agency) and many national scientifically authoritative bodies in different countries. Its slow implementation can be explained by the long time, which was needed to demonstrate adequately the safety and wholesomeness of irradiated food, the lack of readily available radiation facilities and their investment-demanding character, as well as an inadequate awareness of problems, which justify the use of this technology. The safety and nutritional adequacy of irradiated food have been well established (WHO, 1994; DIEHL, 1995), the technology is ready to use technically, and the need to improve the microbial and parasitic safety of food became a major driving force behind the implementation of food irradiation in both developed and developing countries. When irradiation is perceived as an adjunct to, but not a replacement for, GMP, it can serve as a "critical control point" in the Hazard Analysis Critical Control Points system of safety management, a concept becoming mandatory in more and more countries.

The advantage of *high hydrostatic pressure* (HP) treatment (up to 900 MPa [i.e. as high as 9000-times the atmospheric pressure] for several minutes) is that it treats all parts of a high moisture food equally (high isostatic pressure) and it is attractive from

the point of view of food product quality (BARBOSA-CANOVAS et al., 1997). At present its application is limited to a pasteurization-like process for certain foods in which bacterial spores are not a problem (low-pH products) or which have a limited chill shelf-life. Success of its further development depends on effective control by appropriate combination treatments and additional research for careful establishment of performance criteria for reduction of the number of relevant pathogens by a required safety factor (GOULD, 2000). Due to these facts, and because of costs involved in HP pasteurization/sterilization of foodstuffs, this process will remain probably rather specialised and will only be used to preserve foods for which a premium price can be obtained.

High voltage electric field pulses (HELP) to effect non-thermal inactivation of microorganisms in foods was also explored, and these studies led to the development of prototype and industrial scale devices recently (BARBOSA-CANOVAS et al., 1997). HELP treatment is the application of pulses of very high field strength (2–5 V μm^{-1}) for a very short time (microseconds) to foods between two electrodes. The treatment requires fairly complex electronic and fluid handling systems. Studies on combination processes have shown potentially useful synergies because "electroporated" bacterial cells become much more sensitive than untreated cells (e.g. to bacteriocins). The application of HELP will probably be limited to liquid foods or liquids containing small particulates (GOULD, 2000). There are still, however, considerable knowledge gaps that will need to be addressed, and regulatory hurdles to be overcome, before commercialisation of the technology, but HELP products might be in the market place in 10 years' time.

All these technologies might be utilized even more efficiently in rational combinations with other preservative treatment(s).

Among future challenges for food science in the field of more efficient improvement of microbial safety and quality maintenance of food are

- better understanding of factors affecting the establishment, survival and growth of food-borne microorganisms by multidisciplinary research on
  - physiology of microorganisms;
  - development of predictive models for their growth, survival, inactivation or the shelf-life of foods;
  - effect of food structure on its interactions with microorganisms;
  - application of molecular biology for improved characterization, typing and detection of important microorganisms;
- better utilization of synergistic interactions of "combinations"/ "hurdle effects", but avoiding stress adaptation.

Advances in predictive microbiology will assist optimization of existing processes and formulation of alternative processes with regard to their effect on food safety. If the microbiological feasibility of new physical technologies is carefully established and,

particularly, new combination approaches are developed and proven effective, the opportunities for the use of some of the new techniques are likely to expand in the future. From the food industry side, there is a need to "metabolise" the novelty of the aforementioned technologies to appreciate their potential over the hurdles that must be overcome.

Food with improved nutritional value

Until recently, health aspects of food were mainly thought of as the absence of detrimental components, in particular additives and unwanted compounds. In the past years, however, it has become evident that some naturally occurring components of certain foods can help to maintain a state of well being and health through optimising our body functions or to reduce risk of chronic diseases, such as certain cancers and coronary diseases (ROBERFROID, 1999). This is particularly important in that most countries are experiencing a considerable rise in the proportion of elderly people, and proper nutrition can assist us to live not only longer, but in better health (PSZCZOLA, 1999).

In addition, many technological innovations are related to the recognition of the important role of food in promoting and sustaining health, resulting in the concept of "*nutraceuticals*" and "*functional foods*". Nutraceuticals are those food components, which play particular roles in maintaining health. They originate mainly from plants, i.e. they are "*phytochemicals*". Functional foods are foods characterized by the presence of one or more components having beneficial physiological effect and being effective in the maintenance of good health. In one group of functional foods live bacteria (certain lactobacilli and bifidobacteria) provide the main beneficial effect. These foods are called *probiotics* (KNORR, 1998; SANDERS, 1999). Other functional foods contain *prebiotics,* i.e. substances that facilitate growth of the beneficial bacteria in the intestinal tract of the consumer. This interaction between food, nutrition and health is a new challenge for both food science and the food industry and it represents a positive approach to "optimizing" nutrition. It is important, however, that claims on health-promoting effects have sufficient scientific substantiation. It is important that health authorities should establish criteria for mandatory qualification of functional foods. Development of functional food should not be only a marketing claim, but rather a scientific challenge. The presence of a bioactive compound in a food does not necessarily ensure that it will be biologically active when it is consumed. The benefits of the "functional foods momentum" will not be realized unless scientifically sound and non-misleading messages are provided to consumers (MILNER, 1999).

Food can be said to be "functional" if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body beyond adequate nutritional effects, in a way which is relevant to either the state of well-being and health or the

reduction of the risk of a disease (ROBERFROID, 1999). A functional food can be a natural food, a 'modified' traditional food or a 'novel food' as defined in the aforementioned EU Regulation.

The production of functional foods happens by

- eliminating a component causing deleterious effect to the consumer (e.g. an allergenic protein);
- increasing the concentration of a natural component which induces beneficial effects;
- adding a *natural* substance which is not normally present, but for which beneficial effects have been demonstrated;
- replacing a component which causes deleterious effects by a component with beneficial effects;
- improving the bioavailability of food components with beneficial effects.

Functional foods must remain foods, they are not medicaments, but part of nutrition/diet. Because functional food development targets healthy subjects, proving their effects' statistical significance requires a special methodological approach (MÉANCE et al., 1999). It will become increasingly important to

- identify the mechanism of action of the active components in functional foods;
- clarify the impact that commercial processing and home-processing have on bioactive components within foods;
- optimize process parameters for maximal retention and increased bioavailability of beneficial compounds;
- understand the dynamic interactions that occur among the various components of not only the food but with the other constituents of the entire diet;
- investigate the interaction between diet and human intestinal microflora and its implication for health.

Transgenic food

While consumers accept the functional food concept remarkably readily, the acceptance of novel foods as they are defined according to the EU Regulation (EC, 1997), and particularly transgenic food, is controversial.

Conventional breeding can be used to transfer genes only between sexually compatible organisms. Complementary to conventional breeding techniques, gene technology allows the transfer of genes between unrelated species. Thereby, breeding targets can also be achieved more quickly both in plant and animal breeding. This is one of the key, but hotly debated technologies of our times. Important topics that need to be addressed vary from legislation, such as labeling requirements, to safety and

environmental issues. Concerns about application of this agricultural biotechnology are on the ecological impact of growing genetically modified foods, the impact of these crops on biological diversity, and on the safety of food supply, or the development of resistance by insect pests. However, the potential of the agricultural new biotechnologies is enormous also for developing countries. Therefore, questions about agricultural biotechnology must be addressed for people in both developed and developing countries, as we have to address the issue of food security for a world population of some 9000 million people in the year 2050. Furthermore, genetic engineering is not just a new technology for crop improvement, it is a powerful research tool that is helping to provide fresh and better insights of molecular mechanisms involved in biological processes.

It is forecasted that in the next decade about four dozens of agricultural crops will be genetically modified. It is estimated that already 8.3 million ha of genetically modified corn (20% of the total cultivated area) were planted in the US in 1999, and that more than 66 million ha will be used to cultivate transgenic plants by 2005 in North America (GACHET et al., 1999). No less remarkable are the opportunities and research results in the field of food biotechnology by improving microorganisms used in food fermentations, and on the exploitation of microorganisms for the manufacture of food ingredients.

Without venturing more deeply into this enormously complex problem-area where I have no expertise, I should like to limit myself to those aspects of transgenic food or food components which are relevant to the potential improvement of nutritional value and technological functionality of food or food components.

There are already a number of foods on the market which are produced using genetically modified organisms or containing GM ingredients e.g. chymosin used in cheese making, use of GM tomatoes in paste, and GM soybean and corn products. Thus, most of the transgenic products to date have been developed for agricultural and processing efficiency, and not yet with direct consumer benefits in mind, such as improved taste and higher nutritional value. I share the views of those who consider that a "second generation" of transgenic crops should be devoted to achieve these advantageous compositional changes in order to serve the increased interest in functional foods and passing the benefit on to the consumer. Food R&D in the 21st century should also support consumer oriented product development because it has the potential to become part of the health care system. Several transgenic foods are now under development, which aim to develop properties of nutritional significance. Some examples:

- producing rape seed and corn with nutritionally more favourable oil composition (containing negligible levels of erucic acid);
- spinach and lettuce accumulating less nitrate;

- potato richer in starch, thus absorbing less fat when fried;
- certain cereals with increased lysine content;
- certain legumes with increased methionine content;
- rice not producing an allergen;
- "yellow rice" capable of synthesising beta-carotene;
- strawberry and broccoli producing higher levels of anticancer and antioxidant agents (GACHET et al., 1999; HOGAN, 1999; LIU, 1999).

Safety evaluation, however, is a key issue, which must be addressed in relation to the development of novel or improved foods. Guidance on this complex topic is available from a report of a Joint FAO/WHO Consultation (1996) on "Biotechnology and Food Safety" (FAO/WHO, 1996).

Regarding safety assessment of novel food in relation to nutrition, special attention must be paid also to allergenicity of those foods which are produced using this modern biotechnology to avoid the potential appearance of a major food allergen in a product that is normally allergen free.

A summary of results of an EU project on the development of new methods for safety evaluation of transgenic food crops has been presented at the 3rd Karlsruhe Nutrition Symposium in October 1998 (NOTEBORN, 1998), while the International Life Science Institute recently published "Consensus Guidelines" on the safety assessment of viable genetically modified microorganisms used in food (ILSI, 1999).

Principles for approving novel food, particularly genetically modified raw materials, were fundamentally different in North America than in the European Union, which created complications for the food industry and trade. The labeling of genetically modified organisms was not mandatory in the US, where the FDA labeling policy requires "biotech foods" to be labeled only if they are significantly altered. In response to a request from FDA for comments from the public on the Agency's current regulatory regime for food biotechnology and labeling, in formal comments submitted to FDA, the National Food Processors Association (NFPA) called, early in the year 2000, for a compulsory notification process prior to the marketing of new "biotech" food products. The producers of such foods should file with FDA summary documentation to support the determination of safety for the biotech food (NFPA, 2000). NFPA further supports the use of voluntary labeling of foods to indicate the presence or absence of bioengineered ingredients. The European Union "Novel Food Regulation" established a system for formal, mandatory pre-market evaluation and approval for most innovative foods and food production processes, placing particular emphasis on genetically modified products. It requires additional specific labeling of "any characteristic or food property such as composition, nutritional value or nutritional effects, or intended use which renders the food no longer equivalent to its conventional counterpart." Although this EU Regulation provides broad guidance, it leaves the door wide open to

interpretation and it is unlikely to stimulate European innovation and competitiveness (HUGGETT & CONZELMANN, 1997). The recently finalised "White Paper on Food Safety" of the Commission of the European Communities states that "the Community provisions governing *novel foods* have to be tightened and streamlined", and it describes actions to this end (EC, 2000).

This is an area where the future depends on acceptance of rational, science-based weighting of risk with benefits and the provision of accurate and unbiased information to the consumer. The risk of not using technologies and of alternative methods should be also concerned. However, there is a multitude of ethical and social issues to be considered (MOSELEY, 1999). There appears to be an even greater aversion among consumers towards the genetic engineering of animals than towards plant biotechnological programmes (MENRAD, 1998).

To analyse the risks of transgenic foods, the FAO/WHO Codex Alimentarius Commission established an "Ad Hoc Intergovernmental Task Force on Foods derived from Biotechnology" (FAO, 2000), which held its first meeting in Japan at the time when I was preparing this paper. This Task Force uses the concept of "substantial equivalence" established by the Organization for Economic Cooperation and Development as a central to the process of risk assessment and will review other methods for science-based risk assessment. It is expected that the above Codex Task Force will come up with a Codex' standard on transgenic foods. The "precautionary approach" adopted by the recently signed UN Protocol on Biosafety (Cartagena Protocol) can be used by governments in their risk management relevant to the above risk assessment. Identifying and labeling foods as having been derived from biotechnology can form a risk management system that will both protect the health of consumers and promote fair trade practices (FAO, 2000).

In my view, trusting science is one of the key factors for developing new technologies and evaluating new products, regardless of the technology or practices concerned. Unfortunately, however, food irradiation, biotechnology, and confidence in food safety are all media-vulnerable issues. Future developments will depend on more effective and balanced communication to the public, effectively addressing social, ethical and political issues, scientific questions and regulatory needs. It is important to understand the way that people perceive risk psychologically. Consumer acceptance of new processes is likely to be increased when a direct consumer benefit is recognized. However, huge information gaps exist among scientists and particularly between scientists and consumers. To match the promise offered by technological advances and optimize nutrition, the overcoming of barriers in psychological and – as a consequence – political feasibility is required. This needs not only research efforts but education in all stages of the food chain and in all sectors of communication (HOBAN, 1999), because "if we fail to train we fail to convince, if we fail to convince we fail".

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The author gratefully acknowledges the valuable advice and ideas of Prof. F. KÄFERSTEIN, Prof. G. BÍRÓ and Dr. D. BÁNÁTI.

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Acta Alimentaria, Vol. 30 (3), pp. 281–295 (2001)

Preliminary communications

CELLULASE PRODUCTION AND CONVERSION OF RICE STRAW TO LACTIC ACID BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

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(Received: 22 November 1999; revision received: 18 January 2001; accepted: 27 March 2001)

Factors affecting the cellulase production of *Aspergillus niger* using sugar cane bagasse as carbon source were investigated. The highest enzyme activities were obtained, when the culture medium was supplemented with 0.133% tryptone as nitrogen source. The rate of cellulase production was considerably increased when 0.5% Tween 60 was added to the production medium. For FPA and β-glucosidase production pH 5.0, while for CMC-ase pH 5.5 was found to be optimal. The highest cellulase activities were obtained at 30 °C and 300 r.p.m. The highest saccharification degree was achieved, when alkali treated rice straw was used as substrate. The main objective of the present study was to examine the possibilities of lactic acid production from alkali treated rice straw using simultaneous saccharification and fermentation technique with *T. koningii* cellulases and *L. delbrueckii*. The highest conversion of cellulose was obtained using 6% alkaline treated rice straw supplemented with 1.2 mg enzyme/g substrate at pH 4.8 and 45 °C.

Keywords: rice straw, saccharification, SSF, lactic acid, cellulase production, *Aspergillus niger*, *Trichoderma koningii*, *Lactobacillus delbrueckii*

Lignocellulosic biomass is considered to be one of the most important resources for the production of glucose, alternative fuels and chemicals. For instance, the annual production of sugar cane bagasse is about 3.40 million tons in Egypt. Lactic acid has been produced commercially by fermentation since 1881. Today, the amount of lactic acid produced via biological process makes up to 50% of the total lactic acid production (HOFVENDAHL & HAHN-HAGERDAL, 1997). Lactic acid is mainly used in the various fields of food industry as preservative agent. It can also be used as a precursor in the

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0139–3006/2001/$ 5.00

production of other organic compounds such as acrylic acid, acetaldehyde, and ethanol. Recently, lactic acid has drawn a lot of attention because of the achievement in the development of biodegradable poly-lactic plastics. Furthermore, lactic acid has stimulating effects on plant (KINNERSLEY et al., 1990; MULLIGAN et al., 1991; MERCIER et al., 1992; NORTON et al., 1994). A typical process configuration for bioconversion of lignocellulosic biomass to lactic acid consists of two steps i.e. enzymatic hydrolysis of the cellulose content of the raw material and the fermentation of the sugars formed in the hydrolysis to lactic acid using a suitable chosen bacterium (LADISH & SVARCZKOPF, 1991; DEMIRIC et al., 1993; KATZEN & MONCEAUX, 1995; HAHN-HAGERDAL, 1996; KADEMIC & BARATTI, 1996; OLSSON & HAHN-HAGERDAL, 1996). A novel approach, which has been successfully applied in the bioconversion of lignocellulosic substrates to fuel ethanol (LEZINOU et al., 1994; PHILIPPIDIS & SMITH, 1995), is the so-called simultaneous saccharification and fermentation (SSF) technique. During SSF the enzymatic hydrolysis of the raw material is performed together with the fermentative conversion of the produced sugars to lactic acid in one reaction vessel. In general, the SSF has three important advantages compared to the two step separate hydrolysis and fermentation technique. Namely, the capital cost of the process can considerably be decreased, since only one reactor is needed; material losses due to handling can be minimized; and inhibition of the enzymes caused by the liberated sugars can be avoided, since they are converted to the end product in the same instance as they are formed (PARAJO et al., 1997).

In the present work, the cellulase production of *Aspergillus niger* was studied using sugar cane bagasse as carbon source. The effect of quality and quantity of nitrogen source, pH, addition of surfactants, incubation temperature, and agitation speed was investigated. The other main objective of this present study was the examination of lactic acid production applying the SSF process using alkali treated rice straw using *Trichoderma koningii* cellulases together with *Lactobacillus delbrueckii*.

1. Materials and methods

1.1. Microorganism

Freeze-dried cultures of *Aspergillus niger* 00632, *Trichoderma koningii* 2691, and *Lactobacillus delbrueckii* 01357 were obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM) Budapest, Hungary.

1.2. Substrates

In the present study rice straw, cotton stalks and sugar cane bagasse were used as substrates. All raw materials were sun dried for 5 days and their particle size was

reduced to 1–2 cm by manual method. A portion of the dried and ground materials was treated with 2M NaOH at 30 °C for 48 h, while the other portion with 1 wt% H_2SO_4 at 120 °C for 100 min (CARRASCO et al., 1994). The pretreated materials were thoroughly washed with tap water, oven dried at 70–80 °C and milled before used.

1.3. Media and culture conditions

Fungal stock cultures were maintained on standard PDA slants at 4 °C. Three ml of spore suspension obtained from a 7-day old PDA slant of *A. niger* was used to initiate growth in an E-flask containing 50 ml of sterile production medium (pH 5.0) in which the concentration of nutrients were: 7 g l^{-1} $(NH_4)_2SO_4$, 10 g l^{-1} KH_2PO_4, 1.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1.5 g l^{-1} $CaCl_2 \cdot 2H_2O$, 1.5 g l^{-1} urea and 1 g l^{-1} glucose together with 3 wt% sugar cane bagasse as carbon source. Trace elements were also added: 25 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 10.3 mg l^{-1} $MnSO_4 \cdot 4H_2O$, 7 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, and 18.3 mg l^{-1} $CoCl_2 \cdot 6H_2O$ (TÜRKER & MAVITUNA, 1987). Cultures were incubated on a rotary shaker at 30 °C and 100 r.p.m. The fermentation broth was filtered and the enzyme activities were determined. From a 7-day old PDA slant of *T. koningii* the conidia was suspended in 3 ml of sterile water and was used to inoculate an E-flask containing 50 ml of TÜRKER (1987) medium supplemented with acid treated sugar cane bagasse (EL-HAWARY & MOSTAFA, 2001). The culture was incubated at 30 °C and 100 r.p.m. The fermentation broth was filtered, precipitated using cold ethanol, dialyzed, lyophilized and used in the SSF experiments.

Lactobacillus delbrueckii 01357 was maintained on agar slants containing 20 g l^{-1} glucose, 10 g l^{-1} peptone, 10 g l^{-1} beef extract, 5 g l^{-1} yeast extract, 5 g l^{-1} sodium acetate, 2 g l^{-1} sodium citrate, 2 g l^{-1} K_2HPO_4, 0.58 g l^{-1} $MgSO_4$ $7H_2O$, 0.21 g l^{-1} $MnSO_4$ H_2O, 1 ml Tween 80 and 20 g l^{-1} agar. The pH was adjusted to 6.4. The seed culture was incubated at 45 °C for 24 h. (ABE & TAKAGI, 1991). Inoculum for lactic acid production was prepared by transferring 24-h old culture of *L. delbrueckii* to 250 ml E-flasks containing 100 ml of culture medium which had the same composition as described before without any agar added.

1.4. Enzymatic hydrolysis of pretreated materials

The enzymatic hydrolysis of the various pretreated substrates was performed at 2 wt% dry weight content in 0.05M (pH 4.8) sodium acetate buffer solution supplemented with 0.2 mg of protein/g of substrate. The hydrolysis mixture was also supplemented with 0.3 g l^{-1} sodium azide in order to prevent microbial growth. The 25 ml E-flasks containing a total volume of 10 ml reaction mixture were incubated in a rotary shaker at 50 °C and 100 r.p.m. After 48 h of hydrolysis, hydrolysates were centrifuged at 4000 r.p.m. for 30 min and the supernatants were collected. The total

reducing sugar content of the various supernatants were analyzed by using the so-called DNS procedure (MILLER, 1959). Saccharification percentage was calculated using the following equation (MANDELS et al., 1976):

$$\text{Saccharification } (\%) = \frac{\text{Total reducing sugar } (\text{mg ml}^{-1}) \times 0.9 \times 100}{\text{Initial substrate concentration } (\text{mg ml}^{-1})}$$

1.5. Fermentation of hydrolysates

The hydrolysates obtained were supplemented with 6 wt% yeast extract, 0.167 wt% sodium acetate, 0.167 wt% $(NaPO_3)_n$, 0.1 wt% $MgSO_4 \cdot 7H_2O$, 0.005 wt% $FeSO_4\ 7H_2O$ and 0.005 wt% $MnSO_4\ H_2O$. Before inoculation the pH was adjusted to 4.8. In order to prevent acidification during the lactic acid fermentation 5 wt% $CaCO_3$ was added to the medium. Growth was initiated using vegetative cells of *L. delbrueckii* in a 100 ml E-flask containing 50 ml of nutrient supplemented and sterilized hydrolysate. The inoculum contained 10 (v/v)% of the medium. The fermentation experiments were carried out at 45 °C for 6 days.

1.6. Simultaneous saccharification and fermentation

SSF experiments were carried out in 100 ml static E-flasks containing 50 ml of sterilized medium incubated at 45 °C. The composition of the SSF medium was 60 g l^{-1} pretreated material, 60 g l^{-1} yeast extract, 1.67 g l^{-1} sodium acetate, 1.67 g l^{-1} $(NaPO_3)_n$, 1.0 g l^{-1} $MgSO_4\ 7H_2O$, 0.05 g l^{-1} $FeSO_4\ 7H_2O$ and 0.05 g l^{-1} $MnSO_4\ H_2O$. The pH was adjusted to 4.8. To prevent acidification due to lactic acid formation 0.6 g $CaCO_3$/g substrate was added to the medium. The SSF medium was supplemented with 0.2 mg of protein produced by *T. koningii*/g of substrate. The *L. delbrueckii* inoculum contained 10 (v/v)% (ABE & TAKAGI, 1991).

1.7. Measurement of enzyme activities

Filter paper activity (FPA) was measured according to MANDELS and co-workers' (1976) procedure. The reaction mixture containing 0.5 ml of 0.05M acetate buffer (pH 4.8) and 0.5 ml of culture filtrate, was incubated together with a 1×3 cm (25 mg) strip of Whatman No. 1 filter paper at 50 °C for 60 min. The enzymatic reaction was terminated by addition of 1 ml DNS reagent (MILLER, 1959). After 5 min of boiling and addition of 10 ml of distilled water the absorbance was measured at 540 nm.

Carboxyl-methyl-cellulose degrading capacity (CMC-ase) was determined by incubating 0.5 ml of enzyme sample together with 0.5 ml of 1 wt% carboxy-methyl-cellulose in 0.05M acetate buffer (pH 4.8) at 50 °C for 30 min. The hydrolysis was

stopped by addition of 1 ml DNS reagent. After boiling for 5 min and dilution with 10 ml of distilled water the absorbance was read at 540 nm.

For β-glucosidase activity determination 10 µl of culture filtrate was incubated together with 1 ml of 0.67 mM (0.02 wt%) p-nitrophenyl-β-D-glucopyranoside in 0.05 M acetate buffer (pH 4.8) at 50 °C for 10 min. The enzymatic reaction was terminated by addition of 3 ml 0.1 M NaOH solution. The absorbance was measured at 400 nm (RECZEY et al., 1990).

1.8. Determination of lactic acid

Lactic acid was determined according to BARKER and SUMMERSON's procedure (1996).

2. Results and discussion

2.1. The cellulase production of Aspergillus niger

In a set of experiments the effect of quality of the nitrogen source was investigated. Ammonium sulfate and urea of basal media were replaced with urea or ammonium sulfate separately or with other organic and inorganic nitrogen sources in such a way that the amount of final nitrogen concentration in the media remained unchanged. The results are summarized in Fig. 1. It can be seen that the cellulase production of *A. niger* cultivated on untreated sugar cane bagasse as carbon source was higher using tryptone as nitrogen source. Using tryptone the FPA activity was increased by about 20% compared to the control medium, while the CMC-ase and ß-glucosidase activities were increased by 6 and 12%, respectively. The final pH of the fermentation broth was found to be around 5.2 when tryptone and peptone (which was found to be the second best nitrogen source) were used. There might be a relationship between the final pH of the culture medium and the cellulase production. The positive effect of complex, organic nitrogen sources on the cellulase production could be due to the presence of growth promoters, in such amounts, which are optimal for fungal growth and enzyme production. The same observations were reported by DOPPELBAUER and co-workers (1987) and MAGNELLI and co-workers (1996).

Since, tryptone proved to be the best alternative nitrogen source resulting in significantly higher enzyme production compared to the standard medium, the effect of tryptone concentration on the cellulase production of *A. niger* was also studied using untreated sugar cane bagasse as carbon source. Supplementation of the culture medium with 0.133% tryptone resulted in the highest CMC-ase and β-glucosidase activities (Fig. 2), however maximum FPA activity was reached when the tryptone concentration was at 0.176%.

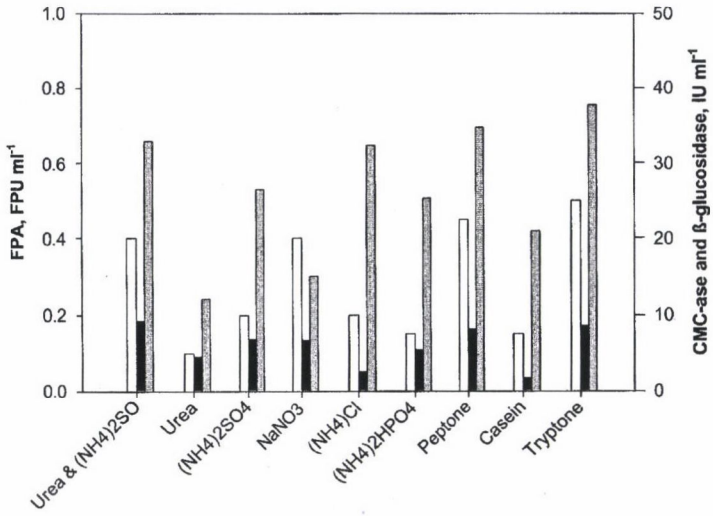

Fig. 1. The effect of nitrogen source on the cellulase production of *A. niger* using sugar cane bagasse. Filter paper activity (FPA): white bars, ß-glucosidase activity: gray bars; CMC-ase activity: black bars

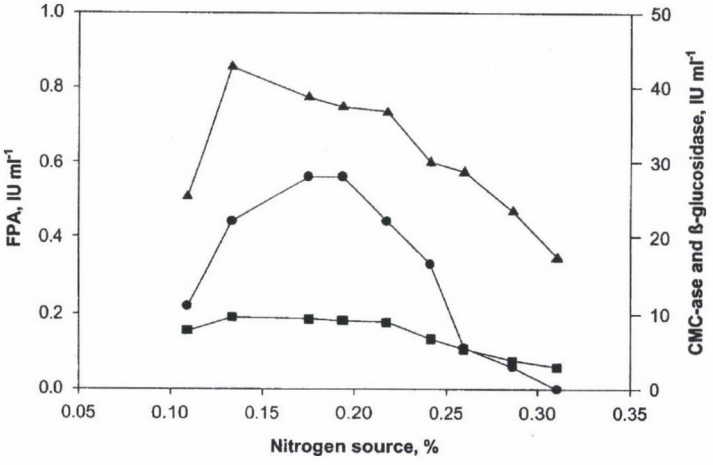

Fig. 2. The effect of nitrogen source concentration on the cellulase production of *A. niger* using sugar cane bagasse. Filter paper activity (FPA): ●; β-glucosidase activity: ▲; CMC-ase activity: ■

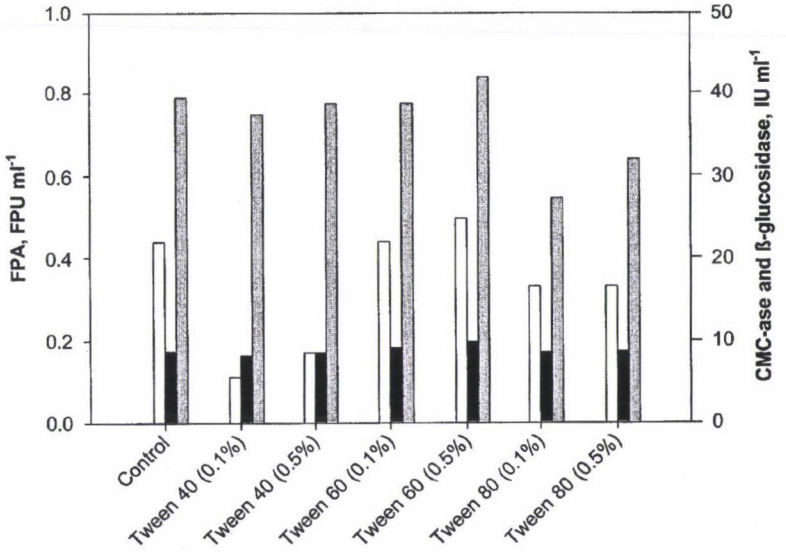

Fig. 3. The effect of surfactants on the cellulase production of *A. niger* using sugar cane bagasse. Filter paper activity (FPA): white bars; β-glucosidase activity: gray bars, CMC-ase activity: black bars

The effect of surfactants addition on the enzyme production was studied by supplementing the production medium with Tween 40, 60, 80. Two different concentrations were applied, i.e. 0.1% and 0.5%, respectively. The addition of Tween 60 at both concentrations stimulated the cellulases production of *A. niger* (Fig. 3). All enzyme activities were significantly higher compared to the reference fermentation in which no surfactants were added. Similar results were obtained by HUNG and co-workers (1988), LONG and KNAPP (1991) and STUTZENBERGER (1987).

The initial pH of the cultivation is considered as an important factor effecting the cellulase production. In a set of experiments the initial pH of the culture medium was varied between 4.0 and 7.0 by addition of either HCl or NaOH. Figure 4 shows the enzyme activities as the function of the initial pH. The highest FPA and β-glucosidase activities were obtained at pH 5.0, however maximal CMC-ase production was observed at pH 5.5. The results showed good agreement with the data obtained by BASTAWDE (1992).

To evaluate the effect of incubation temperature on the cellulases production of *A. niger* shake flask experiments were run at various temperatures between 20 and 35 °C. The optimal temperature for enzyme production was found to be 30 °C (Fig. 5).

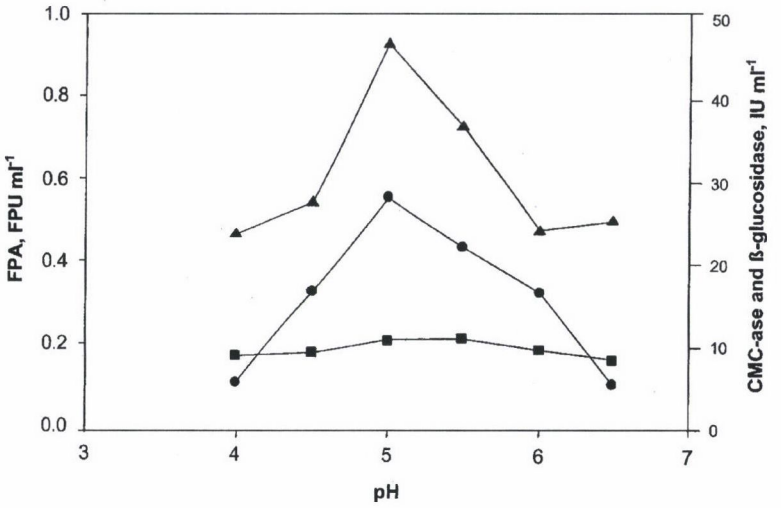

Fig. 4. The effect of initial pH on the cellulase production of *A. niger* using sugar cane bagasse. Filter paper activity (FPA): ●; β-glucosidase activity: ▲; CMC-ase activity: ■

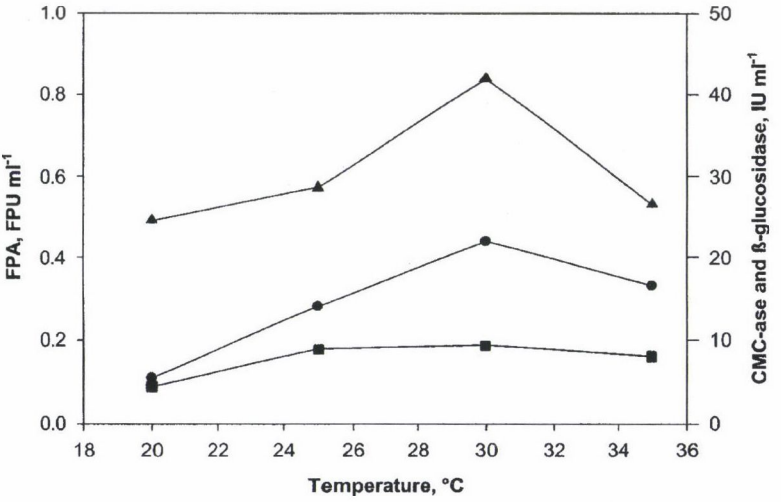

Fig. 5. The effect of incubation temperature on the cellulase production of *A. niger* using sugar cane bagasse. Filter paper activity (FPA): ●; β-glucosidase activity: ▲; CMC-ase activity: ■

Similar results were obtained by KNAPP and LEGG (1986), DOPPELBAUER and co-workers (1987), BASTANDE (1992), KHALAF ALLAH and co-workers (1993).

The effect of agitation speed on the cellulase production was investigated by varying the speed between 100 and 300 r.p.m. Figure 6 shows the results obtained. It can ben seen that the enzyme production was increased 4.4 times at 300 r.p.m. compared to the control cultivation. These results were in agreement with that reported by MAGNELLI and co-workers (1996) TÜRKER and MAVITUNA (1987) BUSWELL and CHANG (1994) and SILVA and co-workers (1995). RECZEY and co-workers (1996) used 350 r.p.m. as a good agitation value for cellulases production by *T. reesei* RUT C30.

2.2. Hydrolytic capacity of cellulases produced by A. niger

The results of hydrolysis experiments obtained with cellulases produced by *A. niger* are summarized in Table 1. It can be seen that untreated materials exerted great resistance towards enzymatic attack, which is due to the strong physical interaction between the components of the naive lignocellulosic materials. Hemicellulose acts as a glue between the lignin and cellulose molecules. By removing the hemicellulose fraction, enzymatic accessibility can be considerably increased.

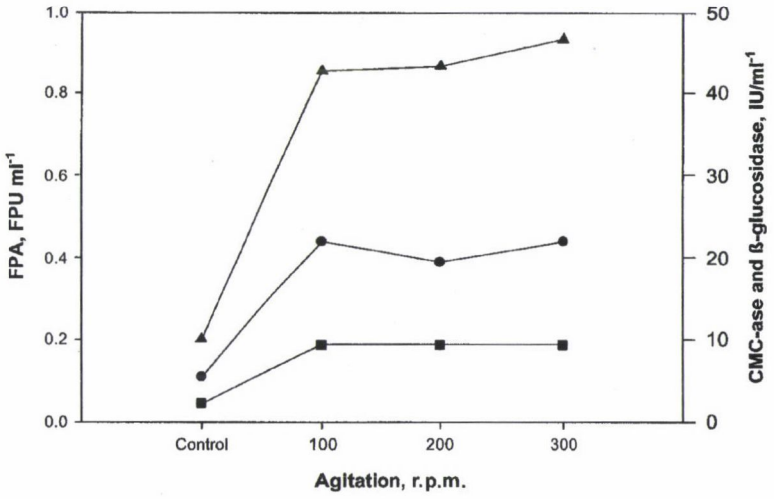

Fig. 6. The effect of agitation on the cellulase production of *A. niger* using sugar cane bagasse. Filter paper activity (FPA): ●; β-glucosidase activity: ▲; CMC-ase activity: ■

Table 1

Effect of A. niger *cellulases on the saccharification process*

Cellulosic substrates		*A. niger* cellulases produced on untreated sugar cane bagasse	
		Glucose (mg ml^{-1})	% Saccharification
Rice straw	Untreated	0.99	4.45
	Acid-steam treated	6.91	31.09
	Alkaline treated	12.13	54.58
Cotton stalks	Untreated	1.38	6.21
	Acid-steam treated	7.57	34.06
	Alkaline treated	11.12	50.04
Sugar cane bagasse	Untreated	1.52	6.84
	Acid-steam treated	4.10	18.45
	Alkaline treated	8.17	36.76

Conditions: 2% substrate, 0.2 mg enzyme/g substrate, 0.3 g l^{-1} sodium azide, 48 h, 50 °C, 100 r.p.m., acetate buffer pH 4.8.

Furthermore, it is a generally accepted fact that during pretreatment reduction in cellulose crystallinity occurs, which results in higher hydrolysis rates and yields (CARRASCO et al., 1994). Acid-steam treatment appears to be an effective method to increase enzymatic digestibility. A saccharification degree of 34% was observed in case of cotton stalks. Treatment with alkaline proved to be the best method and a conversion of 55% was obtained with rice straw, while the same value for cotton stalks was 50%. The successfulness of alkaline treatment is probably due to its complex action on the lignocellulosic biomass. Besides the increase of the available surface for enzymatic hydrolysis and reducing cellulose crystallinity, the partial hydrolysis of hemicellulose and swelling of the cellulose occur.

2.3. Lactic acid production using rice straw

In a set of experiments rice straw was used as substrate for lactic acid production. Two different options were compared, i.e. separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

In the SHF experiments *L. delbrueckii* was cultivated on the hydrolysate of alkali treated rice straw at pH 4.8 and 45 °C. The results are shown in Fig. 7. The concentration of lactic acid in the fermentation broth was about 40 g l^{-1} after 4 days of

incubation, which was considerably higher (about 50%) than reported by PARAJO and co-workers (1996) using eucalyptus wood hydrolysate. After 4 days of residence time, about 84% of the initial glucose was converted to lactic acid. Addition of $CaCO_3$ to the medium prevented acidification due to lactic acid formation, and an average pH of around 4.65 was observed. Significantly higher yields were obtained by MCCASKEY and co-workers (1994) using municipal waste hydrolysates. About 65 g l^{-1} lactic acid concentration was obtained from 100 g l^{-1} glucose containing medium.

The results obtained with SSF experiments using 6 wt% alkaline treated rice straw are summarized in Fig. 8. The concentration of produced lactic acid continuously increased reaching a maximum concentration of 34 g l^{-1}, which means a 57% conversion of rice straw after 5 days of incubation time. Due to the $CaCO_3$ addition the pH of the fermentation did not fluctuate much and was between 4.6 and 4.8. ABE and TAKAGI (1991) using milled newspaper reached a lactic acid concentration of 53 g l^{-1} after 5-day incubation. The conversion of rice straw to lactic acid was about 57% when SSF was used, while only 50% conversion was obtained with the SHF technique. From the results it can be concluded that SSF technique for lactic acid production was advantageous compared to SHF. Therefore, the factors affecting the lactic acid production using SSF were investigated.

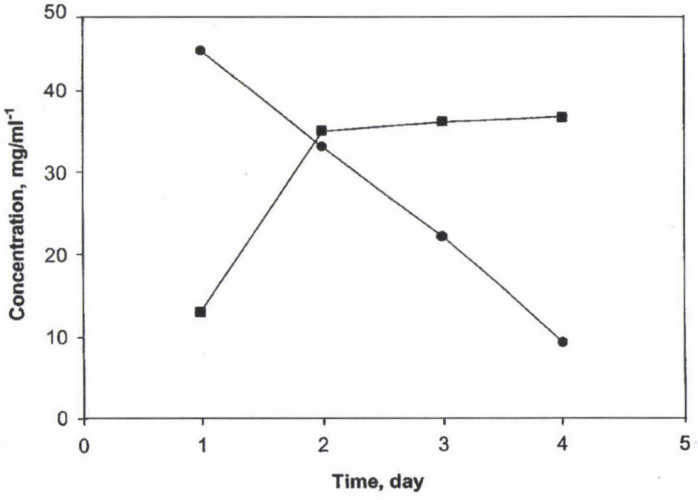

Fig. 7. Lactic acid production using SHF. Glucose: ●; Lactic acid: ■

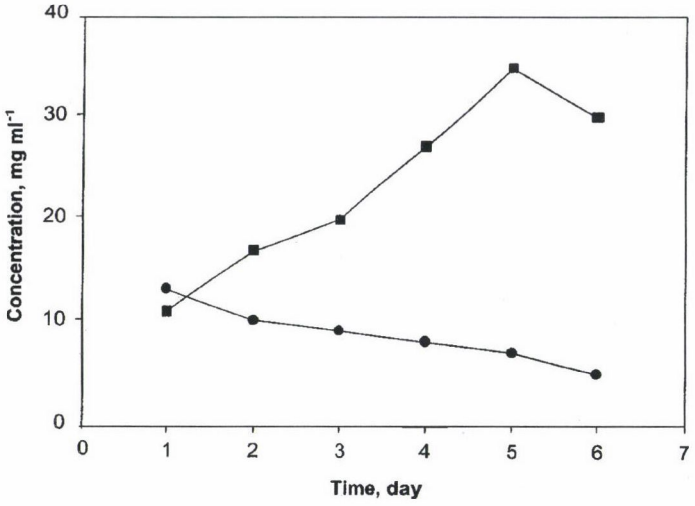

Fig. 8. Lactic acid production using SSF. Glucose: ●; Lactic acid: ■

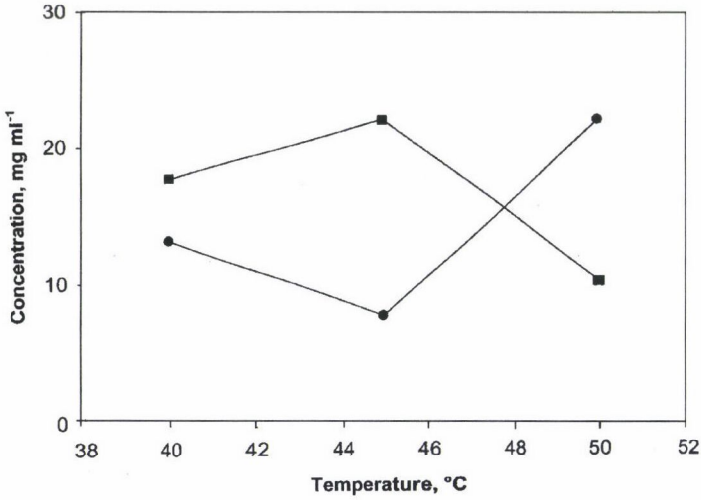

Fig. 9. The effect of incubation temperature on lactic acid production using the SSF technique. Concentrations of glucose (●); and lactic acid (■) obtained after 48 h of residence time

2.3.1. The effect of pH on the lactic acid production. The effect of medium pH as one of the most important factors on the SSF technique was investigated. Calcium carbonate was used to prevent the acidification caused by the lactic acid produced during fermentation. The culture medium was supplemented with 0.6 g $CaCO_3$/g substrate. The obtained results showed that pH 4.8 was the optimal pH for lactic acid production (data not shown).

2.3.2. The effect of incubation temperature on lactic acid production. To investigate the effect of incubation temperature on lactic acid production in the SSF experiments, three different temperatures i.e. 40, 45 and 50 °C were examined. Experiments were run for 48 h. As it is shown in Fig. 9 the optimal temperature for lactic acid production was at 45 °C.

At 40 °C 13% lower, while at 50 °C almost 50% lower lactic acid concentrations were obtained compared to that of reached at 45 °C. These results were not surprising at all because 45 °C was the optimal temperature for the growth of *L. delbrueckii*. These results showed good agreements with the results reported in the literature (PARAJO et al., 1997).

3. Conclusions

For efficient conversion of lignocellulosic materials to lactic acid with SSF technique enzyme preparation with high cellulase activity is required. The cellulase production of *A. niger* using sugar cane bagasse was optimized. The optimal amount and quality of nitrogen source was determined, and tryptone was found to be the best nitrogen source. It was shown that the addition of surfactants to the culture medium increased the extracellular amount of cellulases. The effect of incubation temperature as well as the pH and the agitation speed on the cellulase production of *A. niger* was examined and optimized.

It is known that the amount of cellulases produced by *Aspergillus* strains is not sufficient for efficient conversion of lignocellulosic biomass using the SSF technique. Therefore, in the SHF and SSF conversion of alkali treated rice straw *T. koningii* cellulases were used. It was shown that SSF resulted in good conversion of rice straw to lactic acid.

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Acta Alimentaria, Vol. 30 (3), pp. 297–311 (2001)

DETERMINATION OF ACETONE IN COW'S RAW MILK BY FLOW INJECTION AND GAS CHROMATOGRAPHIC METHODS

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(Received: 15 September 2000; accepted: 22 March 2001)

An automatic flow injection (FIA) and two possible reference gas chromatographic (liquid and headspace sampling GC) methods including appropriate sample preparation were developed and validated for determination of acetone in milk. The methods were tested by preserved raw milk samples.

The FIA results were compared with data obtained by GC determinations. It was found that FIA procedure is suitable for rapid automated measurement of acetone in the range of 0–5 mmol l^{-1} in milk, therefore it is a promising analytical method for ketosis monitoring in dairy farms.

Keywords: acetone, flow injection analysis, gas chromatography, ketone bodies

Ketone bodies are formed mainly in the liver from acetyl-CoA in mammals. Acetone and acetoacetic acid may be clinically termed as oxidised, isopropanol and β-hydroxybutyric acid, respectively, and consequently named as reduced ketone bodies. In late pregnancy and early lactation in ruminants, the synthesis of ketone bodies – as important regulating agents of energy metabolism – can be regarded as a physiological process (FEKETE et al., 1999). However, an inadequate diet or different diseases (i.e. metritis, cystic ovarium disease, etc.) cause ketosis, which means an increasing level of ketone bodies in body fluids i.e. blood, urine or milk. Ketosis has been associated with decreased milk yield, increased risk of clinical ketosis and impaired reproductive performance, while clinical ketosis is accompanied by clinical changes such as anaemia, depression or excitation (JORRITSMA et al., 1998).

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The early detection of disorders, i.e. elevated levels of ketone bodies mainly under subclinical conditions, may reduce the frequency of ketosis – consequently the profit loss in milk production.

Nowadays, in clinical practice acetoacetic acid and β-hydroxybutyric acid content in body fluids are used to diagnose ketosis. In case of subclinical stage, one of the silent signals is the elevated level of acetone in blood, urine and finally in milk.

Analysis of blood and urine requires more sample preparation and special knowledge, while the determination of acetone in raw milk seems to be a quick and a very useful tool in early detection of this illness (TYÖPPÖNEN & KAUPPINEN, 1980; VON DIEKMANN et al., 1986).

Ketone bodies in blood and other tissues are usually analysed by chemical, chromatographic, enzymatic and fluorimetric methods. Several chemical methods have been developed for determination of acetone, acetoacetate, β-hydroxybutyrate, and the total amount of ketone bodies (THIN & ROBERTSON, 1952; STEGER & VOIGT, 1970). These methods are based on oxidation of different ketone bodies to acetone and on the determination of acetone by various colorimetric reactions. These methods are quite annoying and time-consuming, especially if one has to deal with samples containing very small amount of ketone bodies and often has to apply hazardous reagents. Furthermore, the common problems in field studies are the chemical instability of acetoacetate in fluids, the matrix interferences and the low recoveries, especially for β-hydroxybutyrate (64–86%) (THIN & ROBERTSON, 1952). The enzymatic and the fluorimetric procedures are based on enzymatic reactions. Enzymatic kits for determination of β-hydroxybutyrate and acetoacetate are available and are used in human and veterinary clinical practice (WILLIAMSON et al., 1962; DARGEL, 1987; OZAND et al., 1985). These are more specific and rapid methods than the colorimetric methods available, but the sensitivity is the same. Fluorometric methods, based also on enzymatic reaction and fluorescence change of NADH, make it possible to detect very low concentrations of β-hydroxybutyrate and acetoacetate (<0.1 mmol l^{-1}) (YOUNG & RENOLD, 1966).

In order to build up an everyday monitoring system for the cow's populations we have studied the possible ways of automation and cost reduction of acetone determinations in milk. Comparing the methods published in literature, we decided to apply flow injection analysis technique coupled with gas diffusion technique (VON DIEKMANN et al., 1986; TÖMÖSKÖZI et al., 1999) for raw milk samples.

Nowadays, there are no officially recommended or reference methods in the area of dairy analyses. The use of chromatographic methods, especially headspace gas chromatography (HRADECKÝ et al., 1978; VAN STEKELENBURG & DE BRUYN, 1970; ERIKSSON, 1972; LÓPEZ-SORIANO & ARGILÉS, 1985; KIMURA et al., 1985; SIEGEL et al.,

1977; WINTERBACH & APPS, 1991) can solve most of difficulties of methods mentioned above.

In this paper, we report two possible GC methods to use for reference analysis of acetone.

1. Materials and methods

1.1. Materials

The methylethylketone reference substance for gas chromatography and the acetone were purchased from Merck, Germany (catalogue numbers: 109709 and 100020). Ethanol was obtained from Reanal Ltd., (catalogue number: 05031-6-69). All other chemicals were of analytical grade obtained from Reanal Ltd., Hungary.

Samples were preserved (with Broad Spectrum Multitabs II from D&F Control Systems Inc., USA), and raw milk samples were obtained from Hungarian Herd Recording Ltd., Gödöllõ, Hungary.

1.2. Methods

1.2.1. Sample preparations. Sample preparation method for FIA assay. Only homogenisation as sample pre-treatment was used for FIA procedure.

1.2.2. Sample preparation method for liquid phase sampling GC assay. Four ml of milk sample and 0.4 µl of ethanol (internal standard) were pipetted into a 10-ml centrifuge tube. Two hundred µl of 3 mol l^{-1} freshly prepared TCA (trichloracetic acid) solution was added to the mixture. It has been shaken by hand for 1 min, than has been centrifuged at 7000/min (2200 g) for 20 min. The supernatant was passed through a 45 µm filter (cat. number: HVLP02500, Millipore Ltd., Ireland) and 2 µl of the filtrate was injected directly by syringe into the gas chromatograph.

1.2.3. Sample preparation method for headspace sampling GC assay. Ten ml of raw milk sample and 1 µl of methylethylketone (internal standard) were pipetted into a 20 ml bottle, capped with rubber septum and adjusted to 60 °C in the air thermostat. A sampling device with a loop volume of 3 ml (Fig. 1) injected the samples.

1.2.4. Preparation of standard solutions. For FIA and gas chromatographic determinations 10 mmol l^{-1} acetone stock solution was prepared and diluted in the range of 0.05 to 5 mmol l^{-1}. For GC assays, the acetone standard solution contained the needed amount of internal standards (0.1 µl methylethylketone and ethanol per ml sample).

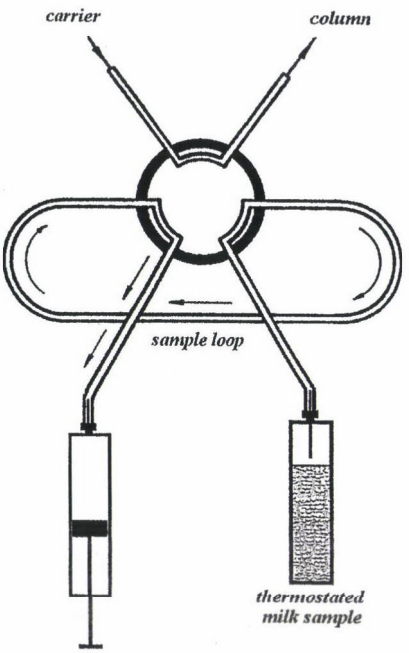


Fig. 1. Sampling device for reduced pressure headspace sampling GC determination of acetone in raw milk

1.2.5. FIA method. The developed method is based on the relatively high volatility of the acetone content of the injected milk sample into the carrier solution, and it can be put through on the gas diffusion membrane and appears in the reagent stream containing indicator and hydroxylamine. The acetone and hydroxylamine react to form acetoxyme. The pH change during the chemical reaction is detected photometrically. According to the principle of FIA method (VON DIEKMANN et al., 1986), a standard flow injection system (Enviroflow 5012 System and Detector System 5042 made by Foss-Tecator, Sweden) with gas diffusion unit (Chemifold V XS, Foss-Tecator, Sweden) and thermostat (FIAstar 5101, Foss-Tecator, Sweden) was applied. The optimised FIA settings are shown in Fig. 2.

1.2.6. Liquid phase sampling GC method. The developed GC methods were used with the same adsorption chromatographic system: Carlo Erba Vega Series 2 (Carlo Erba, Germany) gas chromatograph model with 6 ft. column, packed with Porapack Q 80/100 mesh (from Waters Ltd., USA) and equipped with flame ionisation detector (Carlo Erba, Germany) was used in our experiments. Data were collected with HP 35900 ADC interface and were analysed by HP ChemStation software (Hewlett Packard Ltd., USA).

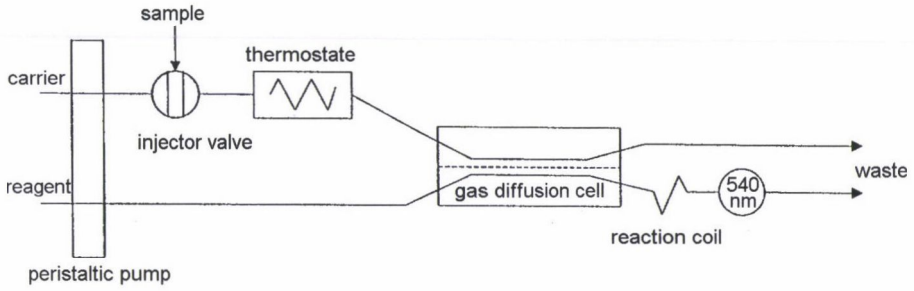


Fig. 2. FIA system for determination of acetone in raw milk. Sample volume: 200 µl; Carrier: 2 ml min^{-1}, phosphate buffer (0.1 mol l^{-1}, pH=7) containing 1.2 g 30% Brij 35; Reagent: 1.5 ml min^{-1}, 100 ml methylorange indicator stock solution (0.25 g l^{-1}) and 150 ml hydroxylamine stock solution (20 g l^{-1}) were diluted with distilled water to 1000 ml; Detection: 540 nm; Cycle time: 80 s; Reaction coil: 0.5·60 cm; Thermostat: 80 °C

In order to protect the column from matrix substance contamination in milk samples a precolumn packed with glass wool was inserted in front of the analytical column.

The applied parameters were as follows:

Column:	Porapack Q
Column temperature:	isotherm, 200 °C
Injection:	on column
Injector temperature:	125 °C
Injected sample volume:	2 µl
Internal standard:	ethanol
Detector:	FID
Detector temperature:	125 °C
Carrier:	He
Calibration/measurement:	The ratio of each peak area to the internal standards was determined and plotted against the concentration ratio.

1.2.7. Headspace sampling GC method. The headspace-sampling device is shown in Fig. 1. Conditions of the analytical determination were:

Column:	Porapack Q
Column temperature:	isotherm, 175 °C
Sampling:	reduced pressure headspace
Sample loop:	3 ml
Internal standard:	methylethylketone
Detector:	FID
Detector temperature:	120 °C
Carrier:	He

The developed FIA and GC methods were investigated to establish their performance parameters. The results were analysed by Statistica.5 (StatSoft Ltd., USA) software.

2. Results

2.1. FIA method

The test range was declared between 1 and 10 mmol l^{-1} acetone concentration, according to the physical range of measurable values published in the literature. The calibration was performed using water solutions, because during the pre-examinations significant differences were not found among the results obtained from water and milk standard solutions. The standard curves were linear in the whole concentration range tested. The slope and the intercept of the calibration curve mainly depended on the status of the gas diffusion membrane used, so it was necessary to perform a new calibration process before each batch. The average sensitivity of the FIA method was characterised by the average slope of linear calibration curves: 225.9±4.74 absorbance unit per mmol l^{-1}. The statistical analysis of variances of the measured absorbance values of standards showed that the variances were constant at α=0.05 significance level. The results of residual analysis and trend-test (no trend, no extreme value, the residuals have normal distribution) also proved the suitability of the calibration method applied.

The detection limit was established on base of average results of blank samples' repeated measurements (0.03 mmol l^{-1}). The reproducibility of FIA method (SD %<2.2) was defined by variance analysis. Date, staff and replicates played a role in variance factors. The results are shown in Fig. 3. At a very low concentration – close to the detection limit of the method – there was no effect of different operators, and this might be explained by the uncertainty of the determination itself. At higher concentrations there was not found an effect of different date, and according to the

raising acetone concentration an increase of the effect of operator and replicates were found. The precision of the FIA assay was determined by recovery percentages of the acetone added to the samples (Table 1). The FIA method is precise enough because of the narrow range of recovery results (98.8–110.6%) given. (At the lowest standard concentration the higher than 105% recovery can be explained by the uncertainty of the acetone addition.) Therefore, it seems to be appropriate for determination of acetone in raw milk samples.

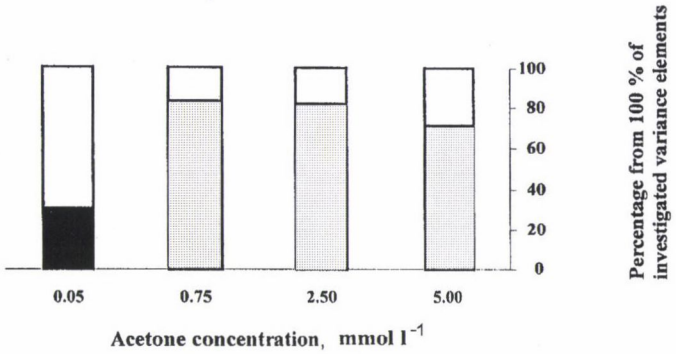


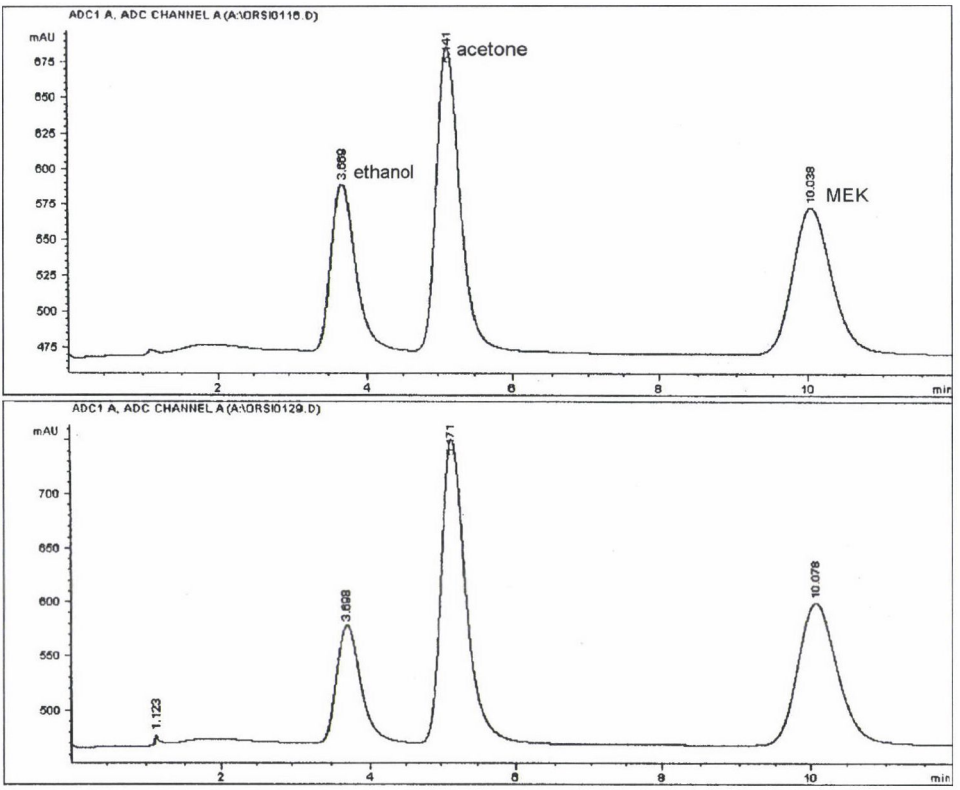
Fig. 3. The reproducibility of FIA method: the influence of the investigated variance elements. □: Effect of replicates; ▨: effect of operator; ■: effect of date

Table 1

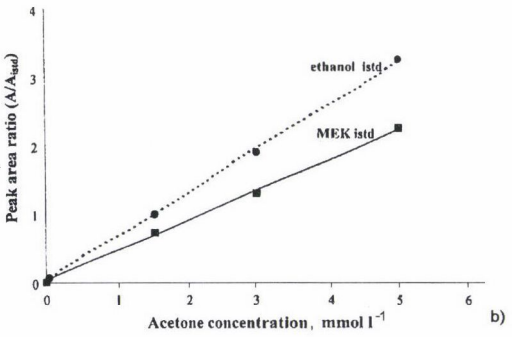
Precision of the FIA assay, determined by recovery percentages of the acetone added to samples

Sample id.	Acetone added (mmol l^{-1})	Recovery (%)	SD (%)
Milk 1	0.05	110.6	1.90
Milk 2	0.10	104.7	2.03
Milk 3	0.50	103.6	1.12
Milk 4	1.00	98.8	0.97
Milk 5	2.50	101.5	0.69

Results represent 5 independent determinations


ADC1 A, ADC CHANNEL A (A:\ORSI0116.D)
mAU
acetone
ethanol
MEK
3.699
5.441
10.038
min
ADC1 A, ADC CHANNEL A (A:\ORSI0129.D)
mAU
1.123
3.698
5.471
10.078
min

a)


Peak area ratio (A/A$_{istd}$)
ethanol istd
MEK istd
Acetone concentration, mmol l^{-1}

b)

⇐ Fig. 4. Headspace chromatograms of 3 mmol l^{-1} acetone standard solution, one of the raw milk samples with ethanol and methylethylketone (MEK) internal standards (a), and calibration graphs for headspace sampling GC (b). Calibration with ethanol istd.: y=0.6503x, R^2=0.9991; with methylethylketone: y=0.4532x, R^2=0.9992

Table 2

Average retention times of the components of interest

Component	Average retention at liquid phase sampling GC (min)	Average retention at headspace sampling GC (min)
Ethanol	1.73	3.56
Acetone	2.47	5.14
Methylethylketone	not detectable in preserved raw milk matrix	10.04

2.2. GC methods

During the calibration process, excellent separation of ethanol, acetone and methylethylketone was obtained by the developed and previously described GC methods (Fig. 4). Comparing the separation of two GC methods during the measurements of the milk samples, it was found that the liquid phase sampling GC was not suitable to separate the methylethylketone peak from the matrix peaks clearly. We have decided using ethanol as an internal standard since it has a rather short retention time. The retention times of components of interest are shown in Table 2.

The standard curves for both methods were linear in the whole concentration range tested. They were plotted from water or milk solutions of the standards. Significant differences were not found between calibrations (Fig. 5) and measurement data of milk samples (Table 3). According to these results, the standards were prepared in water solutions. The errors of peak area ratios of headspace sampling GC are shown in Fig. 6. It was found that the deviation of analytical determinations was related to the concentration of the samples analysed. To define the reproducibility of the injection, two different concentration levels of standard solution were injected into a gas chromatograph 5 times. (Results are shown in Table 4). The standard deviations involve the differences related to the manual injection procedures. The precision of methods developed by the authors was determined by recovery percentages of the acetone added to milk samples. The results of headspace sampling process were better than those of liquid phase sampling GC (Table 5). The correlation diagram of FIA and liquid phase sampling GC method in case of 9 different milk samples are shown in Fig. 7.

Figure 8 display the correlation diagram between FIA and headspace sampling GC method for 20 milk samples analysed. In addition the FIA-results and the two GC-results were compared and analysed by t-test. Significant differences were not found between the result obtained by FIA and headspace sampling GC, but differences were found between FIA and liquid phase sampling GC, however, it must be kept in mind that in second case the number of samples was less.

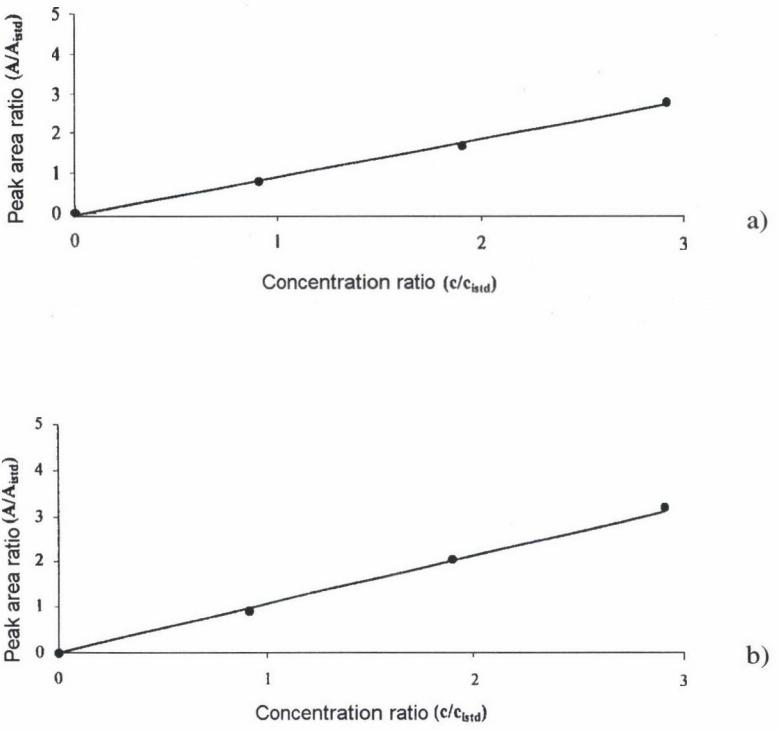


Fig. 5. Plot of calibrations for determination of acetone by liquid phase sampling GC method. Acetone standards were prepared from water standard solutions (y=0.9978x, R^2=0.9982) (a) and from milk standard solutions (y=1.0584x, R^2=0.9963) (b)

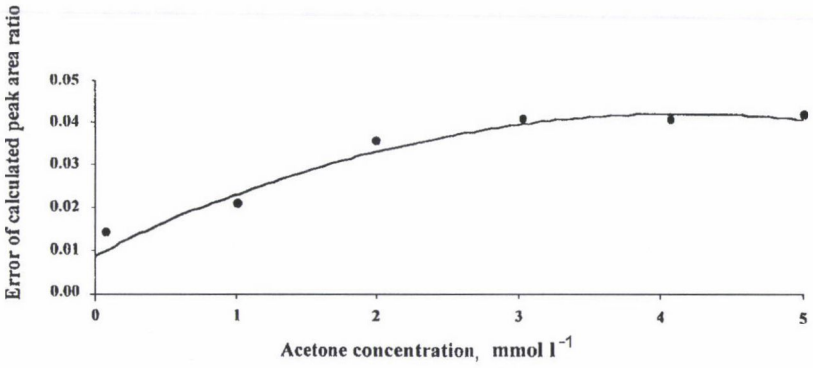


Fig. 6. Relationship between the acetone concentration and the error of analytical determination. Fitted function: $y = -0.0019x^2+0.0162x+0.0086$, $R^2=0.8799$

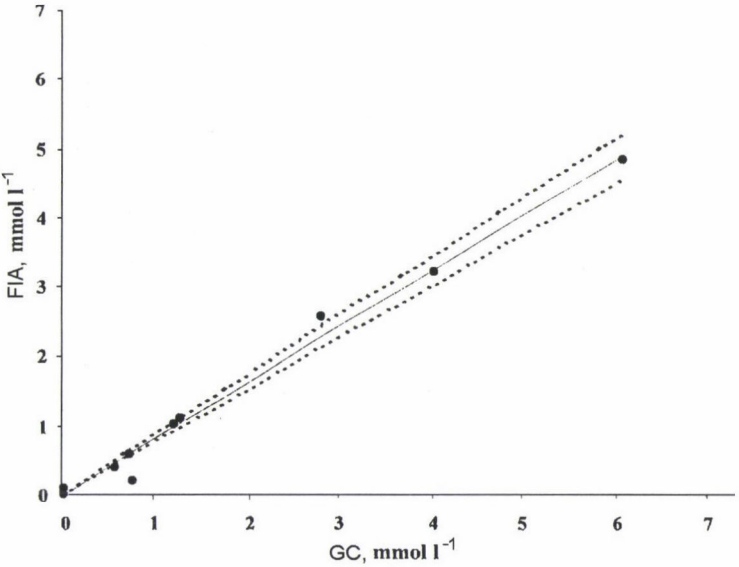


Fig. 7. Correlation between results of FIA and liquid phase sampling GC procedures for 9 milk samples. Fitted function: $y=0.8094x$, $R^2=0.9877$. Confidence band: 95%

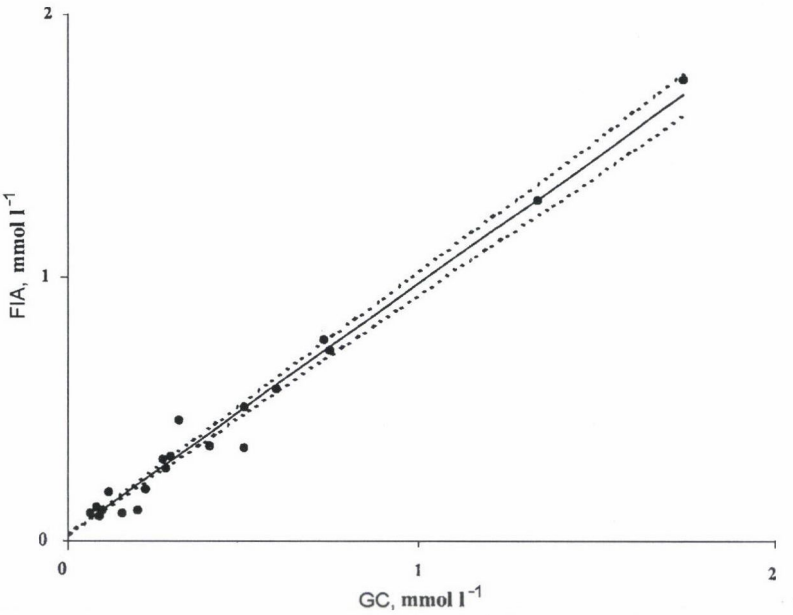


Fig. 8. Correlation between results of FIA and headspace sampling GC procedures for 20 milk samples. Fitted function: y=0.9893x, R^2=0.9807. Confidence band: 95%

Table 3

Liquid phase sampling GC measurements of raw milk samples with water and milk calibration standard solutions. The results represent 3 independent sampling

Sample id.	Average (mmol l^{-1})	SD (mmol l^{-1})	Average (mmol l^{-1})	SD (mmol l^{-1})
Milk 1	3.68	0.052	4.03	0.057
Milk 2	5.56	0.839	6.08	0.918
Milk 3	0.53	0.065	0.58	0.071
Milk 4	4.26	0.856	4.66	0.935
Milk 5	2.59	0.057	2.83	0.063
Milk 6	1.11	0.216	1.22	0.236
	(milk calibration standards)		(water calibration standards)	

Table 4

Results of the investigation of injection procedures

Sample	A/A_{istd}	Average	SD	Sample	A/A_{istd}	Average	SD
0.10 mmol l^{-1} st.	0.075	0.126	0.0708	0.05 mmol l^{-1} st.	0.039	0.059	0.0141
	0.116				0.059		
	0.248				0.065		
	0.110				0.053		
	0.079				0.077		
1.00 mmol l^{-1} st.	0.639	0.648	0.0059	0.50 mmol l^{-1} st.	0.239	0.229	0.0177
	0.651				0.245		
	0.644				0.200		
	0.652				0.224		
	0.652				0.235		
(liquid phase sampling GC method)				(headspace sampling GC method)			

Table 5

Precision of GC methods. The results represent 5 independent determinations

Acetone addition	Average recovery (liquid phase sampling GC method) (%)	Average recovery (headspace sampling GC method) (%)
0.25 mmol l^{-1}	105.26	102.51
2.50 mmol l^{-1}	88.76	100.37
5.00 mmol l^{-1}	102.67	98.64

3. Conclusions

We have launched, optimised and validated a FIA and two possible reference GC methods for the determination of acetone in milk samples. The methods were tested with preserved raw milk samples. The results obtained by optimised FIA method were compared with the results of the headspace and liquid phase sampling GC. We had to take into consideration the results of validation procedure and disadvantages of the liquid phase sampling GC method: it requires too long a time and is very expensive sample preparation method for biological samples such as milk. The advantages of the first are: simple and rapid GC determination can be carried out using by headspace sampling (Table 6). Therefore we suggest using the headspace method as a reference method of acetone determination in raw milk samples in the range of mmol l^{-1} concentrations.

Table 6

Comparison of the different methods

Investigated parameter	FIA	Liquid phase sampling GC	Headspace sampling GC
– Detection limit (mmol l^{-1})	0.03	0.01	0.01
– Linear range (mmol l^{-1})	0–10	0–10	0–10
– Calibration requirement	before each batch	only control	only control
– Sensitivity (slope of linear calibration graph)	225.9 ± 4.74 (Abs/mmol l^{-1})	0.998 (peak/conc. ratios)	0.453 (peak ratio/conc.)
– Error of analytical determinations (SD %)	–	<1	<1
– Reproducibility (SD %)	<2.2	<2.0	<2.0
– Accuracy (aver. recovery %)	103.8	98.9	100.5
– Retention time of acetone (min)	–	2.74	5.14
– Sample preparation	minimal	complex	minimal
– Time/sample (min)	1.5	180	15
– Cost/sample (USD)	0.2	3	5
– Correlation with FIA (corr. coeff.)	–	0.993	0.991

From the investigations presented in this paper the final conclusion is: both, either the FIA method developed should be chosen, – because of its high accuracy, precision, sensitivity and reproducibility – or the headspace GC method can be applied for cow's milk-monitoring. The first is faster and more profitable, especially considering costs per test.

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The authors wish to express their appreciation to Prof. S. KEMÉNY (Budapest University of Technology and Economics, Department of Chemical Unit Operation) for his instructions in the statistical analysis, to Mrs. K. GUNDEL, Mr. S. PRÓKAI and Dr. F. KATONA (Hungarian Herd Recording Ltd., Gödöllő) for making raw milk samples available; to Mrs. Á. MISKOLCZI and Miss A. TARKÓ for their technical assistance (Budapest University of Technology and Economics, Department of Biochemistry and Food Technology).

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Acta Alimentaria, Vol. 30 (3), pp. 313–322 (2001)

EFFECT OF BREED, LIVE WEIGHT ON THE FATTY ACID, AMINO ACID CONTENT AND ON THE BIOLOGICAL VALUE OF BEEF

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(Received: 10 October 2000; accepted: 16 November 2000)

The meat of 21 Hungarian Simmental and 17 Holstein-Friesian cattles was analysed for fatty acid and amino acid content, and also for the biological value of the meat protein. It can be established that the proportion of the saturated and the mono- and polyunsaturated fatty acids compared to each other is not significantly influenced by the breed and the live weight at the various types and weight categories. The increase in the live weight goes together with the increase in the ratio of the monounsaturated fatty acids in the meat in case of both breeds. The amino acid content of the meat was not significantly influenced by the breed, even the live weight didn't demonstrate any effects. The essential amino acid content and the biological value of the meet of the Hungarian Simmental are practically the same as those of the Holstein-Friesian.

Keywords: Meat composition, Hungarian Simmental, Holstein-Friesian, amino acid composition, biological value, fatty acid content

According to the lipid-theory worked out in the 50s (KEYS et al., 1957), the cholesterol and the saturated fatty acids content of the animal fats are the key factors to cause arteriosclerosis, high blood-pressure, frequent apoplexy and cardiac infarct in humans (SZAKÁLY, 1995). According to this theory, the cholesterol level of the blood plasma can be reduced by 10 percent with the help of polyunsaturated fatty acids and with the consumption of food containing cholesterol at a decreased level. A positive correlation was established between the blood cholesterol level and arteriosclerosis at the majority of the individuals examined. The theory significantly contributed to the decrease in the use of animal fats in the whole world due to the research studies of the last more than 40 years, proving the multiple susceptibility to the influence of the high cholesterol level of the plasma.

It is widely known that cholesterol is of vital importance for human body, as it is indispensable to the production of sexual hormones, the group of vitamin D, the

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0139-3006/2001/$ 5.00

hormones of the adrenal cortex and the bile acids, which are needed to digest fats. The cholesterol molecules infiltrate the cell membrane in order to protect the cell and the body from various exterior effects. The daily cholesterol demand of the human organism is 1000–2000 mg, out of which 80% is synthesised by the organism itself and only 20% comes from the food, since a part of the food-originated cholesterol leaves the human organism undigested in the excrement.

Concerning the level of cholesterol in animal-originated foods the brain contains 3000–5000 mg/100 g, eggs and giblets contain 400–450 mg/100 g, and animal fats, meats and meat products contain 80–100 mg/100 g cholesterol. Consequently the organism can take up only a certain proportion of its cholesterol content from meat and animal fats and the major part of our demand is synthesised by the human body.

The fatty acid content of animal fats – as well as cholesterol – and most of the saturated fatty acids are considered to be responsible for provoking the diseases mentioned above. Plants, containing unsaturated vegetable oil in a considerable amount, are considered to be very advantageous in preserving our body's health. KEYS and co-workers (1957) stated that the fatty acid composition is optimal if it contains saturated (SAFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids at a 1:1:1 proportion. OKUYAMA and IKEMOTO (1999) established that concerning the various illnesses it is not the cholesterol content of the consumed food that is important, but rather the ratio of the n6/n3 PUFA, which has to be kept at the lowest level, and which ratio can be significantly modified by changing the n6 and n3 PUFA content of the fodder. DE DECHERE and co-workers (1998) established that the n3 PUFA content of the sea fish reduces the illnesses of the coronary artery and the reduction can be reached by a consumption of 200 mg n3 PUFA per day. According to their studies the n3/n6 PUFA ratio of the sea fish did not really influence the diseases mentioned above.

The beef consumption plays an important role in the balanced nutrition in Hungary due to its high nutrition-biological values. The nutritious components of the meat can be digested easily, the biological value of its protein is high, it is an important source of macro and micro elements, and it is also known that almost all of its components can be biologically digested in an excellent way (BRUCE, 1994). However, the protein and the fatty acid content of the meat have to be taken into serious consideration from the point of view of human nutrition. The question of how to preserve your health through your nutrition came into the centre of interest in the last few decades in Hungary, according to which the consumer's demands on the quality of the meat changed as well. Even facing the detriment of tastiness, consumers laid stress on the consumption of non-fatty meats and on fats which contain higher amount of polyunsaturated fatty acids, as these – being described before – play a primary part in preventing heart and vascular system diseases.

For further support of the meat playing an extremely important part in the human nutrition, the analysis of the fatty acid components of the meat of the most frequently bred cattle of Hungary, the Holstein-Friesian and also the Hungarian Simmental was set as an aim. The ratio of the saturated and unsaturated fatty acids, the amount of protein, amino acid components and biological values were also determined. In our research we would like to present further data for understanding better the composition of meat in order to highlight its role in healthy nutrition.

Unsaturated fatty acids play an important role in creating tastes and aroma (LESEIGNEUR & GANDEMER, 1991) but on the other hand, the increase of their proportion decreases the oxidative stability of the fats of the muscles and so it favours the deterioration processes (SHAHIDI, 1992). Many studies refer (PERRY et al., 1998) to the fact that the proportion of the saturated and unsaturated fatty acids is significantly influenced by the feeding. According to the research of MANDELL and co-workers (1998), the amount of the polyunsaturated fatty acids can be significantly increased by the fattening on the grazing grounds. MALAU-ADULI and co-workers (1998) doing research on Jersey and Limousine cattle, experienced a significant influence of variety, gender and age concerning the amount of the saturated and unsaturated fatty acids. Similarly, RULE and co-workers (1999) demonstrated differences within breed (Hereford, Limousine, Piemont) according to the fatty acid components of the fats.

HUERTA-LEIDENZ and co-workers (1996), determining the fatty acid components of the fatty tissue of Hereford and Brachmann bulls at different age, could demonstrate significant differences considering only their MUFA level. According to their research work, during the fattening the amount of the saturated fatty acids decreased by 10% by the increase of age; the MUFA increased significantly, while the PUFA just slightly increased, and the proportion of the 18:2 and 18:3 fatty acids changed as well. KAZALA and co-workers (1999), studying the fatty acid components of the rib muscle and *musculus longissimus dorsi* of the cross-breed Wagyu cattle, did not find any differences between the MUFA/SAFA proportion of the muscles. They established that the oleic acid content of the fat of the heifer's muscle tissue is higher and its palmitic acid content is lower than those of the bulls at the same age. The amount of the miristic acid increases at both muscles by increasing fat content, while no such relation was found in case of the linoleic acid.

The types of the proteins of the muscles are varying, which significantly influences the amino acid composition of the muscle. The nutritive value of the protein depending on the protein composition of the different tissues is determined above all by the amount and the proportion of the essential amino acids, but it changes by the age and by the live weight, and it can be influenced by the breed as well (PIVA & GUGLIELMETTI, 1978). The consumer' assessment on the value of the meat should not be rendered independent of the amino acid composition of the proteins, because a

negative relation was observed (SZŰCS et al., 1985) between the arginine and histidine content of the meat and its tastiness. MOLNÁR and MOLNÁR (1981) studying the amino acid composition of different muscle groups of Hungarian Simmental cattle differing in sex and age established significant differences in case of methionine, lysine and arginine content. They also established that the amino acid composition of the different muscles changes according to their age, and even the amino acid content is influenced by the stress of the muscles as well.

1. Materials and methods

During our research meat components of 21 Hungarian Simmental and 17 Holstein-Friesian cows were analysed. The average live weight of the animals was 520 kg, which was ranked between a small weight (400–500 kg) and a large weight (501–600 kg) category. When removing the bones after the slaughter, the in-between part of the 11th and 13th rib was removed from the right half part of the animal. After the homogenisation of this slice the determination of the protein content was carried out with the help of a Kjel-Foss fast nitrogen analyser and the fat content was analysed as well. Both determinations were done according to the relevant Hungarian standards. The fatty acid content of the fat was analysed by a Chrompack CP 9000 gas chromatograph; the amino acid content was analysed by a Labor MIM amino acid analyser. When analysing the fatty acid content the results relating to the unknown sample were given as the relative mass percentage of the fatty acid methyl esters (CSAPÓ et al., 1995). During the amino acid determination the protein was hydrolysed in 6 mol l^{-1} hydrochloric acid during 24 h, followed by a simultaneous hydrolysis in which the amino acids containing sulphur, were determined in an oxidised form after performic acid oxidation (CSAPÓ et al., 1986). The biological value of the protein was calculated according to the method of MORUP and OLESEN (1976), where the reference basis was the 2:1 mixture of potato and egg white.

The statistical evaluation was carried out with the SPSS 9.0 statistical program with the general linear model of 2×2 factorial arranged III. Sum of square type, where apart from the two basic effects, the variety, the slaughtering and the weight category were also analysed. When analysing the fatty acid content in the first model the mono fatty acids were grouped according to whether they were once/polyunsaturated or saturated, while in the second model the fatty acids were grouped according to whether they were n3 or n6 fatty acids. Three models were set up in the case of the estimation of the amino acids: the first contained only the essential amino acids, the second contained only the non-essential amino acids and the third contained both the essential and nonessential amino acids and also their biological value.

2. Results and discussion

Table 1 shows the fatty acid content of the meat samples from various breeds between 400–500 kg and 501–600 kg weight categories. Table 2 shows the effects of the variety and the live weight on the fatty acid content, while the amount of the essential and non-essential amino acids is reported in Table 3. The effects of the variety and body weight on the amino acid content and on the biological value were summed up in Table 4.

Table 1

The fatty acid composition of rib samples from different breeds and weight.
(Relative percentage of the fatty acids methyl-esters)

Fatty acids	Hungarian Simmental (n=21)				Holstein-Friesian (n=17)				n=38	
	400–500 kg		501–600 kg		400–500 kg		501–600 kg		Total	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
10:0	0.005	0.03	0.005	0.03	0.005	0.03	0.012	0.04	0.007	0.003
12:0	0.007	0.01	0.007	0.01	0.007	0.01	0.007	0.01	0.007	0.001
14:0	2.88	0.15	3.06	0.15	2.79	0.16	2.7	0.17	2.87	0.01
15:0	0.50	0.04	0.469	0.04	0.578	0.05	0.458	0.05	0.50	0.002
16:0	28.38	1.69	26.92	1.62	29.03	1.79	29.73	1.89	28.39	0.86
16:1	3.94	0.39	4.97	0.37	3.79	0.41	3.75	0.43	4.16	0.21
17:0	1.20	0.1	1.22	0.1	1.17	0.11	1.24	0.12	1.20	0.01
18:0	20.82	1.57	17.80	1.49	21.89	1.65	19.49	1.75	19.91	0.81
18:1	38.33	1.64	42.33	1.56	37.20	1.73	39.35	1.83	39.44	0.87
18:2 n6	2.16	0.12	1.95	0.11	1.96	0.13	1.97	0.13	2.01	0.01
20:0	0.25	0.03	0.15	0.03	0.24	0.03	0.19	0.03	0.20	0.002
20:1	0.15	0.03	0.14	0.03	0.15	0.04	0.19	0.04	0.15	0.002
18:3 n3	0.52	0.05	0.43	0.05	0.36	0.05	0.32	0.06	0.41	0.003
20:3 n6	0.21	0.03	0.19	0.03	0.19	0.04	0.20	0.04	0.20	0.002
20:4 n6	0.37	0.07	0.20	0.06	0.31	0.07	0.22	0.08	0.27	0.003
Saturated fatty acids	54.15	1.92	49.73	1.83	55.81	2.03	54.01	2.15	53.23	1.02
Monounsaturated fatty acids	42.42	1.90	47.43	1.81	41.14	2.00	43.28	2.13	43.75	1.02
Polyunsaturated fatty acids	3.26	0.18	2.77	0.17	2.82	0.19	2.72	0.20	2.90	0.01
Σ n6	2.74	0.19	2.34	0.18	2.46	0.20	2.40	0.22	2.49	0.01

Table 2

The effects of the breed and the live weight on the fatty acids composition

Fatty acid methyl ester (%)	Breed	Live weight	Breed × live weight
10:0	NS	NS	NS
12:0	NS	NS	NS
14:0	NS	NS	NS
15:0	NS	NS	NS
16:0	NS	NS	NS
16:1	NS	NS	NS
17:0	NS	NS	NS
18:0	NS	NS	NS
18:1	NS	NS	NS
18:2 n6	NS	NS	NS
20:0	NS	**	NS
20:1	NS	NS	NS
18:3 n3	**	NS	NS
20:3 n6	NS	NS	NS
20:4 n6	NS	NS	NS
Saturated fatty acids	NS	NS	NS
Monounsaturated fatty acids	NS	NS	NS
Polyunsaturated fatty acids	NS	NS	NS
Σ n6	NS	NS	NS

NS: Not significant
** $P < 0.01$

In the first model neither the variety nor the weight category had any effects on the fatty acid content of the fat. Arachinic acid (20:0) and linolenic acid (18:3n3) turned out account for the greatest proportion (R^2=0.213, and R^2=0.201) in the calculation of the total variance. On the other hand in case of the second model both the variety and the live weight possessed significant impacts. As it can be seen in Table 1, the monounsaturated fatty acid, the oleic acid, and two saturated fatty acids, the palmitic acid and the stearic acid give 90% of the whole fatty acid content. The ratio of the PUFA/SAFA is an average of 0.06, which seems to be the most favourable in the small weight Hungarian Simmental group. The increase in the live weight at both breeds goes together with the increase in the monounsaturated fatty acid ratio and with the decrease in the amount of the saturated and unsaturated fatty acids. In the latter case an increase concerning the n3 fatty acids can only be experienced at the great weight Holstein-Friesian category. According to the data given in Table 2, only the arachidonic acid shows significant differences concerning the variety and only the linolenic acid has similar effects concerning the live weight.

Table 3

The amino acid composition of rib samples from different breeds and weight

Amino acids	Hungarian Simmental				Holstein-Friesian				n=38	
g/100 g protein	400–500 kg		501–600 kg		400–500 kg		501–600 kg		Total	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Essential amino acids	47.2	0.39	46.8	0.3	46.7	0.4	46.2	0.4	46.7	0.20
Arginine	6.11	0.15	6.08	0.1	6.02	0.1	5.9	0.1	6.04	0.00
Phenylalanine	4.14	0.08	4.12	0.0	4.19	0.0	4.23	0.0	4.17	0.00
Histidine	4.24	0.11	4.12	0.1	3.92	0.1	3.96	0.1	4.07	0.00
Isoleucine	4.23	0.11	4.26	0.1	4.4	0.1	4.36	0.1	4.31	0.00
Leucine	8.22	0.10	8.16	0.1	8.34	0.1	7.99	0.1	8.18	0.00
Lysine	9.25	0.17	9.09	0.1	9.09	0.1	8.7	0.1	9.05	0.00
Methionine	1.65	0.15	1.68	0.1	1.73	0.1	1.44	0.1	1.63	0.00
Threonine	4.91	0.01	4.87	0.1	4.58	0.1	4.45	0.1	4.72	0.00
Valine	4.52	0.15	4.43	0.1	4.49	0.1	5.18	0.1	4.62	0.00
Non-essential amino acids	51.6	0.42	52.0	0.4	52.1	0.4	52.4	0.4	52.0	0.21
Alanine	6.64	0.27	6.19	0.2	6.92	0.2	6.91	0.3	6.63	0.14
Aspartic acid	8.92	0.21	9.08	0.2	8.82	0.2	8.71	0.2	8.9	0.10
Cystine	0.85	0.11	0.98	0.1	0.99	0.1	1.19	0.1	0.99	0.00
Glycine	5.65	0.31	6.18	0.3	6.62	0.3	7.03	0.3	6.32	0.17
Glutamic acid	16.8	0.22	17.0	0.2	16.4	0.2	15.8	0.2	16.6	0.13
Proline	4.97	0.25	4.99	0.2	4.86	0.2	5.30	0.2	5.02	0.13
Serine	4.05	0.09	4.07	0.1	3.77	0.1	3.83	0.1	3.94	0.00
Tyrosine	3.77	0.10	3.51	0.1	3.70	0.1	3.65	0.1	3.65	0.00
Biological value	74.4	3.47	73.0	3.3	73.8	3.6	79.1	3.8	74.8	1.75

The analysis of the 3-model set up for the evaluation of the amino acid results shows that the impact of the variety seemed to be significant at the first model, containing essential amino acids, while concerning the other two models neither the impact of the variety nor the live weight seemed to be significant. The interaction of [variety × live weight] could not be demonstrated in either model. From the essential amino acids it was the threonine (R^2=0.290) and the valine (R^2=0.287) which mostly took part in creating the variance, so almost 50% of the total variance was given by these two essential amino acids. Out of the non-essential amino acids glutamic acid (R^2=0.326), glycine (R^2=0.224) and serine (R^2=0.188) played a significant role within the total variance. According to the data in Table 3, the amount of the essential amino acids in the case of the Hungarian Simmental is bigger in both weight categories than in

the Holstein-Friesian's group. Considering all the individuals, the biological value is 74.89, which corresponds very well to the value of about 72–76 given by the literature (HEGEDŰS et al., 1981; ENSMINGER et al., 1995). It deserves attention that the biological value is 79.19 at the heavier (501–600 kg) Holstein-Friesian cattle. Table 4 shows that in case of the amino acid content of the meat of the two breeds, a significant difference was reported in the essential amino acids such as histidine, threonine and valine, while considering the non-essential amino acids it was glycine, glutamic acid and serine which produced no significant differences. Between the live weight categories only leucine produced no significant difference, and regarding the interaction of the two main influences it was valine, turned out to be significant.

Table 4

The effects of the breed and the live weight on the amino acid composition and the biological value

Amino acids (g/100 g protein)	Breed	Live weight	Breed × live weight
Essential amino acids	NS	NS	NS
Arginine	NS	NS	NS
Phenylalanine	NS	NS	NS
Histidine	*	NS	NS
Isoleucine	NS	NS	NS
Leucine	NS	*	NS
Lysine	NS	NS	NS
Methionine	NS	NS	NS
Threonine	***	NS	NS
Valine	*	NS	*
Non-essential amino acids	NS	NS	NS
Alanine	NS	NS	NS
Aspartic acid	NS	NS	NS
Cystine	NS	NS	NS
Glycine	**	NS	NS
Glutamic acid	***	NS	NS
Proline	NS	NS	NS
Serine	**	NS	NS
Tyrosine	NS	NS	NS
Biological value	NS	NS	NS

NS: Not significant
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Comparing the fatty acid content of the beef with the fatty acid content of the pork (CSAPÓ et al., 1999; HUGO et al., 1999), it can be pointed out that the amount of the saturated fatty acids is larger in the analysed meat concerning all the fatty acids, while the oleic acid and linoleic acid content of the pork fat are substantially larger than that of the meat analysed. Comparing the fatty acid content (HERNANDEZ et al., 1998) of the *musculus longissimus dorsi* of the pork with that of the rib, it can be ascertained that it is richer in saturated fatty acids; the oleic acid content is approximately the same, while among the polyunsaturated fatty acids the linolenic acid content is seven times higher and the arachidonic acid content is fifteen times higher than that of the analysed meat. It can be stated as a summary that the saturated fatty acid content of the analysed meat is higher and its unsaturated fatty acid content is lower than that of the lard and the pork.

3. Conclusions

Studying the fatty acid content of the cattle meat of various breeds and live weight we established that according to the valuation of the GLM model the ratio of the saturated and mono- and polyunsaturated fatty acids compared to each other is not significantly influenced by the breed and the live weight in most cases. The ratio of the monounsaturated fatty acid content increases in the meat in line with the increase in the live weight. Regarding the achievements of our research, the amino acid content of the meat in the case of most amino acids is not significantly influenced by the breed and also there is no pronounced impact of the live weight. The essential amino acid content and the biological value of the meat of the Hungarian Simmental individuals practically show no difference from the meat of the Holstein-Friesian breed.

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Acta Alimentaria, Vol. 30 (3), pp. 323–327 (2001)

Book reviews

Diet and health: scientific perspectives

W. J. VEITH

Medpharm, Stuttgart, 2nd ed., 1998, ISBN 3-88763-068-8, 277 pages

The science-based nutrition has developed revolutionary in the last decade, nevertheless there is still much to learn. It is becoming more and more apparent that dietary practices impact directly on the health, and many diseases can be traced directly to the composition and nutritional pattern of foods. Eating habits have also changed drastically in these modern times and highly processed foods, higher intake of animal products have become an established part of the everyday diet of the majority of the people living in industrialised countries. It is a pity, that degenerative diseases are particularly common in these societies. Healthy eating practices are known no guarantee of good health but it might have a main impact on the quality of life. The relationship between the diet and degenerative diseases such as cancer, cardiovascular disease and osteoporosis is well established, it is known what the contributory factors are, but the best alternative lifestyle is still an issue of debate.

The first edition of "Diet and Health" was published at the end of 1993, but the concern about the food safety issues has underlined the need for a comprehensive reassessment of this field of science. Some of the concern expressed in the first edition regarding the safety of animal products in the human diet, such as prion diseases, global increase in food poisonings, attributed to antibiotic-resistant bacteria, genetic engineering have been found to be more than warranted. On the positive side, this book is an attempt at correlating scientific data regarding the causative agents in dietary related diseases, and also provides an insight into the field of alternative lifestyles.

This edition contains information from authentic and highly regarded sources. A wide variety of references are listed. Reasonable efforts have been made to publish reliable data and information for the nutritionists, food scientists, pre-trained consumers, but the author and the publisher could not assume responsibility for the validity of all materials or for the consequences of their use.

The part and chapters are as follows: Part 1- Diet and Health. Chapter 1: Nutritional importance of proteins (amino acids, protein digestion, plant and animal proteins, requirements); Chapter 2: Role of carbohydrate and fibers in human diet and health (carbohydrate in the foods, digestion, diet and control of glucose levels, NSP fiber and digestion of resistant starch); Chapter 3: Fats in the diet and fat related health risks (fats in the diet, digestion and absorption of fats, fats and diseases, processed fats); Chapter 4: Animal products related health risks (ammonia, phenols, PAH, *N*-Nitroso compounds etc. related risks in meat, dairy products related lactose and milk intolerance, animal products and foodborne illnesses as *Salmonella, Campylobacter, Listeria, Escherichia coli, Yersina* infections); Chapter 5: The vegan and vegetarian lifestyle (vegan dietary practices, health aspects of the vegetarian diet, osteoporosis); Chapter 6: Additional dietary components and hazards (vitamins and minerals in vegan diets, food additives, caffeine and alcohol and diet). Part 2 – An alternative lifestyle. Chapter 7: The whole-food alternative based general guideline

to healthful living (what is the whole food, general guidelines, ensuring proper food combinations as acid and alkaline-forming foods, combining fruits and vegetables or grains and legumes, grains, bread, legumes, nuts and oilseeds, seeds, fruits and vegetables, fruits, stone fruits, pip fruits, berry fruits, citrus fruits, subtropical and tropical fruits, vegetables, *Chenopodiaceae*, *Compositae*, *Convolvulaceae*, *Cucurbitaceae*, *Cruciferae*, *Leguminosae*, *Liliaceae*, *Malvaceae*, *Solanaceae*, *Umbrelliferae*). Part 3 – Applying the concept. Chapter 8: Guideline and recipes (introduction to the useful equipment, basic shopping lists, basic recipes).

The detailed Register at the end of the book helps the reader in finding the subject he is interested in.

É. GELENCSÉR

Technology of biscuits, crackers and cookies

D. MANLEY

Woodhead Publishing Ltd, Cambridge, 2000,
ISBN 1 85573 532 6, 480 pages

Duncan Manley's book, the third edition of which was published recently and is richer than the previous ones were, is well known among the specialists of this field. It completely covers the field mentioned in the title. The great many receipts as well as the numerous figures, among them the flow sheets and the simple technical schemas that dwell on the essence, makes the book suitable for getting a considerable role in education, too. Naturally, it deals with technology, but its strong point is that the technical points are also emphasised. Given its character, it contains formulas only moderately, and there are no design manuals and technical sizing work-help in it at all, for it was not made for mechanical engineers and factory constructors but for technologists. The work is very well arranged, which is backed up by a seven-page index.

The publication of the new edition is timely, because nowadays eating out is getting a larger and larger role, and there are a lot of people who prefer the tasty food with smaller mass between the principal meals, which resulted in the development of technology and marketing of many snack products. This phenomenon excited the interest of product-developers and scientists engaged in dietetics as well, and there are more and more of these kind of products on the market. Nowadays, it seems that we can count on the further increase of this interference of tendencies.

Specialists, dealing with this topic, generally know well and have read Duncan Manley's book with benefit, the third edition of which, at the first glance, does not differ in many respects from the previous ones. The essential difference is that in this edition the newer results of the development of production technology are included, and the dietetical judgement of these products is given a larger emphasis as well. The book deals first of all with technology in accordance with its title, but it goes well beyond it. Those aspects are dominant, too, by the help of which nutritional enhancement and technological possibilities occur by using

newer raw materials, subsidiary materials and additives. It is also a novelty that the nowadays indispensable quality control systems are specifically reviewed. An increased interest appears for the previously mentioned topic primarily in field of balanced and guaranteed quality bulk food production.

The structural mode represents a special value by which the author publishes technical sources related to a given question in abundant quantity besides the basic references. This allows a better understanding of field, and in addition it made the profound study in certain minor questions possible. This principle is definitely more pronounced in the new edition.

The book is a useful source of knowledge not only for specialists in practical manufacture, but for those engaged in education as well.

L. SZALAI

Managing frozen foods

CH. J. KENNEDY (ED.)

Woodhead Publishing Limited, Cambridge, 2000, ISBN 1 85573 412 5, 286 pages

Experts from frozen food industry and academia across the European Union collaborated in this work. They took into consideration the many technical and scientific developments being made in the field of frozen foods. It can be realized that the emphasis of their action was to promote the existing best practice as well as to look to the future. Their aim has been to write at a level that will be of use to the professional food technologists or engineers working in the frozen food industry. Furthermore this book is suggested to all who are interested in this field, including students, researchers.

The winning strategy of the frozen food industry is to improve quality continually, in order to consolidate and increase its presence alongside the large fresh, and minimally processed. fruit and vegetable market. The best way to increase the range of frozen vegetable products is to ensure that they have similar characteristics to fresh ones. This depends on many factors, and on two in particular: raw material and technological processing. The raw material quality, which could be notably improved, depends on agronomic factors (e.g. production, sensitivity to disease, suitability for mechanical harvesting) to be considered from development through to harvest. The quality of raw material suitable for freezing needs to be highlighted. Fruits and vegetables are very sensitive to freezing damage. The chemical and physical actions of freezing can be highly detrimental to them, since their texture is mainly ensured by turgor. When the fruits and vegetables reach the processing industry they must have specific attributes defined by the following: technical experience, specific techniques arranged between farmers and industries, quality standards created by expert organisations.

Twelve chapter summarise information on frozen food industry including all the important aspects. Following the introduction in Chapter 2 the main aspects of maintaining safety in the cold chain is summarised. In Chapters 3 to 6 the quality of raw material (fruit and vegetables) in relation to freezing,

pretreatment of fruits, vegetables, meat and fish are discussed. In Chapter 7 the physical and biochemical processes that take part in the deterioration of frozen food products are reviewed. In Chapter 8 the author provided an overview of the freezing of the major categories of processed foods. In particular, ready-to-eat meals, bakery products and icecream are considered. New developments are reviewed in freezer technology by one of the leading technologists in Chapter 9. In Chapter 10 they review the properties of the wide range of materials now in use for the packaging of frozen foods including their handling and processing properties. In Chapter 11 the main steps of the cold chain between the production process and consumption are summarised (storage, shelf-life and products quality, cold store design, monitoring and recording product conditions). In Chapter 12 the main characteristics of a good frozen food retail display cabinet are summarised. In the last Chapter C. J. Kennedy takes the reader along the frozen food chain of the future. The textural quality and the retained nutritional content of frozen foods after thawing will be improved by new manufacturing processes such as pressure shift freezing which allow the production of smaller ice crystals during freezing. Stability will also be improved by formulation and by understanding of mobility and biochemistry in the unfrozen component.

References at the end of each chapter give guideline for the readers to find information concerning a definite subject. The collection of the references has proved the experience of the authors. At the end of the book we can find an index including all important words and phrases help to readers to find a definite subject.

M. NAGY-GASZTONYI

Cereal biochemistry

P. C. MORRIS and J. H. BRYCE

Woodhead Publishing Limited and CRC Press LLC, Cambridge, 2000, ISBN 1 85573 498 2, 252 pages

Genetic modification (GM) is a fairly new scientific field in which the new genetic information is introduced to the genom. The book highlights significant results of genetic transformation e.g. integration of genes of insect resistance or herbicide tolerance, into wheat, maize, rice and barley. The aim of the book is to introduce the reader into the world of GM-technology, product development, risk assessment and current industrial practices.

In the Introduction the most important cereals, their annual, global production, as well as modern plant breeding strategies are reviewed in a concise form. Most of the scientific information and specific terms needed to comprehend the Chapters are clearly presented.

The further Chapters are as follows: Chapter 2: The genetic transformation of wheat and barley (target tissues, delivery of DNA, selection and regeneration, examples), Chapter 3: The genetic transformation of rice and maize (approaches, vectors, agronomically useful genes), Chapter 4: Product development in cereal biotechnology (commercial targets, efficacy screening, molecular quality control,

regulatory issues and risk assessment), Chapter 5: Using biotechnology to add value to cereals (weed control, disease resistance, improved nutritional and processing properties), Chapter 6: Molecular biological tools in cereal breeding (markers, characters, deployment of molecular markers), Chapter 7: Risk assessment and legislative issues (general principles, assessing the impact of genetically modified crops, regulation of biotechnology, public perceptions), Chapter 8: Current practice in milling and baking (composition, requirements for milling, bread baking, biscuit manufacture), Chapter 9: Current practice in malting, brewing and distilling (fundamentals, current practice, limitations), Chapter 10: Current practice in cereal production, Chapter 11: Summary and conclusion (improving cereal production and quality, potential of cereal biotechnology, commercial practice.

Authorized transgenic crops were introduced into the market in the recent past, and special emphasize has been given to future prospects. It is clearly reflected in the list of references at the end of each chapter, that the scientific results could only cover the last decade. Much work has been done to develop existing GM-technologies, which may result in the formation of a new gene-technology driven agro-industry, or a branch of the existing agriculture. Many effects caused by the presence of GM-species in the environment are discussed or unknown today, however, much work is in progress to explore relationships with other areas. The detailed index given at the end of the book, helps the reader to get quick information.

This book is a valuable source for all those, who want to get a deeper insight into this progressive field.

J. SZAMOS

Typeset by
WEGATREND Kft., Budapest

PRINTED IN HUNGARY
PXP Ltd., Budapest

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ACTA ALIMENTARIA

An International Journal of Food Science

Editor
J. HOLLÓ

Volume 30 **December 2001** **Number 4**

Akadémiai Kiadó
Budapest

ISSN 0139-3006
CODEN ACALDI

ACTA ALIMENTARIA

An International Journal of Food Science

Sponsored by the Joint Complex Committee on Food Science of the Hungarian Academy of Sciences and published by financial support of the Ministry of Agriculture and Regional Development.

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Acta Alimentaria is a quarterly journal in English, publishing original papers on food science. The main subjects covered are: physics, physical chemistry, chemistry, analysis, biology, microbiology, enzymology, engineering, instrumentation, automation and economics of foods, food production and food technology.

Distributor:

AKADÉMIAI KIADÓ
H-1519 Budapest, P.O. Box 245, Hungary

Fax: (36-1) 464-8221
E-mail: kiss.s@akkrt.hu
http://www.akkrt.hu

Publication programme, 2001: Volume 30 (4 issues)

Subscription price per volume: US$ 236.00, including normal postage, airmail delivery US$ 20.00.

Acta Alimentaria is published 4 times per annum: March, June, September and December

Acta Alimentaria is surveyed by Chemical Abstracts, Current Contents/Agriculture, Biology and Environmental Science, ASCA, BIOSIS, Nutrion Abstracts and Reviews.

AAlim 30 (2001) 4

ACTA ALIMENTARIA

AN INTERNATIONAL JOURNAL OF FOOD SCIENCE

VOLUME 30
2001

AKADÉMIAI KIADÓ
BUDAPEST

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Acta Alimentaria, Vol. 30 (4), pp. 329–331 (2001)

Editorial

BIOFILM – A CHALLENGE TO THE FOOD INDUSTRY

However adhesion of bacterial cells to different surfaces is rather well known fact, its consequences in the different industries are dealt with only relatively recently. Interest and the necessity brought scientists together in COST projects (COST 511 on "Interaction of microbial systems with industrial materials" /finished/ and the ongoing COST 520 on "Biofouling and Materials"). The latest International Conference on Biofouling and Materials held in Frankfurt, 10–13 June 2001 was aimed at an improved understanding on the underlying processes, their recognition, countermeasures and anti-microbial strategies. The main topics were Biofouling, Biofilm Basics, Biocides, Materials Problems and Monitoring. Unlike the previous conferences, food industrial problems were also discussed, and one of the keynote lectures was dedicated to the public health implications of the biofilms. Biofouling is problem not only to the food processing industry, but also to the drinking water distribution systems.

Biofilms exist as communities of microbial species embedded in a biopolymer matrix on practically any substratum. They are ubiquitous, heterogeneous in time and space, frequently appearing as collections of mushroom-shaped microcolonies with moving water channels between them. The multistep process of biofilm formation has been studied, and a lot is already known about the adhesion, the biopolymer synthesis and its protective role. The microcolonies have defined boundaries which allow fluid channels to run through the biomatrix. This requires higher-level differentiation, quorum sensing, and the highly structured biofilm provides bacteria with a measure of homeostasis, a primitive circulatory system, and among others a large measure of protection from antimicrobial agents. The microbial cells in the biofilm are more resistant to heat, chemicals and sanitizers, and this is attributed partly to the diffusional barrier created by the biomatrix, partly to the very slow growth rates of cells in the biofilms, due to the nutrient depletion.

In the food industry the substrata on which biofilms and biofouling are present, may be the raw food material, food contact surfaces, or non-food contact surfaces (floors, walls). The microbial flora may be resident or transient, actively attached or passively retained on the surface, multiplying or not (biostasis). In all cases, there is a "biotransfer potential", i.e. the possibility to contaminate products during processing

0139-3006/2001/$ 5.00

either with spoilage or pathogenic microbes. The interaction between species within the biofilm may lead to the expression of properties unexpected from studies on individuals; therefore the threat posed to product quality or safety can be underestimated.

The primary colonisation strongly depends on material composition and chemical stability. One of the concerns was the material side of the biofilm problem, that is to develop materials with low bioadhesion, the improvement of surface material hygienic life time by selecting surface materials in combination with cleaning chemicals and cleaning procedures, optimising design of production equipment; development of environmentally friendly cleaning procedures for biofilm removal. Methods were also developed to provide a more realistic scenario for testing the cleanability of surfaces. It was clear that combination of different analytical procedures were necessary to reveal the importance of organic soil in cumulative fouling of food contact surfaces.

The sessile micro-organisms are often present in a "non-readily culturable" form, which necessitates enrichment and resuscitation techniques, and molecular based methods to evaluate the true composition of the biofilm community. The list of the different techniques applied in the biofilm research was very impressive. It was also clear that no single method can be chosen for the detection of the presence and composition of biofilm and the microbial species, but the combination of different techniques such as traditional and modern microbiological/molecular biological methods (viable cell count, fluorescence in situ hybridisation /FISH/ by rRNA targeted fluorescently labelled oligonucleotide probes, microautoradiography /MAR/ for detecting viable single bacteria), different microscopic techniques (direct epifluorescent microscopy and confocal laser scanning microscopy with acridine orange staining, viability staining, atomic force microscopy /AFM/, environmental scanning electron microscopy /ESEM/ combined with energy dispersive X-ray analysis), X-ray photoelectrone spectroscopy /XPS/, Fourier transform infra-red reflectance microscopy /FTIR/, time of flight-secondary ion mass spectrometry /ToF-SIMS/, etc., should be used to get close to reality.

Attempts were also made to develop an integrated anti-fouling strategy in general, where all steps of biofilm formation were considered from the point of view of prevention. Special attention was paid to the physiology of microbes and the possible monitoring systems.

The number of papers on bacterial adhesion and biofilm formation in the food industry is slowly increasing, however, still not enough attention is paid to the problem. On the IuFOST World Congress, held in Seoul in 22–27 April 2001, dedicated to the Paradigma Shift – Harmonization of Eastern and Western Food Systems, just some posters dealt with the investigation of bacterial attachment in the food industry. Until recently, most of the work on adhesion of food-borne pathogens, like *Salmonella* spp., *Listeria* spp., *Klebsiella* spp., *Escherichia coli*, *Enterobacte*r spp. etc., have mostly been

moderately short-term laboratory experiments. Investigations under factory conditions are very difficult, and up to now but a few paper proved the presence of persistent resident flora in food processing plant causing either public health concern or quality defect in poultry. The detection of the food-borne pathogens is a rather tedious work from a microbial community, but with the availability of molecular techniques the probability to track down these organisms is greatly increased however minor part of the total microbial population they are. The competition between species will also occur within the biofilm, and it might have some beneficial role, too. Established natural biofilms may contain a diverse range of micro-organisms, including fungi and protozoa, and probably the industrial environments contain equally diverse microbial communities, however, this area has also received little attention so far.

J. BECZNER

Acta Alimentaria, Vol. 30 (4), pp. 333–342 (2001)

CHROMIUM AND ZINC IN A SERIES OF PLANTS USED IN PORTUGAL IN THE HERBAL TREATMENT OF NON-INSULINIZED DIABETES

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(Received: 13 September 2000; accepted: 18 June 2001)

Chromium (Cr^{3+}) and zinc (Zn^{2+}) are essential micronutrients for humans. Chromium's main action is thought to be the regulation of blood sugar, because chromium deficiency is related with diabetic-like (type 2) symptoms, and chromium supplementation is associated with increased glucose tolerance and insulin sensitivity. Zinc supplementation also alleviates hyperglycaemia of the same type of diabetic patients contributing to improved insulin activity. Some Portuguese plants are commonly used as teas, alcoholic extracts or as powders by diabetic people and as medicines. Although their active principle is not yet known, the importance of their chromium and zinc content in the claimed therapeutic properties should not be disregarded. Thus, the determination of chromium and zinc in some Portuguese medicinal plants was performed by atomic absorption spectrophotometry. Almost all the analysed plants contain chromium and zinc at the normal level for these elements but many plants, which are used for the preparation of teas or other forms of remedies to help those type of diabetic patients, contain them in higher levels.

Keywords: chromium, zinc in plants, medicinal plants, antidiabetic plants

A requirement for chromium to assure normal glucose tolerance in rats was first observed by using the glucose tolerance factor (GTF), which is isolated from brewer's yeast or pork kidney (SCHWARTZ & MERTZ, 1959). In human subjects trivalent chromium supplements improve glucose metabolism (ANDERSON, 1992).

A good supply of chromium is important to keep a daily intake of about 50 μg (range 50–200 μg) (ANDERSON et al., 1992). Lists of foodstuffs with known concentrations of chromium (ADRIAN, 1991), as well as selected foods have been published (ANDERSON et al., 1992). In Iraq, for example, bread from barley flour is traditionally used in the management of diabetes mellitus, and the high content of chromium (5.69 μg/dry wt) in this flour was postulated to explain its beneficial effect (MAHDI & NAISMITH, 1991).

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0139-3006/2001/$ 5.00

Zinc has many different functions. It has been identified in over 200 enzymes, as a component of biomembranes with an important role in structure and function. It stabilizes some hormone receptor complexes, helps to stabilize the structures of RNA, DNA, ribosomes and transcription factors (KING & KEEN, 1994). Alterations in zinc metabolism have been shown to occur in both diabetic humans and experimental animals: hypozincaemia is a relatively common finding in diabetes and Zn supplementation of diabetic patients has been reported to improve immune function (KING & KEEN, 1994). Recommended zinc intake to maintain balance is about 15 mg per day for adult males and 12 mg per day for adult females (KING & KEEN, 1994).

In Portugal some plants are consumed by type 2 diabetic people to alleviate their health problems. In popular medicine those plants are well known in relation to that therapeutic property, although the involved active principle is not known. Maybe compounds containing chromium and/or zinc could also be responsible for their pharmacological effect (FELCMAN & TRISTÃO-BRAGANÇA, 1988; OSÓRIO E CASTRO, 1998). On this premise, some Portuguese plants, commonly employed to reduce sugar in the bloodstream and others without this property, were analysed for these elements, using atomic absorption spectrometry.

1. Materials and methods

1.1. Instrumentation and analysis conditions

For chromium determination a Shimadzu atomic absorption spectrophotometer (flameless atomic absorption spectrophotometer), model 6501, equipped with a Shimadzu graphite furnace, model 6FA-6000, a deuterium lamp background corrector and an autosampling system model ASC-6000 were applied in all determinations. As a light source, a hollow cathode lamp from Hamamatsu Photonics K.K. (Japan) was employed, and pyrolytic coated graphite tubes were used as atomisers.

The conditions for analysis were based on the instructions contained in the instrument manual. Samples of 10 µl were carried to the graphite tube by the autosampling system and submitted to the furnace established conditions (Table 1). The remaining parameters (slit = 0.5 mm, wavelength = 357.9 nm) were set according to manual instructions.

For zinc determination a Shimadzu atomic absorption spectrophotometer, model 6501, was used. Parameters for flame atomic absorption spectrophotometer (slit = 0.5 nm, wavelength = 213.9 nm; mode = B.C.G.; fuel: acetilene, 2 l min^{-1}; oxidant: air, 1.5 l min^{-1}; burner: 10 cm; burner height: 9 mm) were set according to manual instructions with some modifications.

Table 1

Furnace conditions for chromium analyses

Stage	Temperature (°C)	Time (s)	Heat
Drying	120	10	Ramp
Drying	120	10	Step
Ashing	600	10	Ramp
Ashing	600	10	Step
Atomization	2400	3	Step
Atomization	2400	3	Step

1.2. Sample preparation

Some plants were obtained from drug stores, others were collected in this School Farm and only the parts expected to be active were used. The first step in sample preparation was washing with bidistilled water to avoid contamination by dust and/or soil and drying at room temperature, followed by grinding in a porcelain vessel, always avoiding contact with stainless steel. The powder obtained was further dried at 75 °C for 3 h. Calcinations were performed by submitting 0.5 g of material at 500 °C for 3 h (in triplicate) in a porcelain capsule. To the remaining ash 10 ml of a mixture of suprapure $HCl:HNO_3:H_2O$ (1:1:8) was added, and after boiling for 2 min the cool solution was quantitatively transferred to a 25 ml volumetric flask to be used without further dilutions. This procedure was performed (CURTIUS & CAMPOS, 1982) and each sample was calcinated at least twice (always in triplicate) resulting in six acid solutions for repeated further analysis on different days.

1.3. Chromium and zinc analysis

Direct determination of chromium concentration in the prepared samples was performed comparing with aqueous standard chromium (Tritisol, Merck). A stock chromium solution (50 ppm) was prepared and stored in a polyethylene container. Fresh working aqueous standards were made by volumetric dilution of the stock solution to the desired concentrations with deionized water. A calibration curve automatically prepared between 20 and 100 ppb of Cr^{+3} was obtained ($r^2 = 0.9948$).

Direct determination of zinc concentration in the prepared samples was performed by comparison with aqueous standard zinc ($1.000\ g\ l^{-1} \pm 0.002$, Panreac). A stock zinc solution (1000 ppm) was prepared and stored in a polyethylene flask. Fresh working aqueous standards (0, 0.4, 0.8, 1.2, 1.6, 2.0 ppm), diluted to the desired concentrations with deionised H_2O, were used to get a calibration curve ($r^2 = 0.9992$).

To check and validate the obtained results a reference powder plant (lichen material IAEA-336) was used, showing the expected values (in the 95% confidence interval) for chromium and zinc.

2. Results

Chromium and zinc content of some plants with and without claimed type 2 antidiabetic properties were determined by using atomic absorption spectrophotometry (standard calibration method). Table 2 presents results for chromium content ($\mu g\ g^{-1}$ dry wt), for a series of 21 plants almost all from Portugal, (leaves from fruit trees), a few of which are used in the herbal treatment of diabetes (type 2) and included the alga *Fucus vesiculosus* L. It can be seen that chromium concentrations are between 0.2 (plant 21) and 7.5 $\mu g\ g^{-1}$ dry wt (plant 1) and the median of the 21 plants = 1.6±1.7 $\mu g\ g^{-1}$ dry wt. Two groups of plants can be defined on the basis of their chromium levels. One of them, plants 1, 2, 4, 6 and 9, claimed to have hypoglycaemic activity, have much higher concentration of chromium (median: 3.6±2.3 $\mu g\ g^{-1}$ dry wt) than the others (median: 0.97±0.65 $\mu g\ g^{-1}$ dry wt). Particularly, the plant *Syzygium jambolanum* (plant 1), a Brasilian plant normally transformed in an alcoholic solution, and the alga *Fucus vesiculosus* (plant 2), are claimed to have strong non insulinized antidiabetic properties. The plant and the alga have 7.5 and 5.0 $\mu g\ g^{-1}$ dry wt of chromium, respectively, much more than the others described in Table 2. The other marked plants have also been referred to as antidiabetic ones and indeed their chromium content is in the range found for other hypoglycemic plants (OSÓRIO E CASTRO, 1998; FELCMAN & TRISTÃO-BRAGANÇA, 1988). Concerning their relatively high content of chromium, the plants *Ficus carica* (plant 3) and *Corylus avellana* (plant 5), at least, could, in principle, be accepted as important plants to diabetic people, if their content of chromium is important to have therapeutic effect, but such activity was not reported for them. Significant differences ($P = 0.05$) were found for chromium content between the two groups of plants (antidiabetic plants 1, 2, 4, 6, and 9 compared with the remaining 16 plants).

As regards zinc content, 33 plants were analysed (leaves of aromatic plants and others, Table 3) showing zinc concentration values between 8 (plant 33) and 135 $\mu g\ g^{-1}$ dry wt (plant 1) with the median value = 38±21 $\mu g\ g^{-1}$ dry wt. This concentration range includes concentration values also found in other plants (SZENTMIHÁLYI & THEN, 2000), and the median is close to the one found in other plants (16 plants, media = 46) (FELCMAN & PEREIRA, 1998). The antidiabetic plants in which zinc may be important to their therapeutic properties (plants 1, 3, 4, median = 82±38$\mu g\ g^{-1}$ dry wt) were compared with the other 30 plants (median = 33±12), and indicate the possible

importance of zinc in the claimed antidiabetic properties. Particularly, the aromatic plant *Apium graveolens* L. (plant 1) has a much higher concentration of this metal than the others. It should be mentioned that this plant has a very low chromium concentration (OSÓRIO E CASTRO, 1998), so, its therapeutic properties could be due, in principle, to the high content of zinc. Statistical comparison (P = 0.05) of these two groups proves significant differences. The other plants (plants 18, 19, 21, 30, 32 and 33) are also antidiabetic, but this property is attributed to their relatively high chromium content (FELCMAN & TRISTÃO-BRAGANÇA, 1988; OSÓRIO E CASTRO, 1998).

Table 2

Chromium concentration in the studied plants (leaves from fruit trees and others)

N°	Common name	Scientific name	μg g^{-1} dry wt
1[a]	Jambolan	*Syzygium jambolanum* L.	7.5 ± 0.85 (n=10)
2[a]	Sea oak	*Fucus vesiculosus* L.	5.0 ± 0.52 (n=6)
3	Fig	*Ficus carica* L.	2.8 ± 0.23 (n=33)
4[a]	Blackberry	*Rubus fruticosus* L.	2.1 ± 0.32 (n=24)
5	Hazelnut	*Corylus avellana* L.	2.1 ± 0.23 (n=21)
6[a]	Bean plant	*Vigna unguiculata* (L.) Walp	2.0 ± 0.15 (n=44)
7	Plum	*Prunus domestica* L.	1.4 ± 0.11 (n=6)
8	Strawberry tree	*Arbutus unedo* L.	1.3 ± 0.18 (n=6)
9[a]	Strawberry	*Fragaria vesca* L.	1.3 ± 0.17 (n=13)
10	Medlar (tree)	*Eriobotrya japonica* (Thunb.) Lindley	1.1 ± 0.26 (n=21)
11	Vine	*Vitis vinifera* L.	0.9 ± 0.18 (n=15)
12	Cherry tree	*Prunus avium* L.	0.9 ± 0.07 (n=19)
13	Melon	*Cucumis melo* L.	0.7 ± 0.14 (n=18)
14	Peach	*Prunus persica* L.	0.7 ± 0.12 (n=19)
15	Apple	*Malus* Miller *domestica* Borkh.	0.7 ± 0.10 (n=15)
16	Lemon	*Citrus limon* L.	0.6 ± 0.15 (n=16)
17	Quince	*Cydonia oblonga* Miller	0.6 ± 0.10 (n=21)
18	Orange	*Citrus aurantium* L. *sinensis* (L.) Osbeck	0.6 ± 0.08 (n=17)
19	Pear	*Pyrus communis* L.	0.5 ± 0.10 (n=15)
20	Olive	*Olea europaea* L.	0.4 ± 0.15 (n=18)
21	Orange	*Citrus aurantium* L. *amara* (L.) Engler	0.2 ± 008 (n=14)

[a]: antidiabetic plants.

Table 3

Zinc concentration in the studied plants (aromatic plants (leaves) and others)

N°	Common name	Scientific name	μg g^{-1} dry wt
1[a]	Celery	*Apium graveolens* L.	135 (±16.5)n=18
2	Rock-rose	*Cistus ladeniferus* L.	60 (± 2.5) n=9
3[a]	Verbena	*Verbena officinalis* L.	60 (± 4.5) n=6
4[a]	Egypt stars	*Coreopsis tinctoria* Nutt.	50 (± 4.5) n=9
5	Herb of St. Robert	*Geranium robertianum* L.	50 (± 6.0)n=15
6	Coriander	*Coriandrum sativum* L.	50 (± 3.0) n=6
7	Lavender	*Lavandula stoechas* L.	48 (± 6.5) n=9
8	Tomato	*Solanum lycopersicum* L.	45 (± 2.0) n=9
9	Broad bean	*Vicia faba* L.	42 (± 2.0) n=6
10	Grean bean	*Phaseolus vulgaris* L.	41 (± 1.5) n=6
11	Convolvulus	*Convolvulus arvensis* L.	41 (± 3.0) n=9
12	White broom	*Cytisus scoparius* L.	40 (± 1.0) n=6
13	Wild thyme	*Thymus serpyllum* L.	39 (± 3.5) n=9
14	St. John's-wort	*Hypericum androsaemun* L.	37 (± 1.0) n=6
15	Bean plant	*Vigna unguiculata* L. (Walp)	35 (± 6.5) n=12
16	Fennel	*Foeniculum vulgare* Miller	35 (± 2.5) n=6
17	Cypress	*Cupressus sempervirens* L.	35 (± 3.5) n=9
18[a]	Cow-foot	*Bauhinia aculeata* L.	35 (± 2.5) n=9
19[a]	Knot-grass (pigveed)	*Polygonum aviculare* L.	35 (± 6.0) n=10
20	Carque	*Pterospartum tridendatum* L.	34 (± 2.0) n=6
21[a]	White archangel	*Lamium album* L.	33 (± 5.5) n=6
22	Eucaliptus	*Eucalyptus* L.Hér. *globulus* Labill.	33 (± 2.0) n=9
23	Heather	*Erica arborea* L.	32 (± 2.5) n=8
24	Linden (leaves)	*Tilia cordata* Miller	29 (± 1.5) n=6
25	Linden (flowers)	*Tilia cordata* Miller	26 (± 1.0) n=6
26	Centaury	*Centaurium* Hill *erythraea* Rafn.	25 (± 3.0) n=15
27	Gromwell	*Lithospermum* L. *diffusum* Lag.	23 (± 4.5) n=12
28	Rosemary	*Rosmarinus officinalis* L.	20 (± 1.5) n=6
29	Heather	*Erica umbelata* L.	19 (± 1.0) n=8
30[a]	Whortleberry	*Vaccinium myrtillus* L.	17 (± 1.0) n=6
31	Salvia	*Salvia officinalis* L.	16 (± 3.0) n=9
32[a]	Brazil wood	*Caesalpinia ferrea* Mart.	16 (± 1.5) n=12
33[a]	"Pedra H. Kaá" (Brazil)	*Myrcia sphaerocarpa* L.	8 (± 2.0) n=7

[a]: antidiabetic plants.

Zinc concentration in leaves from 18 fruit trees were also determined (Table 4). Zinc concentrations are between 8 (plant 18) and 165 μg g^{-1} dry wt (plant 1) with a median = 37±38 μg g^{-1} dry wt, similar to the one found for plants reported in Table 3.

Plants 1 and 3 present pharmacological activity, and have zinc concentration, median = 115±50 μg g^{-1} dry wt, which is much higher than that of the other 16 plants (27± 21 μg g^{-1} dry wt). Statistical calculations (P = 0.05) show that this difference is, indeed, meaningful. Particularly, the plant *Passiflora* (plant 1) shows a very high content of zinc compared with the others. It is known to have strong antidiabetic properties which could, in principle, be attributed to this metal. Its chromium content is too low (OSÓRIO E CASTRO, 1998) to be important as responsible for its therapeutic properties. The other plant, *Fragaria vesca* L. (plant 3), is also active and its zinc content is relatively high, which could, probably, explain its medicinal activity. The other type 2 antidiabetic plants (plants 12 and 18) have zinc concentration values much lower (19 and 8 μg g^{-1} dry wt, respectively) than the mean (37±38 μg g^{-1} dry wt); this fact may exclude zinc as the factor to explain their activity. Indeed, as was said before, chromium is a better candidate, due to its high relative concentrations (Table 2, plants 1 and 4).

Table 4

Zinc content in the studied plants (leaves from fruit trees)

N°	Common name	Scientific name	μg g^{-1} dry wt
1[a]	Passion flower	*Passiflora* L.	165 (± 4.6) n=18
2	Medlar (tree)	*Eriobotrya japonica* (Thumb.) Lindley	90 (± 6.5) n=12
3[a]	Strawberry	*Fragaria vesca* L.	65 (± 2.0) n=6
4	Hazelnut	*Corylus avellana* L.	60 (± 3.5) n=10
5	Melon	*Cucumis melo* L.	40 (± 3.5) n=6
6	Fig	*Ficus carica* L.	34 (± 4.5) n=15
7	Strawberry tree	*Arbutus unedo* L.	29 (± 4.5) n=6
8	Olive	*Olea europaea* L.	27 (± 2.0) n=6
9	Pear	*Pyrus communis* L.	25 (± 3.5) n=13
10	Almond	*Amygdalus communis* L.	21 (± 3.5) n=6
11	Orange	*Citrus aurantium* L. *sinensis* L.	19 (± 4.5) n=12
12[a]	Blackberry	*Rubus fruticosus* L.	19 (± 3.0) n=14
13	Lemon	*Citrus limon* L.	17 (± 3.0) n=11
14	Plum	*Prunus domestica* L.	16 (± 2.0) n=6
15	Persimmon (dule-plum)	*Diospyros* L.	12 (± 1.5) n=6
16	Vine	*Vitis vinifera* L.	11 (± 1.5) n=6
17	Apple	*Malus* L. *domestica* Borkh	9 (± 1.5) n=6
18[a]	Jambolan (Brazil)	*Syzygium jambolanum* L.	8 (± 3.5) n=11

[a]: antidiabetic plants

3. Conclusions

More than 400 plants with suspected glucose-lowering potential are known (BAYLEY & DAY, 1989) and today hypoglycaemic plant remedies are still prevalent in the third world countries where they have been in use, in most cases for centuries. In western nations, however, such traditional treatments are also experiencing a remarkable come back (EISEMBERG et al., 1993).

There are many organic compounds isolated and identified from plants that previously demonstrated to have hypoglycaemic properties, which are claimed to be due to glycanes, proteins, flavonoids, steroids, triterpenoids, alkaloids, etc. (PEREZ et al., 1998).

It is well known that chromium and zinc are important minerals and are also related to the glucose metabolism. Chromium has been known as an essential mineral for at least 30 years. Its beneficial role is well documented in human health maintenance and its deficiency has been linked with increased incidence of age-related non-insulin diabetes. The beneficial dietary form of Cr is an organometalic compound called glucose tolerance factor which consists of an ion of Cr^{3+} bound to several molecules of niacin and probably glutamic acid, glycine and cysteine. Without Cr^{3+} at its core, GTF is inactive (SCHWARTZ & MERTZ, 1959). About zinc no metalocompound has been isolated but its role in the synthesis, secretion and activity of insulin is well known (KING & KEEN, 1994).

Chromium and zinc deficiency can be caused by inadequate nutrition and age (KING & KEEN, 1994; ANDERSON, 1992). Chromium and zinc contents of individual foods vary widely, and are dependent on the introduction of metals in the growing, transport, processing and fortification of the food. Even well-balanced diets may contain insufficient quantities of chromium (ANDERSON, 1992), which can promote marginal chromium states. Thus, it is possible that teas, alcoholic extracts, or powders prepared from plants with relatively high concentrations of these metals, can complement the diet of healthly or diabetic people.

Some of the analysed plants have already been claimed to have therapeutic properties for diabetic people (non-insulinic diabetes) as referred to above. Perhaps others can be used for that purpose, taking into account their content in zinc and/or chromium. The former have been used with success as teas, alcoholic concentrates or powders, but no studies with animals or humans have been published, except for their mention as antidiabetic plants (OLIVEIRA-FEIJÃO, 1952; FOREY & LINDSAY, 1997), and in oral reports of patients. It remains to be determined if chromium or zinc have any relation to the medical properties of these plants. However, it is known that chromium supplemented to diets reverses symptoms of non-insulin dependent diabetes in laboratory animals and humans (ANDERSON, 1998) and that chromium is better

absorbed in some compounds than in its inorganic state (MERTZ, 1983). It must be complexed with certain ligands to be fully active (TOEPFLER et al., 1977; VINCENT, 1993). It may also be the case that plants with relatively high concentration of chromium with no antidiabetic properties (plants 3 and 5, table 2), form organic complexes not adequate to be active. Concerning zinc, this has been given as a supplement in drinking water with good results on insulin activity (MING-DER et al., 1998), so the plants that contain this metal in relatively high concentrations may due their therapeutic activity to it.

Although it is not proved that therapeutic properties for these plants are due to chromium and/or zinc, these metals should be considered important as they can be used by the plant to produce organometalic compounds, which could be the biologically active molecules.

In conclusion, these results suggest that plants used in Portugal as active agents to alleviate non-insulinic diabetes may have such an activity owing to their high levels of the analysed metals, which should be in a complex form with organic compounds, so as to have the best therapeutic effect.

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The technical assistance for the spectrophotometric readings, performed by Tech. Eng. C. VITORINO, is strongly recognized.

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Acta Alimentaria, Vol. 30 (4), pp. 343–354 (2001)

COULD WE FIND A SUITABLE METHOD FOR ASSESSMENT OF AVERAGE DIETARY INTAKE? IN DOUBT BETWEEN SCYLLA AND CHARYBDIS (A REVIEW)

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(Received: 4 October 2000; accepted: 21 May 2001)

For the sake of comparison of dietary surveys carried out in different countries, it is needed to find a common basic method for the dietary assessment. The suitable method should be relatively simple, easily practicable, inexpensive, suitable for the analysis of desired nutritional parameters in several target groups and comparable independently of the country where it is done. The paper gives a short review and appraisal of the most frequently used dietary assessment methods: 24-h dietary recall, dietary record, food frequency questionnaire and diet history. The background of useful evaluation seems as follows: the skill of interviewers, the common coding system of foodstuffs, the reliable conversion of food to nutrients (comprehensive food composition tables). On the basis of above mentioned criteria the repeated 24-h recall seems to be the first candidate for the common dietary assessment method.

Keywords: dietary assessment methods, comparison of dietary surveys

The method for assessment of average dietary intake in large European populations of different ethnicity, manifold nutritional habits must be

– suitable for the purposes of study, i.e. comparable independently of the country where it is done,

– suitable for the assessment and the analysis of desired parameters, e.g. foodstuffs, food groups, nutrients, non-nutritive ingredients, food supply, food security, nutritional habits, food safety (?),

– suitable for evaluation of nutrition in several target groups (e.g. children, adolescents, elderlies, pregnant and lactating women) or for that of cross-sectional characteristics of the whole or adult population,

– time-saving both for the investigators and the investigated subjects,

– requiring an ordinary skill in nutritional practice from the investigators,

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0139-3006/2001/$ 5.00

– matched to financial resources, i.e. inexpensive,
– adequately sensitive, specific, of high predictive value (THOMPSON & BYERS, 1994).

There are some other significant requirements as prerequisite of a well-organized nutrition trial:
– the target population should be definitively identified,
– the selected sample should represent the target population; the sampling criteria are widely discussed in the special literature (COLE, 1991; HALL, 1983), the critical elements are the design and the sample size; increasing sample size or number of observed days improve the precision; already a single-day dietary intake data may be used for comparison, if the sample is sufficiently large,
– nothing but the findings of a well designed survey can be extrapolated confidently to the larger population of which the study sample is a part,
– an appropriate quality control is needed,
– analysis of nutrient intake requires an adequate food composition data bank.

On the basis of above we have to evaluate the strengths and the weaknesses of the most frequently used methods. First of all we wish to emphasize that the comparison of food items in the European countries seems to be difficult because of divergent meal patterns, differences in preferred foodstuffs and meals, in processing and cooking technology. In our opinion the nutrient content of diet and maybe the main food groups could serve as background of the comparison.

A short review and appraisal of the most frequently used dietary assessment methods

The twenty-four-hour dietary recall

The 24-h recall originally attributed to WIEHL (1942) means an interview. The investigator asks the respondent to enumerate the foods and beverages consumed in the preceding day or in the preceding 24 hours, including their quantity. The interview might be carried out by call over the phone or in person. The latter appears to be the better manner. The interview, the collection of data may be computer-assisted and thus facilitated. The critical points of the method are the well-trained interviewers skilled in the choice of available foods and meals, in preparation practices used generally or in certain regions and by certain ethnic groups. They should be familiar with the nutritional habits (e.g. the foods eaten usually together) and consequently they are able to correct or to complete the answers and to control the accuracy of data.

The recall is based on the memory of investigated subject. The interviewer should help to recall all the food eaten, but should pay attention to avoid influencing the answers. One deemed advisable the use of a preformed protocol and a series of

photographs or outlines demonstrating different food, meal quantities, they are a living guide to both the interviewer and the responders. For an efficacious elaboration and evaluation a coding system of foodstuffs and meals, moreover a computerized program is needed (BEATON et al., 1979; BINGHAM & NELSON, 1991).

Advantages. The personal contact contributes to the reliability of the collected data. The time between eating and recall is relatively short, thus the ratio of forgotten foods is low or zero. The 24-h recall is applicable for a wide population, non-respecting e.g. the illiteracy. It is relatively quick, for an interview roughly 15–20 minutes is necessary.

Disadvantages. The individuals' diet varies day by day and in addition the responders may forget some food items. The food eaten during 24 hours does not represent really the whole nutrition. This is why the recall cannot identify as well the individuals whose intake is too high or too low. But it characterizes the average intakes of a group or the population. Repeated recalls enhance the accuracy. The 24-h recall is inappropriate to analyse the association between nutrient intake and biochemical markers of supply or other health indices (JONNALAGADDA et al., 2000; VALENCIA et al., 1983).

Dietary record

In accordance with this method the responder records the foods and beverages consumed during one day. Also the record of their amounts is needed. The amounts consumed may be measured by household utensils (e.g. cups, tablespoons, scale) or estimated. For the latter manner a collection of pictures seems useful. In general a record of 3 consecutive days is necessary for reliable information. The reporting must be done at the time of consumption on paper or using a dictaphone. Before fact-finding the persons investigated will be trained in the adequate describing of their diet regarding the specification of foods, amounts, cooking methods etc. The report may be combined with a personal interview at least after the first day and at the end of reporting. A skilled interviewer can make the reports more accurate, clarify the entries and add to the omitted items or amounts. Also according to the Hungarian experiences, the self-filling up of a mailed questionnaire is not reliable enough.

Advantages. The dietary record is fairly accurate respecting the foods consumed, thus the method is often regarded as the "golden standard" among the dietary assessment methods used generally. It is more precise than the 24-h recall. Its adoption looks rather easy, but the well-trained professionals are indispensable.

Disadvantages. The record method requires a good co-operation on part of the responders who should be motivated and moreover literate (when paper is used for recording). There are limiting factors. The reliability of records decreases over 4 days, mainly over 7 days because of responders' fatigue. If the investigated subject does not

record the meals immediately as they are being eaten, the number of omitted foods or other faults increases. The investigator can hardly control the responders from this point of view: the quality control is difficult. Undoubtedly the requirement of recording may influence the choice of foods when the responder is somewhat familiar with the principles of healthy nutrition. Of course this phenomenon may be favourable for the individual nutrition, but unfavourable for the reliable actual data. Underreporting may be frequent in general but especially in obese women (ANDERSON, 1988; BINGHAM & NELSON, 1991).

Food frequency questionnaire

In the case of food frequency questionnaire (FFQ) the heart of matter is to ask the subject investigated on his/her usual frequency of consumption of foods listed in questionnaire for certain periods. For estimation of quantities of foods eaten and/or nutrient intakes several FFQs include questions regarding the portion size (semiquantitative FFQ, SQFFQ). Sometimes the term "semiquantitative dietary history" is used for FFQs allow a limited quantification of portion size. The nutrient intake may be estimated/calculated by summing of nutrient content of each food taking into consideration the reported quantity and frequency.

Advantages. FFQ seems to be a useful tool to estimate the usual foods eaten and to clear the nutritional changes in comparison with an earlier report. FFQ provides data to make groups of individuals according to usual consumption of foods or nutrient intake (if SQFFQ is used) and gives information on the relationship between a specific food and nutrition-related diseases (e.g. consequences of alcohol consumption). The FFQ may be self-administered and it requires fairly little time to be completed by an interviewer. They are in general easily scannable, the cost of the data entries is low like that of data collection and processing, the computerization is fairly easy. There is not a negligible aspect that the respondents' burden is small. There are no observer biases. The FFQ may be used for large population surveys.

Disadvantages. Some factors of inaccuracy exist during the use of FFQ method: the consequences of an incomplete list of foods - food groups, errors in the report of frequency and serving size. On the base of a too short list the consumption and the intake will be underestimated, and inversely, a too long list leads to overestimation and that raises the respondents' burden. A list drawn up for general use is inappropriate for ethnic groups or people with distinct eating pattern, also the children need a particular one. For a quantitative approach the SQFFQ provides only approximate data. The development of the food list is crucial to a successful and reliable data collection. It is difficult to develop a comprehensive list including enough but not too much food items, which gives the opportunity to all responders of very different eating habits to find the right answer. Each type of questionnaire should be validated. The FFQs or SQFFQs

may be self-administered, but the authenticity is lower than in the case of interview (BOEING et al., 1989; NELSON, 1991).

Diet history

The original diet history attributed to BURKE (1947) starts with an interview to determine the usual meal pattern, most frequently with a 24-hour recall. The second step is a food frequency questionnaire and the third one a 3-day dietary record. Thus it is a combined method and the strengths and the weaknesses of each method will be partly equalized. It requires a large, skilled staff, a lot of labour, time and burdens the responders. It is used infrequently.

Selecting appropriate dietary assessment method for data collection in Europe and for comparison of results

Which criteria determine the selection of method? One of the criteria is the objective of the research, including the accuracy needed and type of data needed. The next issue is the study population (sample size, willingness for co-operation, time limits). Important preconditions are the skilled interviewers and the skill for coding the foods and at last but not at least the financial sources and the adequate, complete, accurate nutrient database.

One of the most widely used methods for dietary assessment is the 24-h recall. It is logical, logistically simple and suitable for large groups. The choice of the method depends on the stated targets of the study. In Europe a method is needed for a reliable comparison of large population groups' nutrition. We are not seeking unspecified dietary components that may be related to health status but general features of food and nutrient intakes. The 24-h recall is less burdening to the population and relatively less costly. The method yields good information on the average dietary intake of a large population but it is not suitable for determining individual nutritional risk. There are differences between population groups regarding its usefulness. Elderly people are less good subjects (but they excellently record the diet). Women know better the serving sizes, the cooking methods and the ingredients used for preparing the meals than men. Children might forget any food item; the parents (above all the mothers) can help them, although the presence of parents may influence the answers of children.

The interviewers must be trained on the base of common principles. The interviewers' skill plays an important role to have a reliable data collection and to avoid the errors. It seems necessary to compile a "European guideline on data collection for 24-hour recall". A complete 24-h recall should contain the following issues: time of consumption (hour, minute), the food item consumed (brand name, popular name, kind,

type, additions), the portion size, the preparing, cooking procedures (if any) including the used ingredients (e.g. fresh, chilled, deep-freezed raw material, lard, olive oil, sunflower oil, salt, flour, sour cream etc.) in the case of meals prepared at home, the source of other meals, the place of consumption or purchase (e.g. restaurant, fast food restaurant, mass catering, take-away meal). There are in many countries cookery books containing the exact recipes for meals frequently cooked in mass catering establishments. They may be often used for the household meals, too. Concerning the portion size a visual aid is necessary, namely a collection of photos or drawing illustrating the quantity of different food items.

The interviewers should very well know the kinds and types of local foods, the items consumed together (e.g. meat and customary garnishment), they should ask the adequate questions to complete the recall. Moreover, a good interviewer is familiar with creating connection with responders, he/she must be well trained in this field, too. A checklist of interview should be made available for the investigators: the introductory questions (to obtain the responder's benevolence), the questions regarding point of time, food items, conditions of preparing etc. (SEVENHUYSEN et al., 1990).

Another crucial issue is the common coding system. Each repast should have a code: breakfast, brunch, lunch, dinner, supper, repasts in intermediate time. According to some statistical data about 38 000 food items exist in Europe. For their coding the EUROCODE 2 system developed by EUROFOODS and the interchange proposals from INFOODS may be used to establish compatibility between national databases but the differently cooked meals are out of this system. The food coding and descriptor system is based on 14 major food groups subdivided into 2500 subcategories. Is the marking or coding really necessary and useful? If those are not marked, the comparison of different diets is difficult, if not impossible. On the other hand the enormous number means a nearly unmanageable thesaurus. It seems expedient to consider, whether the selection of foods that are most important in decreasing or increasing nutritional risk, could solve the contradiction. Thus the coding of a limited number of foods could be needed. The solution gives opportunity to compare the most significant nutritional risk factors observed in different countries and based on food consumption.

The next step is the conversion of foods to nutrients. The prerequisites for this process are the food composition tables and databases. The goals of the calculation of daily nutrient content are (1) to estimate the adequacy of the dietary intake of population or population groups, (2) to compare the nutrient supply among groups, regions, countries, (3) to study the relationships between the nutritional status and the risk of diet-related diseases, (4) to evaluate the level of nutritional knowledge in the population, to have feedback on the efficacy of nutrition education, information, intervention. The nutrient intake calculated on the base of dietary recall data provides the way for an objective, reliable comparison of nutrition.

Many European countries have their own national food composition tables. The foods usually available in the given country should be included in the tables, but with the globalisation of trade several other foods are imported from abroad in considerable quantity. In this case their nutrient content could be obtained from other sources, i.e. from other tables or by analysing the food. Each food, each meal ingredient registered in recall must be calculated, not a single one may be omitted. For a good estimation only 24-h recall does not give sufficient data. The required number of days depends on the nutrient and it is shown in the next table.

Number of days required for classifying 80% of the population into tertiles of nutritional intake with 95% confidence

Nutrient	Range in days
Energy	3–7
Total fat	5–9
Cholesterol	17–18
Protein	5–7
Carbohydrate	2–4
Fibre	5–10
Vitamin A	46–64
Vitamin C	6–14
Thiamin	6–11
Riboflavin	7–10
Iron	12–19
Calcium	3–5

Source: KARKECK (1987)

The table shows that at least threefold 24-h recall is required. NELSON and co-workers (1989) think that the number of day needed is higher.

The use of national food composition tables has many advantages:
– the tables contain the most popular foodstuffs consumed in the given country,
– their nutrient content meet the characteristics of foods locally produced; the nutrient content depends on species of plants and animals, on agricultural technology, on climatic conditions, on processing and storing conditions and they vary in different countries,
– the naming of food and in the case of cooked dishes the raw material used for preparing the meal would be easily identified.

The international usefulness of national food composition tables considerably depends on range of nutrients included in them. For comparing the nutrient intake on international level a common structure of them together with a list of nutrients is

essential. It may be a limited one for basic issues and a comprehensive one for detailed scientific evaluation. The list of ingredients would include macronutrients (protein, fat, carbohydrate), micronutrients (vitamins, macroelements, microelements), non-nutritive ingredients (e.g. dietary fibre, antioxidants, prooxidants, naturally occurring toxic substances, enzyme inhibitors etc.). The quality of analytical methods employed for investigations of foodstuffs should be emphasized (FAO, 1975; IRELAND & MØLLER, 1999; KOHLMEIER, 1993).

The conversion to nutrients renders possible to determine the main sources of favourable and unfavourable nutritional factors and to compare them.

For constructing national food composition tables or data bank it may be proposed to consult the guidelines prepared by GREENFIELD and SOUTHGATE (1991). A conference organized by the United Nations University was held in 1983 in order to create an international network of food data system (INFOOD). In 1991 the INFOOD guidelines for describing foods was published. An initiative of EUROFOODS started in the late eighties for creating national databases in Europe suited the international requirements. At that time other regional INFOODS databases have been established: LATINFOODS, ASIAFOODS, ASEANFOODS, OCEANIAFOODS, AFROFOODS, NORAMFOODS. It follows from this foregoing that there are useful tools for the above mentioned purposes. We may not neglect that a bibliography of food composition tables was published by FAO in 1975 and by INFOODS Secretariat in 1988 (HEINTZE et al., 1988). Of course they should be updated. At the present time the crucial sources of international data on food composition are the tables published by FAO for various regions (Africa, Eastern Asia, Near East, moreover for amino acids) and by other organizations, institutions (Caribbean, Central America, Eastern Africa, Western Africa). There are some other pathways to find out a complete food classification system. The Codex Alimentarius is a basic source of information regarding foods. The Confederation of the Food and Drink Industries of the EEC has developed the CIAA Food Categorization System. It was designed originally to serve as an allocation tool for food additives, but the system covers all foodstuffs even those that may not require additives. The Harmonized Commodity Description and Coding System compiled by the World Trade Organization suit the requirements of international trade. Also the Food Balance Sheets used by FAO, OECD and EUROSTAT is of practical importance similar to the PROCOME scheme based on the European Combined Nomenclature and used in household budget surveys, in EU DAFNE Project. The Langual thesaurus is used in Europe and the USA. Most recently the European COST Action 99 have elaborated recommendations for food data interchange (SCRIMSHAW, 1997).

In the course of the calculation of nutrients one has to pay attention to nutrient losses and gains during the processing and preparation. The changes depend on the technology and/or method applied during the mentioned procedures. But if a guideline

will be placed at disposal in European nutritional surveys, the evaluation and the comparison will be made much easier. Another complicated issue is the bioavailability, which can be defined as the proportion of macro- or micronutrients that will be utilized in the body. The bioavailability is a property neither of a nutrient nor of a diet by itself, but is the result of an interaction between the nutrients and other food ingredients and it is determined by the total diet. Thus the databases are unable to give the exact value for bioavailability of a single nutrient (DWYER, 1994; MARGETTS, 1991; PERLOFF et al., 1990; WAHRENDORF et al., 1989; WEST & STAVEREN, 1991).

The general use of 24-hour dietary recall

The method is used worldwide very largely. It has been applied in the course of National Health and Nutrition Examination Surveys (NHANES), the Hispanic HANES conducted by the United States Department of Agriculture, furthermore in Canada, in the Ten-State Survey, in the European dietary investigations among others in Poland, Hungary, Russia, Ukraine, Kazakhstan, the former GDR, Czechoslovakia, Yugoslavia, USSR. In Western Europe the food record is preferred (British National Diet and Nutrition Survey Program, Dutch National Food Consumption Surveillance System, Denmark). On the Flair Eurofoods-Enfant Project Meeting in 1992 a European Health and Nutrition Examination Survey was proposed that would resemble NHANES III including 24-hour recall. The determination of a "Food consumption basket", the calculation of the nutrient composition and the dietary adequacy was carried out in several rural communities of Mexico on the basis of dietary recall (BIRÓ, 1997; BRIEFEL, 1994; BRIEFEL et al., 1997; CHERZEWSKA, 1994; PIETINEN & OVASKAINEN, 1994; RIZEK & PAO, 1990).

The validity of 24-hour dietary recall

The validity means on one hand whether the findings of a study give a reasonable representation of the true situation (external validity, it relates more to the interpretation of epidemiological findings), on the other hand whether a measure of exposure or outcome actually measures that exposure or outcome (internal validity). The external validity does not exist without the internal validity. Measures of sensitivity, specificity and predictive value relate to the latter type.

In the case of dietary recall the correlation between recalled and observed nutrient intake (external validity) according to KARVETTI and KNUTS (1985) is in the range 0.58–0.74. The validity is unsatisfactory on the individual level and satisfactory on the group level. The best validity has been observed in the middle-aged group. FANELLI and STEVENHAGEN (1986) emphasize the stability of results (like the 1-day food record). A divergent situation is shown in preschool children. The data obtained by dietary recall

correlated highly with the children's weighed food intake, but they poorly correlated on two consecutive days of data collection (KLESGES et al., 1987). The results are similar to the previous report, which indicated that dietary intake of preschool children is greatly variable. In the study of JOHNSON and co-workers (1996) children of age 4–7 years were investigated by means of dietary recall regarding the energy intake, as well as total energy expenditure determined by doubly labelled water method. Data from three times 24-hour recalls were sufficient to estimate the energy intake in a group, but insufficient for individual measurements. BEATON and co-workers (1979) reported on the sources of variance in 24-hour recall. The variance changes by gender, by the day of week, by nutrient. Knowledge of its partitioning may be favourable also in diet-lipid-heart diseases studies. In Germany the former BGA (Bundesgesundheitsamt, Federal Health Office) published the recommendations for the analysis and the measurement of errors in nutritional epidemiological studies.

Conclusions

"Certainly, an ideal method for epidemiological purposes should provide an adequate degree of accuracy about the foods or nutrient consumed by individuals to test the hypothesis of interest in a powerful fashion" (KOHLMEIER, 1994). Undoubtedly, the 24-hour dietary recall does not perfectly fulfil the requirements of an ideal method. But – in spite of its insufficiencies – the method is easily practicable in all age groups, relatively inexpensive, useful for groups, the elaboration of data and their evaluation are rather simple. The burden of investigated subjects is not too great, from this point of view it has a positive cost-benefit ratio, needs little time. If the data collection meets the generally accepted guidelines, the results are well comparable. The 24-hour recall means a suitable method to clarify the nutritional features, the nutritional habits, the nutritional risk in large population groups, but for more confident results it is reasonable to repeat it at least three times.

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Acta Alimentaria, Vol. 30 (4), pp. 355–361 (2001)

THE DISTRIBUTION OF IRON IN IRON-ENRICHED CELLS OF *SACCHAROMYCES CEREVISIAE*

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(Received: 25 October 2000; accepted: 21 June 2001)

Fresh or freeze-dried iron-enriched bakers' yeast (5% of total solids composed of iron) were fractionated, and the distribution of iron was examined. After centrifugation of fresh yeast creams, 89% of total iron was found in the supernatant, which contained only 23% of the total solids. Results suggest that only 13% of the iron is bound to cells in the fresh yeast suspension. Most of the cell-located iron was found on the cell wall, whereas the cytoplasm contained proportionally (iron content of total solids) almost 3 times less iron than the cell walls. Freeze-drying of the iron-enriched yeast had marked effects on the distribution of total solids and iron (in the fractionation procedures that were carried out following their rehydration). The freeze-drying process induced binding of free iron to the yeast cell wall, and twice as much iron was thus found on freeze-dried cells. In the freeze-dried product, it was estimated that 27% of iron was bound to cell fractions.

Keywords: adsorption, drying, minerals, yeast

Iron has important functions in the human body. It is needed to form hemoglobin in blood and myoglobin in muscles. A deficiency in iron may result in anaemia (NESTEC, 1987).

Inorganic iron salts as inorganic chromium salts (HEGÓCZKI et al., 1997) may not be as readily absorbed or biologically active as organic forms of minerals. Micro-elements from organic sources, particularly yeast, have better absorption quotients and are less toxic than those obtained from inorganic sources (HEGÓCZKI, 1994). Microorganisms accumulate metals by a number of different processes such as uptake by transport, biosorption to cell walls and entrapment in extracellular capsules,

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0139-3006/2001/$ 5.00

precipitation and oxido-reduction reactions (BRADY & DUNCAN, 1994). The organically bound form of chromium found in yeasts is known to be both assimilable and efficient as a dietary source of chromium (HEGÓCZKI et al., 1997).

Many mineral-enriched yeasts, also called organic mineral yeast, are available on the market. The production of mineral-enriched yeast often requires specific growth conditions (NAGODAWITHANA & GUTMANIS, 1985), but most processes are unpublished and proprietary. Little is known on the distribution of the minerals in or at the surface of the cell. Various metals are assimilated by *Saccharomyces cerevisiae* (BRADY & DUNCAN, 1994) and some, such as selenium, are incorporated into amino acids (MATNI et al., 1995). There is little information, however, on the distribution of iron in commercial iron-enriched *Saccharomyces cerevisiae* cells.

The aim of this study was to evaluate the rate of iron binding to cell organic compounds and to determine the effect of drying on iron bound to *Saccharomyces cerevisiae*.

1. Materials and methods

1.1. Yeasts

A fresh concentrated (20% solids) commercial *Saccharomyces cerevisiae* TC1 suspension (Lallemand Inc. Montréal, Canada) was supplemented by 48.4 g l^{-1} of $FeSO_4 \cdot 7H_2O$, and incubated 1 h at 25 °C under rapid stirring. Half of the yeast cell suspension was used immediately while the other was frozen at –40 °C in a cabinet freezer and then freeze-dried at 24 °C for 72 h (Lyo San Inc., Lachute, Canada) under a vacuum of at least 100 μm of Hg (0.1 Torr). The powder was rehydrated in deionized water at 20% solids before fractionation.

1.2 Fractionation of yeasts components

The fractionation steps are presented in Fig. 1. Yeasts suspensions were centrifuged at 15 000 g during 15 min. The pellet was washed with deionized water and centrifuged again for 15 min. The supernatant and the wash water were pooled and the material was thoroughly stirred. This material was pre-filtered on a 8 μm Whatman (No. 2 filter paper) membrane and further processed by ultrafiltration (UF) using a tangential filtration system (Minitan Filter plates, Millipore, Bedford, USA) with membranes having 1000 Da molecular cut-off. A 500 ml supernatant solution was used, and the UF process was stopped after the recovery of 300 ml of filtrate.

The cell pellet obtained from the first centrifugation was resuspended with deionized water and cell disruption was conducted in a mixer mill type MM2 (Retsh, Haan, Germany) in nylon 180 ml vessels according to the method used by MORIN and co-workers. (1993). Disruption was made at 100% intensity during 25 min.

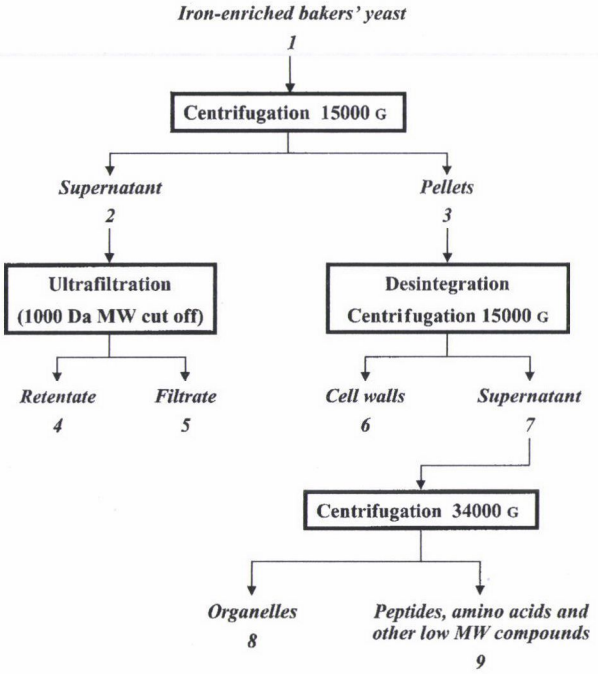

Fig. 1. Fractionation steps of the iron-enriched bakers' yeast cell suspension

Differential centrifugation based on the methodology of VARIMO and co-workers. (1983) was used to separate disintegrated yeast components. Disrupted cells solutions were centrifuged at 15 000 g during 15 min and the supernatants were again centrifuged at 34 000 g during 15 min with the aim of separating organelles, such as mitochondria and microsomes, from other constituents. The supernatant should contain soluble proteins, nucleic acids and peptides, amino acids, salts and other small molecular weight compounds (VARIMO et al., 1983). Fractionation of fresh iron-enriched yeast cells was completely replicated two times. One assay was carried out for the dried product.

1.3 Chemical analyses of yeast cell fractions

1.3.1 Solids content. Total solids content of all fractions were obtained by dry weights after incubation at 105 °C for 16 h. The assays were done in duplicate on each sample and the mean of the obtained values was used. Relative standard error on mean for the solids analyses was <2.1%. Fractionation of fresh iron-enriched yeast cells was done two times and relative standard error on mean on data of the two experiments was less than 9%.

1.3.2 Iron concentration determination. Crucibles were pre-soaked in hydrochloric acid (HCl, 10%) for two hours, rinsed with deionized water and dried in a 550 °C oven for one hour. They were removed from the oven and placed in a desiccator for 30 min. Samples (2 g) were added to the cooled crucibles. Samples were dried at 105 °C and then ashed at 550 °C for at least 16 h. The ash-containing crucibles were cooled in a desiccator for 30 min and weighed when they reached room temperature. The ashes were dissolved in 10 ml of concentrated HCl, which was then evaporated to dryness on a hot plate. The residue was redissolved by adding 10 ml of concentrated HCl. The iron concentration was measured in this solution by atomic absorption with a Varian SpectrAA-100 (Malgruve, Victoria, Australia) unit. A specific hollow cathode lamp (model 3QNY-Fe, Cathodeon Limited, Cambridge, UK) and 372.0 nm wavelength were used to determine iron concentration in the solution. The assays were done in duplicate on each sample and the mean of the obtained values was used. Relative standard error on mean was less than 5.4% for the measurement of iron when multiples analyses were conducted on a given sample. Fractionation of fresh iron-enriched yeast cells was done two times and relative standard error on mean on data of the two experiments was slightly less than 12%.

2. Results and discussion

2.1. Fresh yeast cell suspensions

The distribution of iron and total solids in fresh yeast cell fractions is shown in Table 1. Total iron in initial yeast creams was 10 g l^{-1} and represented the 100% level. After centrifugation of fresh yeast creams, most (89%) of total iron was found in the supernatant, while this fraction constituted only 23% of total solids. Ultrafiltration results of the supernatant fraction suggest that the iron did not bind to a great extent to high-molecular weight components, since the percentage of iron of the total solids was lower in the retentate (fraction 4) than in the filtrate (fraction 5). The sample that had the highest proportional content in iron was the filtrate (fraction 5). Therefore, most of the iron added to the yeast cream was not bound, and it was found that only 13% was attached to or absorbed by the yeast cells. It will be noted that the sum of iron in samples 2 and 3 was greater than that in the original sample by 2%, and this is a reflection of the experimental error in the atomic absorption analyses.

A significant portion of the iron was absorbed, although an incubation of only 1 h was carried out, since the cytoplasm solids (fraction 7) contained 3% of the iron added to the cell suspension. Most of the cell-located iron was found on the cell wall (fraction 6).

Table 1

Distribution of iron and total solids contents in different yeast cell fractions

Fraction[a]	Fresh yeasts					Dried yeasts				
	Iron		Total solids		Fe %	Iron		Total solids		Fe %
	g l^{-1}	%[b]	g l^{-1}	%	of TS[c]	g l^{-1}	%	g l^{-1}	%	of TS
1	10	100	200	100	5.0	10	100	200	100	5.0
2	8.9	89	46	23	19	7.0	70	48	24	15
3	1.3	13	150	75	0.8	2.7	27	130	65	2.1
4	3.1	31	38	19	8.1	3.8	38	52	26	7.3
5	5.3	53	26	13	20	2.2	22	34	17	6.5
6	0.9	9.0	82	41	1.1	2.2	22	96	48	2.2
7	0.3	3.0	72	36	0.4	0.6	6	42	21	1.0
8	0.03	0.3	10	5.0	0.1	0.08	0.8	0.1	0.5	80
9	0.3	2.5	54	27	0.4	0.4	3.5	42	21	0.8

[a]: Fractionation steps are listed in Fig. 1

[b]: percentage of the quantity initially added

[c]: Fe% of TS = percentage of total solids represented by iron in the given fraction

These results are in accordance with those of MOCHOBA and co-workers (1996a) who demonstrated that Fe^{++} appeared at higher concentrations in the mannoprotein fractions than in the intracellular fractions of brewing strain of *Saccharomyces cerevisiae*. The storage site of iron in brewing strain was proposed to be the mannoprotein layer of the cell wall (MOCHOBA et al., 1996a). The cell wall is an important site of initial biological interaction between cells and external metal ions (MOCHOBA et al., 1996b).

With respect to the cytoplasm, iron constituted a lower proportion of total solids in the organelles (fraction 8) than in the rest of the cytoplasm. This suggests that most of the iron that was absorbed by the yeast was still free inside the cell.

2.2. *Effect of freeze-drying*

Freeze-drying of the iron-enriched yeast cell suspension had marked effects on the distribution of total solids and iron in the fractionation procedures that were carried out following rehydration (Table 1).

It can first be observed that the supernatant obtained from the initial centrifugation (fraction 2) of the freeze-dried cells has a lower iron content than that of the fresh yeasts. Thus only 70% of the iron initially added was found in the supernatant of the rehydrated cells (fraction 2), while iron constituted 89% of the fresh yeast suspension total solids. Inversely, a higher iron content was found in the cell pellet of the rehydrated cells.

A slightly lower iron content in solids is found in the supernatant obtained following the first centrifugation (fraction 2) with the previously dried cells (19% for fresh VS 15% for dried). Presumably, freezing and drying of the cells damaged the cell walls and enabled leakage of intracellular components. This hypothesis finds support in the fact that a significant reduction in solids was noted for the intracellular portion (fraction 7) when the cells had been dried. Furthermore, a greater amount of solids are recovered in the UF filtrate (fraction 5) of the supernatant. These results are in line with those of BARRETTE and co-workers, (1999) who had shown cell lysis following freezing of bakers's yeast suspensions.

It is noteworthy that supernatants (fraction 2) obtained from the dried culture had a lower iron content than that of the fresh yeast (Table 1). This is accompanied by a lower iron content in the UF filtrate (fraction 5) of the rehydrated cells, which represents the <1000 mol.wt portion of the supernatant. These data suggest that the freeze-drying process has induced binding of free iron to the yeast cell wall. Indeed, iron content of the cell pellet (fraction 3) was more than twice as high when the cells had previously been freeze-dried (Table 1). Furthermore, data shows that this iron increase in cells that were freeze-dried (fraction 3) was due to binding to the cell walls (fraction 6) as well as the intracellular content (fraction 7).

Drying may impart a change in molecular conformation of the yeast cells and specific sites for metal chelation may become more available. AVERY and TOBIN (1992) as well as VOLESKY and MAY-PHILLIPS (1995) have shown that the uptake of strontium and uranium respectively, are more important in denatured yeast biomasses than in live yeasts.

The UF retentate (fraction 4) of the fresh cells proportionally (Fe% of TS) contained less iron than the filtrate (fraction 5), but the opposite was found with the corresponding fractions obtained from the freeze-dried cells. This suggests that drying not only increased adsorption of iron on the cells but also on the compounds having mol.wt >1000. The solids of the UF retentate contain both the unconcentrated low molecular weight compounds (<1000, fraction 5) and the concentrated high molecular weight components. If the proportional iron content of fraction 5 is subtracted from that in fraction 4, this would enable an estimation on the amount of iron bound to the soluble components (>1000 mol.wt) of the yeast suspension. For the freeze-dried cells, this represents approximately 16% of the iron. Thus, our results suggest that, in the freeze-dried product, 42% of the iron is bound, of which 28% is in the cell fractions. In view of the fact that there was only 13% with fresh yeast, the freeze-drying process significantly increases the degree of iron binding in the product.

4. Conclusion

Incubating yeast cells for one hour in the presence of high concentrations of iron enabled the binding of iron to cell components. Further studies on drying methods (spray-drying or other) or conditions (temperatures) on metal binding to yeast cells are warranted, since the effect of drying proved important in increasing the binding of iron to cells and soluble compounds of mol.wt >1000.

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The technical and scientific support of N. GARDNER, J. CONWAY and R. DEGRÉ was greatly appreciated.

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Acta Alimentaria, Vol. 30 (4), pp. 363–372 (2001)

LIGNIN-MODIFYING ENZYMES OF *PLEUROTUS OSTREATUS* GROWN ON AGRO-RESIDUES

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(Received: 14 November 2000; accepted: 27 June 2001)

The activity of lignin peroxidase (LiP) and laccase produced by *Pleurotus ostreatus* in culture media composed of agro-residues was measured by spectrophotometry. The overall enzyme activity and its dependence on the composition of culture media were determined by using spectral mapping technique followed by non-linear mapping. The relationships between the parameters of enzyme production and the composition of culture media and fermentation time were assessed by stepwise regression analysis. It was established that *P. ostreatus* did not produce LiP. The lowest enzyme production was observed in culture media containing extract of wheat straw. This finding indicates that the use of other agro-residues as substitutes for wheat straw is justified. It was further established that the enzyme production was also influenced by the pH of the culture media. It was found that enzyme activity quadratically depended on the fermentation time.

Keywords: *Pleurotus ostreatus*, spectral mapping, nonlinear mapping

Lignin is the earth's second most abundant polymer ranking only behind cellulose in quantity as a natural biopolymer (CRAWFORD, 1981). It is composed primarily of phenylpropanoid monomeric units interconnected by a complex array of stable carbon-carbon and carbon oxygen bonds (ADLER, 1977). Lignin is highly stable under natural conditions, therefore, its biodegradation is one of the rate-limiting steps in the global carbon cycle.

Mushrooms generally produce a wide range of extracellular enzymes which degrade complex lignocellulosic substrates into soluble substances of considerable nutritive value. White-rot fungi are the most efficient ligninolytic microorganisms in nature. They have evolved a unique mechanism to accomplish the degradation of lignin

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employing extracellular enzymes to generate oxidative radicals (EVANS et al., 1994). This enzyme system is highly nonspecific, therefore, these fungi can oxidise a broad spectrum of structurally diverse compounds (FIELD et al., 1993). Oxidation process involves various enzymes such as lignin peroxidase (LiP), manganese-dependent and manganese-independent peroxidase, laccase, etc. (KIRK & FARRELL, 1987).

LiP attacks both phenolic and non-phenolic aromatic structures the latter giving rise to cationic radicals that fragment spontaneously (KERSTEN et al, 1985). Laccase, a copper-containing phenoloxidase catalyses the four-electron reduction of oxygen to water and it is accompanied by the oxidation of the phenolic substrate (THURSTON, 1994). However, the substrate range of laccase has been recently extended to include non-phenolic lignin subunits in the presence of readily oxidizable primary substrates (mediators). It was found that in the presence of 2,2'-azinobis-(3-ethylthiazoline-6-sulfonate) as mediator laccase oxidizes non-phenolic model lignin compounds (BOURBONNAIS & PAICE, 1990) and polycyclic aromatic hydrocarbons (COLLINS et al., 1996), and enhances the delignification rate of pulp (BOURBONNAIS et al., 1995).

Pleurotus ostreatus, an edible basidiomycete is also able to degrade wood. Its growth and fruiting are dependent largely upon the capacity to utilise cellulose, hemicellulose and other ligno-cellulosic raw materials as a nutritional source. Because of the outstanding importance of lignin decomposition the possible application of *Pleurotus ostreatus* in various biotechnological processes is of both theoretical and practical importance.

The objectives of the study were the determination of the production of LiP and laccase activity during the complete life cycle of *P. ostreatus* cultivated in liquid media composed of agro-industrial residues as the sole source of carbon and nitrogen; the application of multivariate mathematical statistical methods for determining the overall activity and selectivity of enzyme production; and the elucidation of the relationships between the composition of the fermentation media and the characteristics of enzyme production. The results may contribute to the better understanding of the underlying biochemical and biophysical processes and can find potential applications in the biotransformation of lignocellulose biomass.

1. Materials and methods

1.1. Materials

Sodium tartarate and hydrogen peroxide were purchased from Merck KGaA (Darmstadt, Germany); 2,6-dimethoxyphenol (99%, GC) and 3,4-dimethoxybenzyl alcohol (veratryl alcohol) were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Each chemical was used as received.

1.2. Organism and culture conditions

The strain of *P. ostreatus* was taken from the collection of the National Agronomical Station (Oeiras, Portugal). It was maintained on potato dextrose agar (PDA) (Merck KGaA). Dry industrial residues of the production of red pepper (*Capsicum annuum*, dry matter 85.66%) and potato (dry matter 91.15%) (further pepper and dry potato) were used to substitute wheat straw generally used for the commercial scale production of *P. ostreatus*. Extracts of pepper (350 g l^{-1} tap water), dry potato (560 g l^{-1} tap water) and wheat straw (45 g l^{-1} tap water) were prepared by cutting the raw materials into small pieces (approximately 4 mesh) and adding the appropriate quantity of water. The use of higher concentrations of pepper and dry potato for the extraction procedure was motivated by the fact that higher concentration of wheat straw cannot be used because it adsorbs all water. The use of higher concentrations of pepper and potato residues may result in elevated enzyme production of *P. ostreatus*. The mixtures were let to stay for 24 h at 18±2 °C without stirring. After extraction the suspensions were filtered on a Whatman No 1 filter paper then were centrifuged at $2 \cdot 10^4$ g for 20 min. The use of supernatants instead of the whole broth was motivated by the consideration that the sampling of liquid cultures is more precise than that of a broth, containing both liquid and solid phases. In order to determine the nutritive value of the culture media, the concentrations of total phenolics, total and reducing sugars were measured. The composition and initial pH of the culture media are listed in Table 1. Initial pH of the culture media was measured with a pH/mV Meter Digit 501 (Crison, Barcelona, Spain) between glass and kalomel electrodes. Liquid medium for the cultivation of *P. ostreatus* was prepared by mixing 200 ml of extract and 200 ml of distilled water in an 1-l Erlenmeyer flask. The medium was sterilized at 121 °C for 20 min, cooled to 26 °C and inoculated by adding a piece of agar of about 10 mm diameter with micelium. The cultures were incubated at 24±2 °C in the dark for 30 days then were transferred to a fructification room and held at 18±2 °C, 12 h light/day for 33 days. Each seventh day sample was taken from the culture medium under sterile conditions and was centrifuged at 20.000 g for 20 min, and the activity of LiP and laccase was determined by visible spectrophotometry. Each measurement was performed with a UNICAM 87000 Spectrophotometer (Cambridge, England) at 30 °C in a cuvette of 10 mm length. As the pH exerts a considerable impact on the enzyme production, the pH of the samples were also determined by the method described above.

1.3. Determination of enzyme activities

Laccase = reaction mixture contained 10 mM 2,6-dimethoxyphenol in 100 mM sodium tartarate (pH 5.0) and 300–600 µl extracellular culture fluid in a total volume of 1 ml.

Table 1

Composition and initial pH of culture media

No.	compositions	Total phenols ($\mu g\ ml^{-1}$)	Total sugars ($\mu g\ ml^{-1}$)	Reducing sugars ($\mu g\ ml^{-1}$)	pH
I	0.5 straw extract, 0.3 pepper extract, 0.2 potato extract	30.2	201.9	136.3	7.20
II	0.5 straw extract, 0.2 pepper extract, 0.3 potato extract	21.8	158.0	85.6	7.69
III	0.5 straw extract, 0.4 pepper extract, 0.1 potato extract	35.8	228.4	195.5	7.22
IV	straw extract	3.4	9.2	4.2	7.84
V	pepper extract	85.8	563.2	399.5	6.31
VI	potato extract	15.6	290.4	118.2	7.64

The absorbance was measured at 469 nm after 5 min of incubation at 30 °C. LiP = reaction mixture contained 0.54 mM H_2O_2 and 0.4 mM veratryl alcohol in 100 mM sodium tartarate (pH 3.0), and 500 µl of extracellular culture fluid in a total volume of 1 ml. Absorbance was measured at 310 nm after 5, 10 and 15 min of incubation at 30 °C.

1.4. Evaluation of the enzyme activity data by multivariate mathematical-statistical methods

No LiP activity was observed during the fermentation of *P. ostreatus,* therefore, the following calculations were performed only on the activity data of laccase. Data matrix for laccase activities contained the sampling days as variables (together 9 variables) and the composition of culture medium as observations (together 6 observations). In order to determine the overall activity and selectivity of enzyme production according to the composition of culture medium. Spectral mapping technique (SPM) was employed (LEWI, 1976; LEWI, 1989). The method divides the information into two matrices, using the logarithm of the original data. The first one is a vector containing the potency values related to the overall enzyme activity (quantitative measure of the effect of the composition of culture media). The second matrix (selectivity map) contains information on the spectra of activity (qualitative characteristics of the effect). As the evaluation of the multidimensional selectivity maps is difficult with traditional methods their dimensionality were reduced to two by non-linear mapping technique (NLMAP) (SAMMON, 1969). The procedure projects the variables and observations (in our case fermentation media and fermentation time) on a plane in such a manner that the distances between the points should be approximately

the same as in the original multidimensional space. The iteration was carried out to the point when the difference between the last two iterations was lower than 10^{-8}. The potency values calculated by SPM refer to the overall enzyme production of culture media, whereas the distribution of culture media on the map reflects the selectivity of their effect on the enzyme production.

In order to study the effect of the duration of fermentation on the overall enzyme production and on its selectivity the same calculation was performed on the transposed matrix, too. The calculation of selectivity maps was carried out as described above. In this case the potency values refer to the overall enzyme production at the individual sampling times, whereas the distribution of the sampling times on the two-dimensional non-linear map reflects the selectivity of the enzyme production at different sampling times.

The relationship between the results of SPM, fermentation time and the composition of culture media was elucidated by stepwise regression analysis (MAGER, 1982). In the common multilinear regression analysis the inclusion of independent variables that exert no significant impact on the dependent variable lessens the significance level of independent variables that significantly influence the dependent variable. Stepwise regression analysis overcomes this difficulty by eliminating automatically from the selected equation the insignificant independent variables enhancing in this manner the information power of the calculation. The dependent variables were separately the potency values and the first and second coordinates of the two-dimensional non-linear selectivity maps calculated from the original and transposed data matrices, the independent variables being the days of fermentation, the composition and pH of the culture media. As the relationships between the calculated parameters and the days of fermentation were markedly non-linear, the square of the days of fermentation was also included. The number of accepted independent variables was not limited, the acceptance level was set to 95% significance level.

Softwares for SPM and NLMAP were prepared by Dr. Barna Bordás (Plant Protection Institute of Hungarian Academy of Sciences, Budapest, Hungary). Software for stepwise regression analysis was purchased from Compudrug, Ltd. (Budapest, Hungary).

2. Results and discussion

LiP activity was not detected in any culture media during the fermentation process, indicating that *P. ostreatus* does not produce LiP in detectable amount. The activity values of laccase are compiled in Table 2. The activity values show high variations, indicating that both the composition of culture media and the fermentation time exert a considerable influence on the activity of laccase. Similar results have been

previously obtained, however, the authors used a different method for the determination of the laccase activity, therefore, the results cannot be compared (SANNIA et al., 1986). The potency values reflecting the effect of the composition of culture media and fermentation time on the overall activity of laccase are compiled in Table 3. The results entirely support our previous qualitative conclusions, the potency values show high differences according to the composition of the culture media and the fermentation time. Furthermore, the results indicate that the effect of the composition of culture media and that of the fermentation time are commensurable.

The two-dimensional non-linear selectivity map of culture media is shown in Fig. 1. Culture media containing straw extract form a clear-cut cluster, whereas the points characterizing potato and pepper extracts are on the opposite end of the map. This finding suggests that the presence of straw extract in the culture media play a considerable role in the determination of the selectivity of the laccase production of *P. ostreatus*.

Table 2

Laccase production of Pleurotus ostreatus *(nanomol* $min^{-1}ml^{-1}$*) and the pH of the samples. Roman numbers refer to culture media in Table 1*

Laccase production

No. of culture media	Fermentation time (days)								
	7	14	21	28	35	42	49	56	63
I	0	1.53	9.27	12.78	31.45	13.35	26.41	27.42	34.11
II	1.53	5.20	39.69	33.87	47.98	16.94	24.23	40.28	16.33
III	2.16	9.79	21.37	28.23	59.48	19.35	56.25	31.53	34.68
IV	1.92	0.31	15.93	2.70	1.10	0.081	0.82	0.44	0.73
V	5.50	39.58	21.41	70.97	72.72	50.89	35.89	34.07	58.43
VI	1.32	5.65	47.50	61.69	79.92	57.18	56.05	42.74	56.45

Sample pH

No. of culture media	Fermentation time (days)								
	7	14	21	28	35	42	49	56	63
I	7.18	7.15	7.00	6.97	6.92	6.90	6.89	6.89	6.80
II	7.69	7.66	7.62	7.6	7.57	7.55	7.55	7.52	7.49
III	7.22	7.20	7.18	7.15	7.12	7.10	7.08	7.00	6.94
IV	7.80	7.75	7.68	7.57	7.55	7.47	7.40	7.39	7.35
V	6.28	6.25	6.23	6.19	6.17	6.15	6.12	6.10	6.10
VI	7.60	7.58	7.56	7.49	7.45	7.40	7.35	7.32	7.28

Table 3

Effect of the composition of culture media and fermentation time on the activity of laccase production of Pleurotus ostreatus. *Potency values (arbitrary units) calculated by SPM. Roman numbers refer to culture media in Table 1*

No. of culture media	Potency	Fermentation days	Potency
I	52.11	7	5.07
II	74.35	14	25.34
III	87.61	21	62.12
IV	8.01	28	85.83
V	129.82	35	119.47
VI	136.17	42	64.42
		49	81.51
		56	72.05
		63	81.95

The two-dimensional non-linear selectivity maps of the fermentation time are shown in Fig. 2. The days of fermentation form two loose groups according to the length of fermentation. This finding suggests that the selectivity of enzyme production is similar in the first 21 days of fermentation then it changes and remain the same until the end of the fermentation process.

Stepwise regression analysis found significant linear relationships between the overall enzyme production (potency values), its selectivity (first and second coordinates of the two-dimensional non-linear selectivity maps = spm_1 and spm_2) and the composition of culture media and fermentation time.

Culture media (n = 6):

Potency = 133.3–124.7±15.3). amount of straw extract (1)

$$r_{calc.} = 0.9711 \; r_{99\%} = 0.9172$$

Spm_1 = –520.3+(93.0±17.7).pH of the culture media (2)

$$r_{calc.} = 0.9343 \; r_{99\%} = 0.9172$$

Spm_2 = 142.9–(130.6±33.9).amount of straw extract (3)

$$r_{calc.} = 0.8873 \; r_{99\%} = 0.8114$$

Days of fermentation (n = 9):

Potency = $-31.4+(5.71\pm1.66).day -(6.57\pm2.31).10^{-2}.(days)^2$ (4)

$$b'_1\% = 54.75; \; b'_2 = 45.25; \; F_{calc.} = 8.61; \; F_{99.9\%} = 5.14$$

No significant relationship was found between spm_1, spm_2 and the days of fermentation.

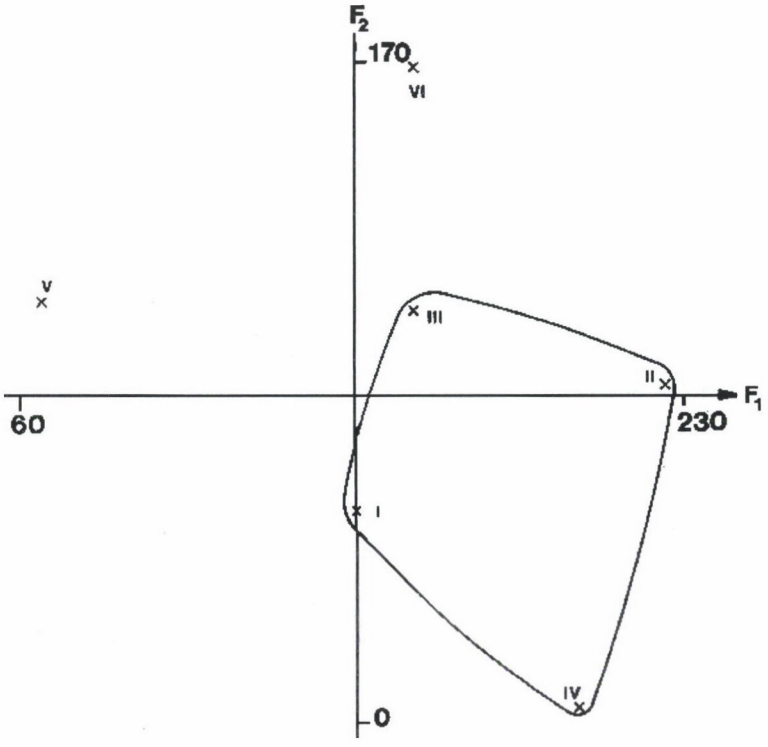

Fig. 1. Two-dimensional non-linear selectivity map of culture media based on the activity of laccase. Number of iterations: 119; maximum error: $2.57\ 10^{-2}$. Roman numbers refer to culture media in Table 1

The data clearly show that the presence of straw extract decreases the overall activity of laccase. This finding indicates that the use of other agro-residues instead of the wheat straw may result in the increase of the enzyme production, therefore, their application as substitutes for wheat straw is justified. This finding is in accordance with the nutritive values in Table 1, where the nutritive value of wheat straw was the lowest. The negative regression coefficient indicates that the presence of straw extract significantly decrease the overall activity of laccase. The results further demonstrate that the selectivity of the laccase production depends on both the pH and the amount of straw extract of the culture media.

A quadratic correlation was found between the days of fermentation and the laccase production, the activity increased at shorter fermentation times and decreased near the end of fermentation process.

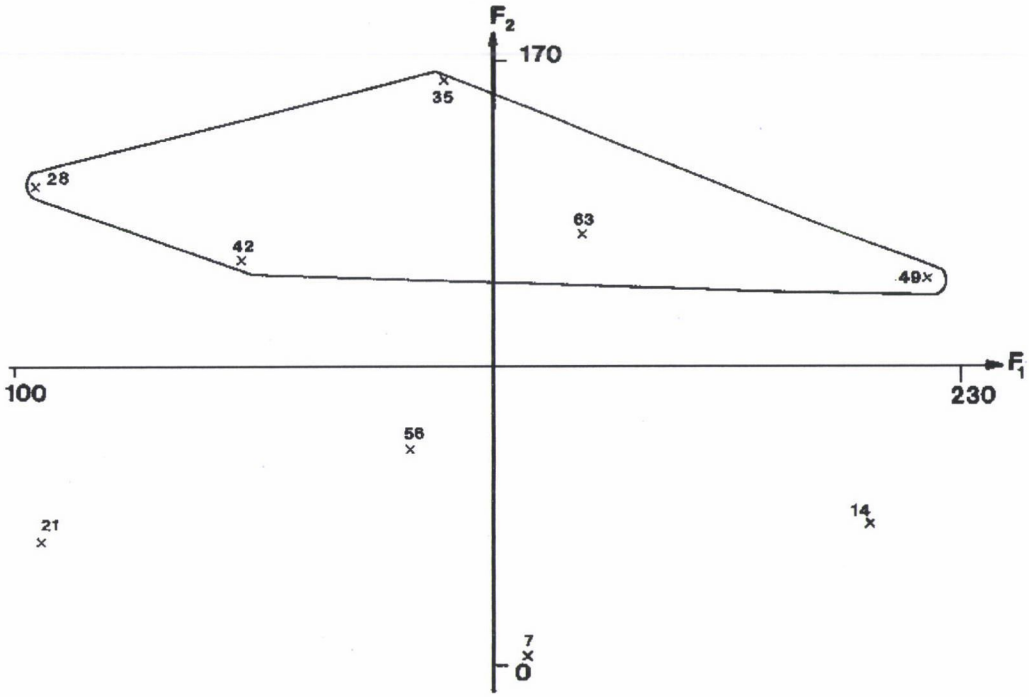

Fig. 2. Two-dimensional non-linear selectivity map of fermentation time based on the activity of laccase. Number of iterations: 89; maximum error: 3.89 10^{-2}. Numbers refer to fermentation days

3. Conclusions

The results proved that *Pleurotus ostreatus* produces laccase enzymes which is able to degrade lignin and related complex molecular structures. Because of this capacity *P. ostreatus* may find application in a variety of biotechnological processes. Calculations further proved that the overall enzyme production and its selectivity can be regulated by the composition of the culture media, containing only agro-residues as carbon and nitrogen sources.

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This work was supported by the Portuguese-Hungarian co-operation project 'Development of new methods and their application for the assessment of the effect of environmental conditions on the stability of color pigments in foods'.

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Acta Alimentaria, Vol. 30 (4), pp. 373–380 (2001)

IDENTIFICATION AND QUANTIFICATION OF SOME CAPSAICINOIDS IN PADRÓN PEPPER (*CAPSICUM ANNUUM* L. VAR. *ANNUUM*) FRUITS

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(Received: 15 November 2000; accepted: 15 June 2001)

Capsaicinoid and phenolic contents were determined at different maturation stages of the pepper fruit, with an increase being observed in the levels of both components. The increase in total soluble phenolics probably reflects the accumulation of capsaicinoids seen throughout development. Four different capsaicinoids and their precursor vanillylamine were identified in the cultivars studied. Capsaicin, dihydrocapsaicin and nordihydrocapsaicin showed the same accumulation patterns in all stages, but homodihydrocapsaicin appeared only in the last stage.

Keywords: pepper, *Capsicum annum* L, capsaicinoids, phenolics, HPLC determination

Phenolic compounds constitute one of the most important groups of natural products, which possess an aromatic ring bearing one or more hydroxyl groups (HARBONE, 1980). Some phenolics, such as hydroxycinnamic acids and flavonoids, are found in all the plant groups, but in very different quantities. However, other phenolics are exclusive to a particular genus and species (SWAIN, 1979; MACHEIX et al., 1990).

These compounds are present in all plant tissues and are often the most abundant secondary metabolites in fruits, where they sometimes reach high concentrations. Phenolics are also important because of their contribution to the sensory quality of fruits- color, astringency, bitterness, and flavor (MACHEIX et al., 1990). The levels of phenolic compounds during growth and maturation vary strongly depending on the availability of the precursors of phenolic molecules and the activity of the enzymes involved in the biosynthesis of these precursors in interconversions between phenolic molecules or in their degradation (LUCKNER, 1980).

There is a group of phenolic compounds characteristic of some fruits of the genus *Capsicum,* capsaicin and other related compounds commonly called capsaicinoids

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(BENNET & KIRBY, 1968). The main capsaicinoids in hot peppers are capsaicin, dihydrocapsaicin and nordihydrocapsaicin. A great number of naturally occurring capsaicinoids have been identified as minor components (JURENTISCH et al., 1979).

The capsaicinoids are synthesized by the condensation of vanillylamine with a short-chain branched fatty acid. The short-chain branched fatty acid moiety is biosynthetically derived from valine, while the vanillylamine moiety comes from L-phenylalanine, via the phenylpropanoid pathway (LEETE & LOUDEN, 1968). This pathway begins with the deamination of phenylalanine to produce cinnamic acid, which flows through this pathway to yield *p*-coumaric, caffeic and ferulic acids. The first part leads to the formation of a wide range of secondary metabolites such as coumarins, flavonoids and lignin. The latter part from ferulic acid to capsaicin is only found in the fruits of the genus *Capsicum*.

Many studies have reported the accumulation of capsaicinoids in *Capsicum* fruits in relation to fruit age, size and stage of development (IWAI et al., 1979; SALGADO-GARCIGLIA & OCHOA-ALEJO, 1990; SUKRASNO & YEOMAN, 1993). The results are similar given the fact that capsaicinoids begin to accumulate in the early stages of fruit development and this accumulation achieves its maximum rate as the fruit approaches the end of its growth phase (HALL et al., 1987). However, the concentration of capsaicinoids varies depending on the different pepper cultivars examined (GOVINDARAJAN et al., 1987).

The Padrón pepper (*Capsicum annuum* L. var. *annuum*) is a commercial cultivar available in the region of NW Spain, Galicia, and widely accepted for fresh market consumption. The fruits are commercialized when immature and their flavor is not very hot, because they contain only low levels of capsaicin, the main compound causing pungency in peppers. This low capsaicinoid content is the most important fruit quality attribute. Phenolic compounds may contribute greatly to the organoleptic characteristics of the fruit. The purpose of this study is to examine the changes in the levels of soluble phenolics and capsaicinoids in the pepper fruit, cv. Padrón, during growth and ripening.

1. Materials and methods

1.1. Plant material

Plants of *Capsicum annuum* L cv. Padrón were grown in a greenhouse on the campus of the University of La Coruña from April to September. Anthesis began *ca* 3 months after germination; individual flowers were numbered and the date of flowering recorded. Pepper fruits were harvested every 7 days from 14 (stage 1) to 42 (stage 5) days after flowering and individual weights of the whole peppers were measured. The fruits were subsequently stored at –30 °C until processed.

1.2. Extraction and quantification of capsaicinoids by HPLC

Capsaicinoids were extracted from Padrón pepper fruits using the technique described by COLLINS and co-workers (1995) with modifications (ESTRADA et al., 1998).

Pepper fruits were oven-dried at 60 °C for 2–5 days, their dry weight (DW) determined, ground using a laboratory mill and stored in sealed plastic tubes at room temperature prior to extraction. The capsaicinoids were extracted from 1.0 g of the ground pepper in 10 ml of acetonitrile by heating to 80 °C for 4 h. The suspended material was allowed to settle and the supernatant was extracted and centrifuged at 100 g for 10 min and then filtered (0.45 μm Whatman on a 10 ml disposable syringe) into a 2 ml glass sample vial, capped and stored at 5 °C until analyzed. A 10 μl aliquot was injected into the HPLC column.

The samples were analyzed using a Waters LC616 System equipped with a Waters 717plus Autosampler, a Waters Temperature Control Module, a Waters 996 Photodiode Array Detector and Millennium Software for data processing. Reverse phase HPLC was carried out on a Spherisorb C_{18} column (5 μm particle size, 150 mm× 46 mm). A precolumn guard cartridge, Spherisorb C_{18} column, was also used. To determine the capsaicinoids, the HPLC operating conditions were: 25 °C, a flow rate of 1 ml min^{-1}, and a 14 min run. The mobile phase was isocratic with 50% solvent A (100% acetonitrile-HPLC grade) and 50% B [10% acetonitrile (by volume) in water].

The vanillylamine and the capsaicinoids were identified on the basis of their retention times and analysis of the spectra of the different peaks. The mean retention time under these conditions was: vanillylamine 4.40 (±0.04) min, nordihydrocapsaicin, 7.00 (±0.06) min, capsaicin 7.42 (±0.05) min, dihydrocapsaicin 10.50 (±0.05) min and homodihydrocapsaicin 11.50 (±0.04) min.

1.3. Extraction and determination of soluble phenolics

Soluble phenolics from Padrón pepper fruits were extracted by the same procedure as capsaicinoids. Total soluble phenols were determined using the Folin-Ciocalteu reagent according to the method of SINGLETON and ROSSI (1965). The content of the soluble phenols was calculated from a standard curve obtained using different concentrations of ferulic acid.

2. Results

2.1. Soluble phenolic and capsaicinoid content

Figure 1 shows the changes in soluble phenolics and capsaicinoids that the pepper fruit underwent during the maturation process. Soluble phenolics were already detected at the first stage, 14 days after flowering. Their levels were similar during the second, third and fourth stages – 21, 28 and 35 days after flowering, respectively. The Padrón pepper showed a moderate increase in these levels at the end of development, with the highest values found in the 5th stage, 42 days after flowering.

The pattern of capsaicinoid accumulation was similar to that of soluble phenolics. Their levels remained low for 21 days, but 28 days after flowering capsaicinoids increased moderately, and finally, the cv. Padrón pepper reached their highest levels in the last stage, 42 days after flowering (Fig. 1). However, the increase in capsaicinoids was higher than in soluble phenolics.

2.2. Identification of some capsaicinoids

Table 1 shows the different capsaicinoids detected in the Padrón pepper fruit. Capsaicin, dihydrocapsaicin and nordihydrocapsaicin, showed the same pattern of accumulation, with the latter being of low concentration. The other homologue of capsaicin, homodihydrocapsaicin, was detected only 42 days after flowering and in significantly lower levels when compared to the other compounds. Vanillylamine, the precursor of capsaicin before condensation with 8-methyl-6-nonenoic acid, could be detected 14 days after flowering, and its content moderately increased until the 35th day. Furthermore, the pepper fruit showed a great increase in this compound at the end of development, with the highest values found 42 days after flowering (Table 1).

Table 1

Vanillylamine and capsaicinoids in whole pepper during growth[a]

D.a.f.	CAP	DHC	NDHC	HDHC	VAN
14	64.3 ± 8.0	47.2 ± 4.0	21.5 ± 0.8	nd	26.0 ± 0.7
21	57.0 ± 5.0	38.2 ± 5.0	19.7 ± 0.7	nd	44.0 ± 0.8
28	161.5 ± 10.0	105.0 ± 8.0	40.2 ± 0.6	nd	48.0 ± 0.9
35	96.0 ± 6.0	56.4 ± 5.0	22.0 ± 0.9	nd	55.4 ± 1.0
42	426.0 ± 20.0	275.0 ± 10.0	95.5 ± 1.0	39.0 ± 0.7	93.0 ± 0.9

[a]Data are expressed in ng g^{-1} DW±SD. D.a.f.: Days after flowering; CAP: capsaicin; DHC: dihydrocapsaicin; NDHC: nordihydrocapsaicin; HDHC: homodihydrocapsaicin; VAN: vanillynamine; nd: not detected

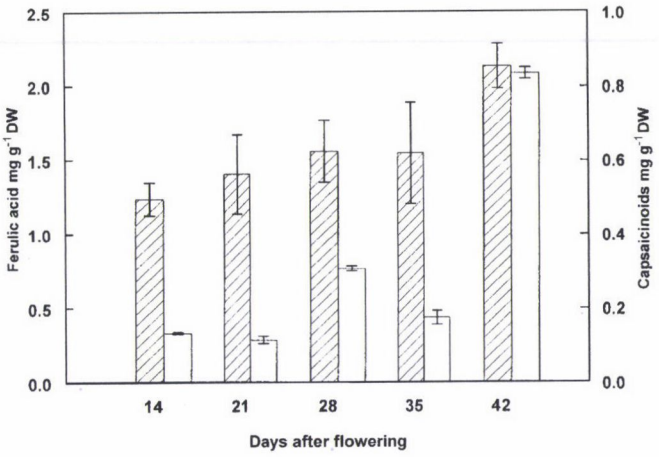

Fig. 1. Changes in soluble phenolics and capsaicinoids in pepper fruits at different stages of development, expressed in mg eq. ferulic acid/g DW. Values are mean of 30 fruits in three different extractions. Bars show SD ▨: soluble phenolics, □: total capsaicinoids

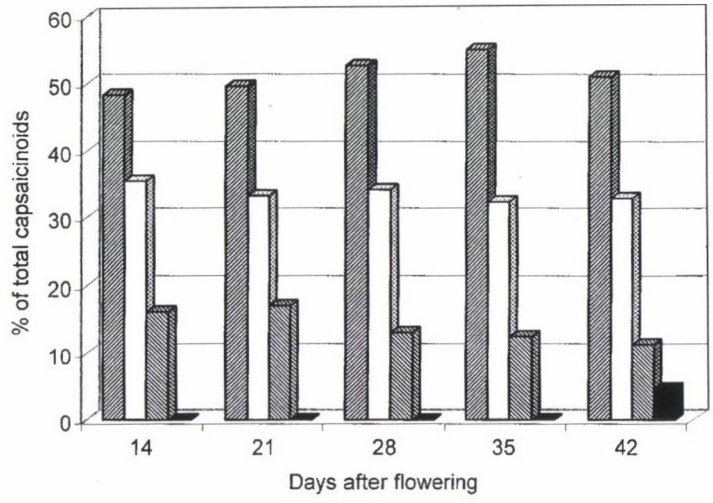

Fig. 2. Percentage of the individual capsaicinoids over the total capsaicinoid content. ▨: capsaicin, □: dihydrocapsaicin, ▧: nordihydrocapsaicin and ■: homodihydrocapsaicin

There were no significant changes in the proportion of the capsaicinoids during the course of development. Capsaicin was always the major component, followed by dihydrocapsaicin and nordihydrocapsaicin in all the stages studied (Fig. 2). However, it is noteworthy that the percentage of nordihydrocapsaicin decreased over the course of development, with the lowest value found 42 days after flowering.

3. Conclusions

Changes of the phenolic pool are observed during the maturation of different species of fruits. Since the first research carried out on *Prunus domestica* (SWAIN & HILLIS, 1959) there have been numerous investigations confirming that soluble phenolics have the highest levels during the initial stages of development, and that these levels fall during growth (MACHEIX et al., 1990; MAYR et al., 1995). In some cases, however, as in red fruits, the levels of phenolics rise again, due to the accumulation of flavonoids such as in grape berries (PIRIE & MULLINS, 1980), or anthocyanins. The latter is the case of the tomato fruit, in which the increase in total phenol content is caused mainly by a strong increment in naringerin and other unidentified compounds in the skin (WARDALE, 1973). This situation is similar to that of pepper, cv. Padrón, whose increase in the content of soluble phenols during maturation probably reflects the formation of the major compounds, the capsaicinoids, since they are also detected by the Folin Ciocalteau reagent (BAJAJ, 1980). The increase in capsaicinoids and the more direct precursor, vanillylamine, mask the drop in the other free phenolics such as protocatechuic acid, chlorogenic acid and cinnamic acid observed in Padrón pepper (ESTRADA et al., 2000).

The relative concentrations of the three principal homologues, capsaicin, dihydrocapsaicin and nordihydrocapsaicin, found in all species have been considered characteristic of the species and subspecies. JURENITSCH and co-workers (1979) proposed a chemotaxonomic classification based on the capsaicinoid composition which agreed, to some extent, with the traditional classification based on flower morphology and seed characteristics. The key to the identification of the individual *Capsicum* species is based on the sum of the three principal individual capsaicinoids and their levels. The proportions found in the cv. Padrón are the ones that correspond to *Capsicum annuum* var. *annuum* in this key. As regards homodihydrocapsaicin, it could be detected during the last flowering stage. This minor capsaicinoid has been found only in a few cultivars of different species of *Capsicum* (IWAI et al., 1979; COLLINS et al., 1995).

When the total amount of capsaicinoids in cv. Padrón were analyzed, it was observed that in the last stage, this value was about 0.83 mg per g of dry weight (0.083%). If these results are compared with other hot varieties of *Capsicum annuum*

(Table 2) (COLLINS et al., 1995; CONTRERAS-PADILLA & YAHIA, 1998), it can be seen that the content of capsaicinoids in the Padrón cultivar is very low even in the last stage. The pepper fruits vary considerably in their pungency according to capsaicinoid content per g of dry weight, covering a range of values evaluated as a mild variety, 0.1 to 0.2%; medium, 0.2 to 0.4%, hot 0.4 to 0.6% and very pungent variety, over 0.6% (GOVINDARAJAN et al., 1987). In conclusion, if we take the capsaicinoid content of the Padrón pepper into account, this cultivar may be considered to be mild, and therefore suitable for fresh consumption, especially during the early stages.

Table 2

Maximum concentration of capsaicinoids in different cultivars of Capsicum annuum *L*

Cultivar	Capsaicinoids mg/g DW
Padrón	0.83
Pasilla[a]	3.70
Cascabel[a]	1.37
Cubanella[a]	12.17
Jalapeño[a]	20.37
Nuevo Mexico[a]	0.81
De árbol[b]	80.52
Piquin[b]	53.71

[a] COLLINS et al., 1995; [b] CONTRERAS & YAHIA, 1998

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This work was supported by Xunta de Galicia (XUGA 10301/A/95). The authors are grateful to Mrs. M. LEMA (S.X.A.I.N., Univ. of A Coruña) for her technical assistance.

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Acta Alimentaria, Vol. 30 (4), pp. 381–393 (2001)

EFFECT OF κ-CARRAGEENAN AND NaCl ON THERMAL PROPERTIES OF FROZEN SURIMI PREPARED FROM ADRIATIC PILCHARD

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(Received: 6 December 2000; accepted: 4 June 2001)

Samples of surimi were prepared under laboratory conditions from Adriatic pilchard *(Sardina pilchardus)*. Water content in surimi was 81.5% before mixing with NaCl and κ-carrageenan, which were added in the range of mass fraction from 0 to 10%. Relative apparent specific enthalpy ($\bar{H}$), initial freezing point T_i, density ρ and thermal conductivity k of surimi in the temperature range from –25 °C to 10 °C were determined by differential thermal analysis (DTA), gravimetric method and line-heat source technique, respectively. For determination of relative apparent specific enthalpy ($\bar{H}$) the mathematical model of enthalpy based on orthogonal collocation approximation of DTA was applied. Redistributions of apparent enthalpy in the freezing range as functions of mass fractions of added substances were determined. Increase of mass fraction of added substances resulted in the increase of mass fraction of bound (unfreezable) water and lowered initial freezing point T_i, which has effects on the decrease of thermal conductivity k and increase of apparent specific enthalpy ($\bar{H}$) in the temperature range from –25 °C to T_i. This effect was more pronounced for samples where surimi was mixed with NaCl.

Keywords: thermal properties, frozen surimi, κ-carrageenan, NaCl

Mathematical models of thermal properties of food are important for the determination of process parameters, design of process units, development of new technologies, and are necessary for the application of numerical methods for determining heat transfer in frozen foods. Thermophysical properties of food can be determined experimentally or estimated from mathematical models which are functions of temperature and food composition (especially water content). There are various methods for the experimental determination of thermal conductivity, which may be generally classified as steady state and transient methods (TAIT & HILLS, 1964).

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Due to experimental problems associated with steady state methods, described by REIDY and RIPPEN (1971), for the determination of thermal conductivity of food the line-heat source technique is applied most frequently. The line-heat source technique measures thermal conductivity, assuming unsteady-state heat conduction from an infinitely long line-heat source immersed in an infinite and homogeneous medium. The theoretical bases of the method are given by Hooper and Lepper and Nix and co-workers (SWEAT & HAUGH, 1974). For small samples of frozen food the thermal conductivity probe constructed by SWEAT (1986) is used most often. To apply this method for foods in the temperature range below T_i, two conditions must be met: linearity of the temperature versus ln(time) plot must be satisfied and temperature increase after heating must be limited to assure measurement at the correct temperature and to minimize the effect of latent heat of fusion (WANG & KOLBE, 1990). Since the construction of the probe is not yet standardised, the relation between the two conditions and the probe design to minimise the difference between theoretical and experimental conditions is still subject to numerous investigations (MURAKAMI et al., 1996). This consideration resulted in the formation of an ad-hoc committee of the North Central Regional Project (NC-136) to address the issue of probe design (MURAKAMI et al., 1995). For determining thermophysical properties (T_i, H, c_p) techniques of differential scanning calorimetry (DSC) and differential thermal analysis (DTA) are applied, which are according to "ICTA" (Nomenclature Committee of the International Confederation for Thermal Analysis) methods based on change of apparent enthalpy (FINDLAY & BARBUT, 1990). DTA is commonly used as a method for determination of phase change temperatures, degree of sample purity, thermal stability and for identification of chemical or biochemical reactions during heat treatments (CUNNINHAM & WILBURN, 1970). Since the DTA method is not standardised (geometry and material of measurement cell, reference substance, experimental conditions) it is considered as a semiquantitative technique, opposite to DSC, which is an accurate quantitative method. However, analysis of DTA curve by application of heat balance by the parabolic partial differential equation provides quantitative determination of heat (enthalpy), which is released or absorbed during the first and second order phase changes (CUNNINHAM & WILBURN, 1970).

In this work, for the determination of thermophysical properties of surimi samples mixed with NaCl and κ-carrageenan, a laboratory apparatus was constructed for differential thermal analysis (DTA) and for determining thermal conductivity with a thermal conductivity probe. A numerical method based on orthogonal collocations for approximating the partial differential equation for interpretation of DTA curve is derived. Measurements were performed in the temperature interval from –25 °C to 5 °C. In order to derive mathematical models, measured values of thermophysical properties were correlated with mass fractions of added substances. The results were validated by available literature data.

1. Materials and methods

1.1. Materials

Surimi samples were prepared in laboratory from Adriatic pilchard (*Sardina pilchardus*) according to the technique by LEE (1984) with details given by SYCH and co-workers (1990). Samples were divided into two groups and mixed with: a) κ-carrageenan and b) NaCl. Mass fractions were in the range from 0 to 10% determined as percent of total mass. Moisture content was 81.5% determined by the A.O.A.C. method (1980) for meat products before addition of the added components. Total proteins mass fraction was 16.3% determined with 1 g samples by Kjeldahl method; (Kjeltec System, model 1002 Distilling Unit, Tecator Inc., Boulder, Colorado, U.S.A). Samples were packaged in polyethylene bags and quickly frozen in liquid nitrogen and stored at –25 °C. Average storage time was 6 weeks before experimental treatment.

As a reference substance for DTA measurement, 30% water solution of $CaCl_2$ was used. Distilled water was used as a calibration substance for correcting of initial freezing point and for the thermal conductivity probe.

1.2. Methods

Density of samples above initial freezing point T_i was determined experimentally by gravimetric method. A known mass (ca. 5 g) of sample (at temperature of 24.2±1 °C) was added to a 60 ml volumetric flask which was filled to volume with distilled water (22 °C). Density was calculated using the following equation:

$$\rho = \frac{m}{60 - V} \tag{1}$$

where ρ (g ml^{-1}) is density, m (g) is mass of sample; V (ml) is volume of water added (procedure recommended for meat samples (SZCZESNIAK, 1983)). The procedure was completed within 30 s, and any moisture absorption by the sample was minimised.

Thermal conductivity was determined by the line-heat source technique by a thermal conductivity probe having a design described by SWEAT (1986). The probe was obtained from Sweat's laboratory at Texas A&M University, Department of Agricultural Engineering. The measurement system for on-line data acquisition and software for statistical evaluation of thermal conductivity were constructed and developed at the Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia (KOVAČEVIĆ & KURTANJEK, 1995).

In the same laboratory DTA apparatus was constructed (KOVAČEVIĆ & KURTANJEK, 1993) and used for measurement of initial freezing point and determination of relative apparent specific enthalpy in the freezing range.

1.3. Evaluation methods

Above initial freezing point T_i densities of samples were experimentally determined by gravimetric method, and below T_i by Levy's equation (2), as reported by SUCCAR (1985):

$$\rho = 966.33 \cdot [0.882 + (T_i - T)] / [0.836 + (T_i - T)] \tag{2}$$

Experiments for measurement of thermal conductivity were conducted in the temperature range from –25 °C to 10 °C at 2.5 °C h^{-1} rate of thawing. Impulses were applied in the power range from 2.25 to 4.2 W m^{-1}. Duration of impulses ranged from 30 to 60 s. Powers applied were lower than those used by SWEAT and HAUGH (1974) and WANG and KOLBE (1990), which was enabled by use of high sensitivity instrument amplifier and accurate A/D conversion. Low energy of impulses resulted in reduced disturbance of distribution of unfrozen water in samples. Lower power was applied in the temperature range close to initial freezing points. Maximum amplitude in temperature was restricted to 0.8 °C in the temperature range below –10 °C and 0.4 °C in the range up to the initial freezing points. For each sample about 40 experiments were performed and in each experiment about 400 data points were taken in the range of linear temperature increase. A measurement system for determining thermal conductivity, which enables one-dimensional treatment of heat transfer was designed. For determining thermal conductivity k from experimental data obtained by the method of line-heat source, a mathematical model of least squares in the linear range of temperature was applied (KOVAČEVIĆ & KURTANJEK, 1995), which is:

$$k = \frac{\overline{T \cdot \ln(t - t_0)} - \overline{T} \cdot \overline{\ln(t - t_0)}}{\overline{\ln^2(t - t_0)} - \overline{\ln(t - t_0)}^2} \tag{3}$$

where t (s) is time; t_0 (s) is initial time.

DTA measurements of surimi samples mixed with the added substances (total 21 samples) were conducted in the temperature range from –25 to 5 °C. Each DTA diagram is corrected only for constant error of +0.045 °C, which was determined from calibration with distilled water. The design of the DTA apparatus is in agreement with assumptions on which the mathematical model for the quantitative determination of apparent enthalpy is applied. The geometry of the test chambers enables one-dimensional treatment of the heat transfer process. Slow rate of temperature increase, 15 °C h^{-1}, makes quasi-steady state approximations applicable, and gives well defined and reproducible DTA diagrams.

Apparent enthalpy is determined from DTA curves in intervals of 40 mK as proposed by KOVAČEVIĆ and KURTANJEK (1993) which has the discrete form given by:

$$H_{k+1} = H_k + \frac{k_s(T_s)}{\rho_s(T_s)\cdot\alpha(T_r)}\cdot \left[T_{r,k+1} - T_{r,k} - \frac{4\cdot\alpha(T_r)}{R^2}\cdot DTA_k\cdot\Delta t\right] \tag{4}$$

where ρ_s (kg m^{-3}) is density of sample; k_s (W m^{-1} K^{-1}) is thermal conductivity of sample; T_s (°C) is temperature of sample; T_r (°C) is temperature of reference substance; R (mm) is radius of a test chamber; DTA (°C) is difference between temperature of sample and reference; k and k+1 is sampling index; Δt (s) is sampling period. For the calculation the following parameters are required: thermal diffusivity of reference substance, thermal conductivity and density of samples and continuous signals for DTA and reference temperature. The zero of relative apparent specific enthalpy is set at $T = -25$ °C. Thermal diffusivity of the reference substance as function of temperature was correlated from data by IBELE (1973):

$$\alpha(T) = 0.1323 - 3.046\cdot10^{-4}\cdot T \tag{5}$$

2. Results and discussion

In the temperature range above initial freezing point, based on 5 replicate measurements, average value of $\rho = 1019$ kg m^{-3} was determined. Values of $\rho_s(T_s)$ in the equation for apparent enthalpy (4) were determined by equation (2).

Thermal conductivities were determined by linear regression for the temperature range corresponding to (3), and linearity was checked by linear coefficient of determination, which was in all experiments in the range $r^2 = 0.97$–0.99. On Fig. 1 results of surimi thermal conductivity measurement with 8% κ-carrageenan concentration are presented. As it can be observed on Fig. 1, values of k at temperatures slightly below the T_i were not determined. This was due to the fact that disturbance of sensitive phase equilibrium by heat impulses from the line-source method can not be neglected. This is the temperature range in which large changes of latent heat and thermal diffusivity occur. A numerical solution of the partial differential equation may be used to evaluate how apparent specific heat affects the conductivity measurement, and to correct the error, as suggested by Schwartzberg. Another way is to use a steady state measurement technique such as a guarded hot plate (WANG & KOLBE, 1990).

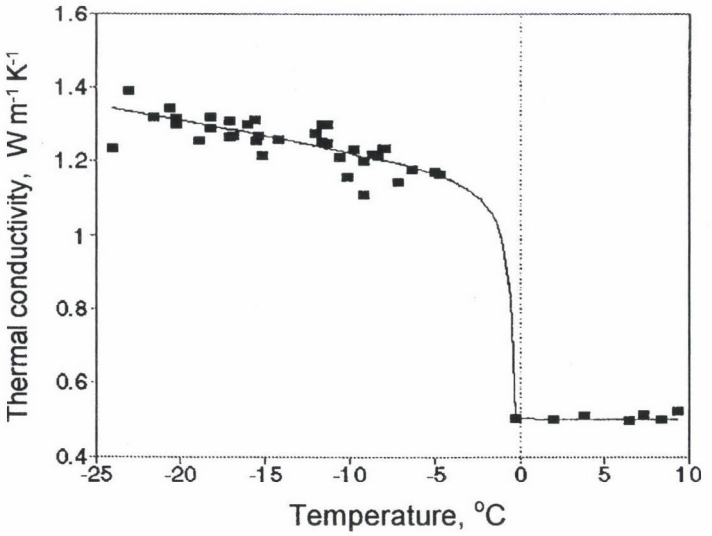

Fig. 1. Thermal conductivity of surimi with added κ-carrageenan ($w = 8\%$). ■: experimental data; –: model data

The parameters of the Schwartzberg model for thermal conductivity (SCHWARTZBERG, 1977), k'_f and B, were estimated by the least squares method from the linearized model expression given by:

$$\left(k - k_f \cdot \frac{T_i}{T}\right) = k'_f \cdot \left(1 - \frac{T_i}{T}\right) + B \cdot (T_i - T) \tag{6}$$

where k'_f (W m^{-1} K^{-1}) and B (W m^{-1} K^{-2}) are parameters in the regression of thermal conductivity; k_f (W m^{-1} K^{-1}) is thermal conductivity at T_i. In (6) T_i was determined from DTA and k_f was obtained by linear regression of the data above the initial freezing point. Values are given in Table 1, but k_f for samples with different added substances levels were not statistically different. Standard deviation σ for k_f for samples of surimi with added κ-carrageenan is 1.3% and for samples with NaCl is 1.1%.

Obtained values of the thermal conductivity for samples of surimi with κ-carrageenan are in close agreement with data of WANG and KOLBE (1990). For example, for sample with 8% κ-carrageenan and 81.5% mass fraction of water standard deviation obtained in this work is 4.9%. For samples with 8% mass fraction of added substances and 80.3% of water WANG and KOLBE report average standard deviation of 5.9%.

Table 1

Values of k_f (W m^{-1} K^{-1}) for samples of surimi with different mass fractions of the added substances

Mass fractions of added substances w, %	k_f, W m^{-1} K^{-1}	
	Surimi + κ-carrageenan	Surimi + NaCl
0	0.499	0.511
1	0.506	0.499
2	0.505	0.504
3	0.511	0.497
4	0.509	0.507
5	0.497	0.499
6	0.506	0.506
7	0.512	0.499
8	0.501	0.494
9	0.495	0.487
10	0.509	0.510

Regression analysis was performed in two stages. First the parameters k'_f and B were estimated from experiments with each mass fraction of the added substances. In the second stage the parameters were correlated with the mass fraction by the linear models:

$$k'_f = a_0 + a_1 \cdot w$$
$$B = b_0 + b_1 \cdot w \tag{7}$$

where a_0 (W m^{-1} K^{-1}), a_1 (W m^{-1} K^{-1}), b_0 (W m^{-1} K^{-2}) and b_1 (W m^{-1} K^{-2}) are parameters in the regression of thermal conductivity; w (%) is mass fraction of the added substances. The estimates of the coefficients in the linear regressions (7) are given in Table 2.

The parameter B, which is related to the linear dependence of thermal conductivity on temperature, decreases with increasing mass fraction of κ-carrageenan and NaCl (Table 2) due to increased level of bound water (WANG & KOLBE, 1990). It is in agreement with the prediction of k values by equation (6), and is also illustrated in Fig. 2. Increase in mass fractions of the added substances on temperatures below T_i resulted in the decrease of k values of surimi. It may be interpreted as reaction of added substances with myofibril proteins of surimi and dipole water molecules, resulting in the increase of mass fraction of bound water, which is unfreezable in the measurement range of temperature from –25 °C to T_i.

Table 2

Linear regression coefficients of the parameters k'_f and B mass fraction of the added substances

Added substance	a_0	a_1	b_0	b_1
κ-carrageenan	1.075	0.12063	0.01429	–0.00487
NaCl	1.138	0.00733	0.01326	–0.00079

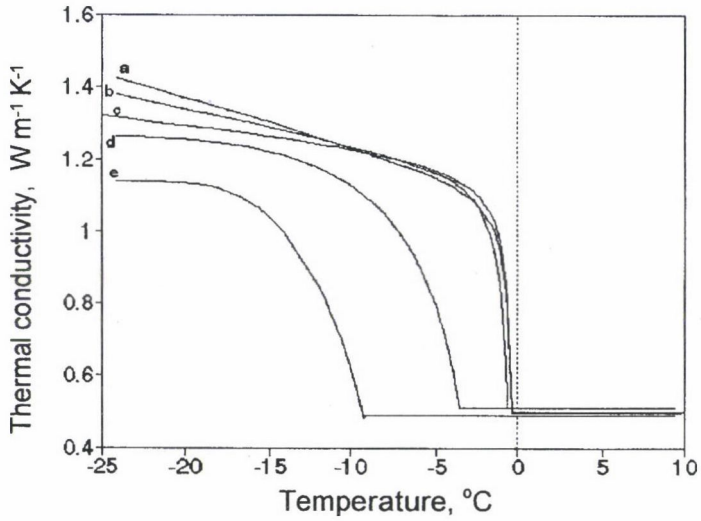

Fig. 2. Thermal conductivity of surimi with added κ-carrageenan and NaCl. a: w (added substances) = 0%; b: w (κ-carrageenan) = 5%; c: w (κ-carrageenan) = 9%; d: w (NaCl) = 5%; e: w (NaCl) = 9%

Interaction of added substances with proteins is also demonstrated by data presented on Fig. 3. Comparison of the initial freezing points T_i for samples of surimi and water solution of NaCl and κ-carrageenan as a function of mass fraction of NaCl and κ-carrageenan calculated on total mass of water show differences, which supports the assumption of essential interactions of added substances and myofibril proteins.

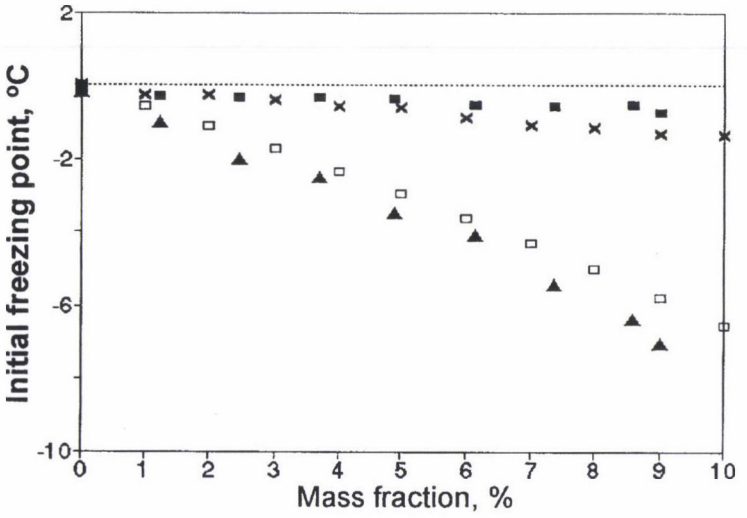

Fig. 3. Comparison of depedencies of T_i for surimi and water solution of NaCl and κ-carrageenan on mass fraction w of added substances calculated on total mass of water. ■: Surimi + κ-carrageenan; ▲ : surimi + NaCl; X: water solution of κ-carrageenan; □: water solution of NaCl

From DTA diagrams the peak points were read off as the initial freezing temperatures. Data for the initial freezing points were reported by KOVAČEVIĆ & KURTANJEK (2000). Values of the initial freezing points T_i were applied in the models for calculation of ρ, k and $\bar{H}$.

The relative apparent specific enthalpies were measured in the temperature range from –25 °C to T_i and are presented as continuous curves as function of the sample temperatures (Figs 4 and 5). The given values are apparent enthalpies resulting from sensible enthalpy (related to temperature) and phase transformation (latent heat). The obtained results for enthalpy are also verified by an independent experimental method, i.e. by differential scanning calorimetry (DSC) measurements conducted by WANG and KOLBE (1991). In Fig. 4 experimental values for enthalpy determined by DTA are given for surimi samples without the cryoprotecting mixture, together with correlation data from the CHANG and TAO (1981) and Dickerson data (HELDMAN, 1981).

The Chang-Tao model is given by:

$$H_r = b_1 \cdot (T_r + 273.15) + (1 - b_1) \cdot T_r^{b_2}$$
$$H_r = \frac{H}{H_i} \quad T_r = \frac{(T + 273.15) - 227.6}{(T_i + 273.15) - 227.6} \tag{8}$$

where H_i (kJ kg^{-1}) is specific enthalpy at T_i. The parameter values $b_1 = 0.295$ and $b_2 = 22.455$ were recalculated by the models proposed for meat group products with experimentally determined mass fraction of water in surimi samples. Data by Dickerson are taken from the table (HELDMAN, 1981) for fish and cod meat. When the specific enthalpy curve determined by DTA is compared with the literature data, it can be observed that enthalpies are in very good qualitative and quantitative agreement with data. For example, measured value of specific enthalpy in the temperature range from –25 °C to T_i for sample without added substance is 244 kJ kg^{-1}, while WANG and KOLBE (1991) have obtained 242 kJ kg^{-1}.

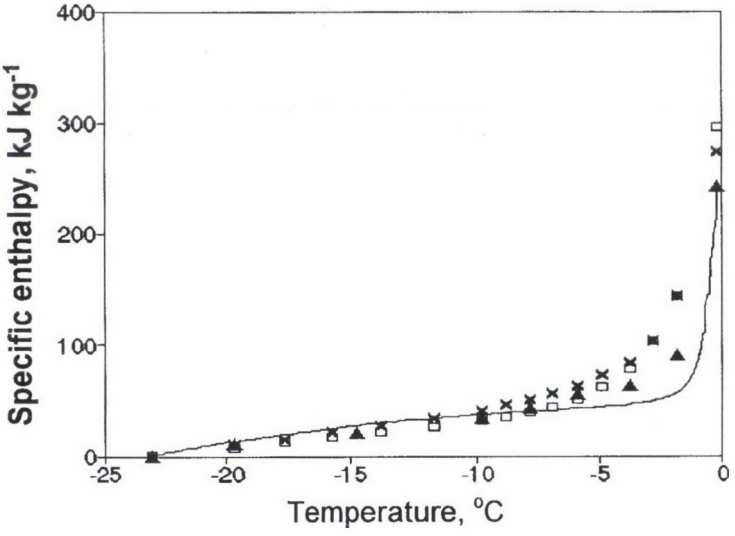

Fig. 4. Relative apparent specific enthalpy of surimi without added substances measured by DTA, compared with data from literature. –: Measured data; ▲: WANG & KOLBE data; □: CHANG & TAO model; X: Dickerson data

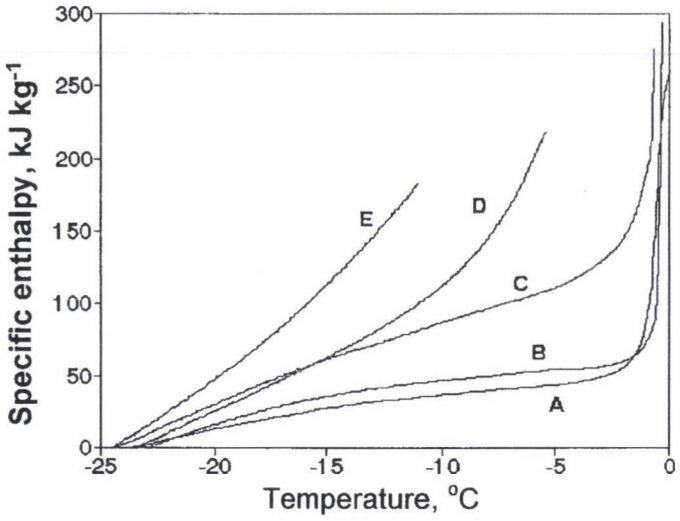

Fig. 5. Comparison of relative apparent specific enthalpy for samples of surimi with different mass fractions of κ-carrageenan and NaCl. A: w (added substances) = 0%; B: w (κ-carrageenan) = 6%; C: w (κ-carrageenan) = 10%; D: w (NaCl) = 6%; E: w (NaCl) = 10%

Results show change in relative apparent specific enthalpy, i.e. increase at lower temperatures with increased mass fraction of the added substances. From comparison of effects of NaCl and κ-carrageenan on specific enthalpy $\bar{H}$, presented in Fig. 5, it is evident that samples with added NaCl have higher values of $\bar{H}$ at the same values of mass fractions of the added substance w and temperature T. It is the result of stronger effect of NaCl on cryoscopic depression of T_i, i.e. nucleation and crystal growth are depressed to lower temperatures. During thawing process such samples absorb more latent heat, which results in the increase of apparent specific enthalpy $\bar{H}$ at lower temperatures.

3. Conclusions

Quantitative treatment of DTA curves (by use of collocation technique) has provided enthalpies of surimi in the temperature range from –25 °C to initial freezing points T_i. Relative apparent specific enthalpy $\bar{H}$ determined by DTA was verified by data from correlation models based on calorimetric measurements. The results show the

effect of added substances NaCl and κ-carrageenan on thermal properties which, are due to their interaction with myofibril proteins in surimi samples. Thermal conductivity k and apparent specific enthalpy $\bar{H}$ are functions of mass fractions of added substances. In the measured temperature interval from –25 °C to T_i increase in mass fractions of added substances results in the increase of the amount of bound (unfreezable) water, lowers initial freezing point T_i, decreases thermal conductivity k and increases apparent specific enthalpy $\bar{H}$. These effects are more pronounced for samples with added NaCl.

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This work was financially supported by the Ministry of Science and Technology, Republic of Croatia, project 058201. Authors express their appreciation to Prof. V. E. SWEAT for providing the line source heating element.

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Acta Alimentaria, Vol. 30 (4), pp. 395–405 (2001)

A CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF CAROTENOIDS AND CHLOROPHYLLS IN VEGETABLE PRODUCTS

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(Received: 7 December 2000; accepted 20 June 2001)

Pigments, such as carotenes, xanthophylls and chlorophylls, were extracted from both vegetables and their industrial products by using a 2:1 (v/v) dichloromethane/methanol solution. To separate and quantify the components in the extraction mixture, a HPLC analysis on reversed phase C_{30} column and binary gradient, made of methanol/water solution and dichloromethane, was employed. This gradient appears to have some advantages over other reported methods, which utilize reversed phase C_{30} column, in terms of resolution and analysis time. The linearity range of the detection response, the chromatographic resolution of a standard mixture constituted of lutein, zeaxanthin, trans-β-apo-8'-carotenal, β-cryptoxanthin, chlorophyll-b, α-carotene, chlorophyll-a, β-carotene, lycopene and the conditions for the complete extraction of those substances from the vegetable matrix were investigated. Both retention time and peak area reproducibility showed an average variation coefficient of about 2% for all the analyzed compounds. As a consequence of the good chromatographic separation of chlorophylls from carotenoids, sample saponification was found unnecessary when analyzing green vegetable products. Finally, to illustrate the applicability of the method, the presence of carotenoid esters in tomato and orange products was examined.

Keywords: carotene, carotenoids and HPLC, chlorophyll, lycopene, tomato, orange products

Carotenoids represent a wide class of natural pigments which have received considerable attention in the last years for their possible role in the prevention of many human diseases. The carotenoids can be classified in two main groups, i.e. carotenes, which are unsaturated hydrocarbons, and their oxygenated derivatives, xanthophylls (or

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0139-3006/2001/$ 5.00

oxycarotenoids). From biological point of view, one of the main properties attributed to carotenoids is that of being anti-oxidant. This arises from the high number of conjugated double bonds in these molecules which quench singlet oxygen atoms, thus inhibiting the peroxide formation in the cellular membrane lipids. It has been found that carotenoid pigments can prevent or slow down the growth of induced skin tumors and it has been reported that the risk of cancer in human beings may be inversely correlated both with the level of retinol in blood and with the dietary intake of β-carotene (PETO et al., 1981; STAHL & SIES, 1996). Another important carotenoid function is that of being physiological precursors of vitamin-A. However, for this function the precursor carotenoid has to have an unsubstituded β-ionone ring and uncharged side-chain. Chain lengthening decreases the provitamin activity. β-Carotene, the most widespread provitamin-A, with two β-ionone rings, shows the highest precursor activity and one molecule could generate two molecules of vitamin-A through the central cleavage. Many carotenoids found in fruits result vitamin-A precursors. Noticeably, lycopene, which is particularly abundant in tomato products, does not possess provitamin-A activity. However, among the natural carotenoids, lycopene is the most efficient singlet oxygen quencher (DI MASCIO et al., 1989; CONN et al., 1991) and more active than β-carotene in suppressing cell proliferation (LEVY et al., 1995). Many chromatographic methods for the analysis of carotenoids in vegetable matrices, which utilize either isocratic or gradient elution, have been developed. The isocratic systems have a lower resolving power and can be useful to separate carotenoids which are in a rather narrow range of polarity (FISHER & ROUSEFF, 1986; QUACKENBUSH & SMALLIDGE, 1986; GUILLOU et al., 1993). Gradient systems have a wider range of applications and have been used for carotenoid analysis in vegetable sources such as orange juices (ROUSEFF et al., 1996), olive oil (PSOMIADOU & TSIMIDOU, 1998) or other biological samples (FERRUZZI et al., 1998). In this paper a simple and efficient chromatographic procedure, that employs a binary gradient made of methanol/water solution and dichloromethane, is reported. This method can be useful in food industry quality control for the determination and quantification of the principal carotenoid compounds.

1. Materials and methods

1.1. Reagents and standards

Potassium hydroxide, sodium hydroxide, anhydrous sodium sulfate, butylated hydroxytoluene (BHT) and triethylamine (TEA) were purchased fron Sigma-Aldrich, Italy, as well as methanol, methylene chloride, diethyl ether and chloroform which were of HPLC grade. Standards of lycopene, β-carotene, α-carotene, trans-β-apo-8'-carotenal, chlorophyll-a and chlorophyll-b were obtained from Sigma-Aldrich, Italy;

lutein (xanthophyll) from Fluka, Italy; zeaxanthin and β-cryptoxanthin were from Extrasynthese, France. The standard solutions were prepared by dissolving the substances in chloroform containing 0.1% (w/v) BTH and, when stored under nitrogen at –20 °C, they were found stable for at least 15 days. Chlorophyll-a and chlorophyll-b standard solutions were prepared as above but with the addition of a known amount of β-carotene, as a further protection against light and oxidation. Because of the carotenoid instability and tendency to undergo stereomutation, photo and thermolability and easiness toward oxidation, all analytical operations were carried out under dim light and at temperatures not higher than 35 °C. The sample and the standard solutions were stored at –20 °C under nitrogen. Solvents contained 0.1% BTH (w/v).

1.2. Sample extraction

Ten ml of non-concentrated orange juice, or 10 ml of tomato puree, or 5.0 g of tomato paste were generally subjected to the extraction procedure. Instead, in the case of orange juice beverages, 50 ml of product was previously concentrated to a volume of 10 ml and then subjected to the extraction procedure. The samples of green mandarin essential oil were analyzed directly without extracting, after filtration with 0.45 μm nylon filters. When extracting carotenoids from *Spirulina* alga, 1 g of starting material was found sufficient for the successive analyses.

The samples were introduced into a 250 ml separator funnel and added with 30 ml of dichloromethane/methanol 2:1 (v/v). After shaking, the underlying organic layer was separated. This procedure was repeated until the starting material was colorless. The pooled organic layers were evaporated to dryness at a temperature not higher than 35 °C. When it was necessary to obtain saponification of the carotenoid esters, the residue was suspended in 6 ml of ethyl ether, made up with 6 ml of 10% KOH in methanol and allowed to stand for 20 h in the dark at room temperature. Afterwards, the mixture was transferred into a 250 ml separator funnel, made up with 20 ml of diethyl ether and then with 100 ml of 10% (w/v) aqueous NaCl. After shaking, the aqueous layer was discarded and the ether layer was washed with water until the washes became neutral to phenolphthalein. Finally, the ether layer was desiccated over anhydrous sodium sulfate and evaporated to dryness.

1.3. HPLC analysis

The dried samples obtained from the extraction procedure were dissolved in different volumes of chloroform and filtered through 0.45 μm nylon filter immediately before the chromatographic analysis. The HPLC analyses were performed on a Waters Alliance chromatograph equipped with photodiode array detector (PDA) mod. 996. The data were collected by a computer and processed by the Waters Millennium software,

which operated on the data collected at 450 nm when analyzing carotens and xanthophylls, and at 428 nm for the chlorophylls. The analyses were made on a reversed phase column YMC-Pack C_{30} (250×4.6 mm i.d.) filled with 5 μm average particle size. The column was thermostated at 30±0.1 °C. The elution was performed at a flow rate of 1 ml min^{-1}. The injection volume was 10 μl. The elution was performed with a linear gradient of solvent A (methanol-water, 95:5, v/v, containing BHT 0.1% and TEA 0.05%) and solvent B (methylene chloride, containing BHT 0.1% and TEA 0.05%). The gradient, starting at sample injection, was from 5% B in A to 70% B in A in 35 min.

2. Results and discussion

Many procedures for extracting carotenoids from a vegetal matrix, in which the cloroplasts of the vegetal cells are predominant, have been reported (for a comparative study see TAUNGBODHITHAM et al., 1998). In these methods different organic solvents, such as tetrahydrofuran (THF), petroleum ether, methanol, acetone, either alone or in mixtures, were employed with or without previous pulp precipitation. Carotens, being highly hydrophobic substances, are better dissolved in apolar solvents such as THF. On the contrary, xanthophylls are better dissolved in more polar solvents, such as methanol. A 2:1 (v/v) dichloromethane/methanol mixture was employed, which is known to extract both neutral and polar lipids with high efficiency (CHEN et al., 1981) and we observed that the vegetal matrices employed were rapidly decolorized by this solution. In fact, in the case of tomato pulp, which strongly retains its pigments, it was observed that two successive treatments with this solvent extracted 98.4% of the pigments. Also in the case of tomato juice two extractions were sufficient to completely decolorize the product. To avoid the oxidation of carotenoids, which are easily oxidized after extraction from vegetal matrix, 0.1% BHT was added to the extraction solvent (HART & SCOTT, 1995).

To estimate the extraction recovery, we used tomato puree with the following carotenoid composition (mg/100 ml): lutein 0.06, α-carotene 0.03, β-carotene 0.24, lycopene 28.9. To three identical aliquots of this sample different amounts of lutein, α-carotene, β-carotene and lycopene were added as reported in Table 1. Each measurement was repeated three times. The recovery was 99.1% for lutein, 99.3% for α-carotene, 98.7% for β-carotene and 101.5% for lycopene.

The extraction mixture, soon after filtration through a 0.45 μm nylon filter, was analyzed by HPLC, as reported in Materials and methods chapter, using a reversed phase C_{30} column with elution gradient made of methanol-water and dichloromethane.

Table 1

Scheme of the carotenoid addition (mg/100 g) to tomato puree samples for the recovery estimate

Sample	1	2	3
Lutein	0.05	0.08	0.10
α-carotene	0.05	0.08	0.10
β-carotene	0.10	0.15	0.20
Lycopene	5.00	10.0	15.0

This elution gradient was previously employed to separate ergosterol from a complex lipid matrix (DE SIO et al., 2000). Figure 1 reports the chromatograms obtained from the analysis performed on the mixture of the standard compounds. In Fig. 2, a typical chromatogram obtained from a sample of dried tomato is reported. In chromatogram "b" of Fig. 2, peaks 1, 2, 3 and 4 correspond to lutein, zeaxanthin, α-carotene and β-carotene in the tomato sample. In chromatogram "a" of Fig. 2, the analysis for the quantification of the lycopene (peak 5) in the same tomato sample is reported. This analysis was conducted on the same solution, whose analysis is reported in chromatogram "b", and was diluted to 1:30. Lycopene, in fact, is so abundant in tomato, in which it represents about 85% of the total carotenoids (LEONI, 1993), that a strong dilution is necessary to make its chromatographic peak quantifiable. Unfortunately, the dilution necessary for lycopene analysis is excessive for the other compounds, thus, for the correct quantification of all components, two analyses of the same sample at different dilution are necessary. On the other hand, we found that, especially for lycopene, a suitable dilution is also necessary inasmuch as a marked deviation from the linearity of the instrumental response as a function of lycopene concentration was observed. This is probably due to high light scattering of lycopene solutions also at relatively low concentration. For this reason, only solutions with lycopene concentration lower than 15 ppm were analyzed. Up to this concentration, in fact, the instrumental response was found linear.

As it can be seen in Fig.1, the standard compounds are analyzed with complete resolution in less than 35 min. Due to the rather fast elution gradient employed, another advantage of this chromatographic analysis is that also the most retained compounds, such as lycopene, emerge as sharp peaks. As a consequence, a noticeable increase in the sensitivity ensues. This advantage was already observed when a similar gradient was used for the chromatographic analysis of phospholipids (SERVILLO et al., 1997).

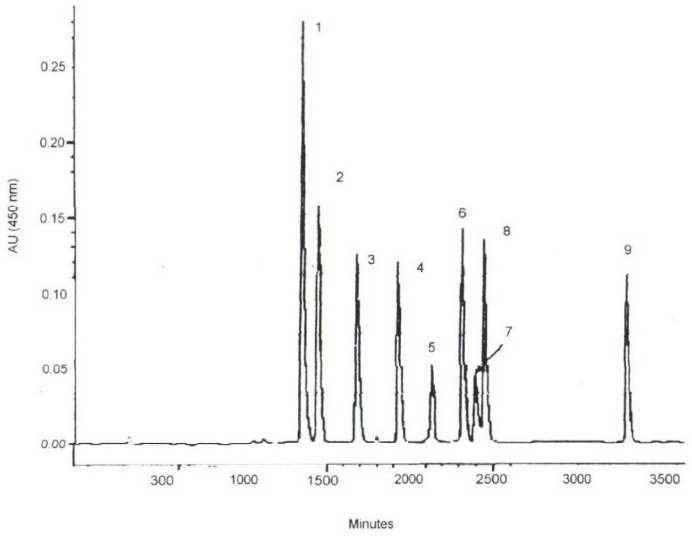

Fig. 1. Reversed phase HPLC separation of carotenoid standard mixture. Peak 1: lutein; peak 2: zeaxanthin; peak 3: trans-β-apo-8'-carotenal; peak 4: β-cryptoxanthin; peak 5: chlorophyll-b; peak 6: α-carotene; peak 7: chlorophyll-a; peak 8: β-carotene; peak 9: lycopene. Chromatographic conditions are reported in Materials and methods chapter

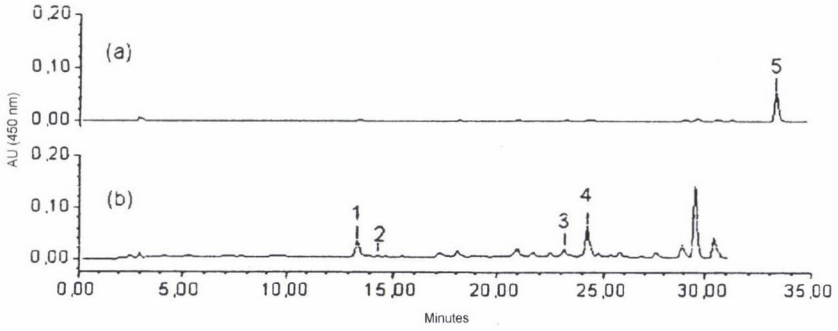

Fig. 2. Reversed phase HPLC analysis of the pigments extracted from a dried tomato sample. The starting solution (chromatographic analysis in chromatogram "b"), was diluted 1:30 to determine lycopene. The chromatogram of the diluted solution is reported in chromatogram "a". The analysis of the starting solution, reported in chromatogram b, was interrupted before the elution of the peak 5, corresponding to the most abundant lycopene, in order to maintain the other components in the same scale. Peak 1: lutein; peak 2: zeaxanthin; peak 3: α-carotene; peak 4: β-carotene; peak 5: lycopene. The component quantification is reported in Table 2

It is worth to note that, with the gradient we used, the analysis time is noticeably reduced, with no loss of resolution, with respect to the method reported by ROUSEFF and co-workers (1996). They used a reversed phase C_{30} column and a rather complex gradient made of methanol, water and MTBE to analyze saponified orange juices carotenoids. In this analysis, the lutein retention time is about 19 min and β-carotene retention time is about 39 min. With the gradient we use, the lutein and β-carotene retention times are about 14 min and 24 min, respectively. Moreover, in our analysis, lycopene retention time is about 33 min and, although ROUSEFF and co-workers (1996) did not report the analysis of this compound, it is very likely that, with the gradient they used, the lycopene would emerge from the column as a highly retained broad peak.

Table 2 shows the content of carotenoids in several sources such as vegetables, vegetable industrial products, the blue alga *Spirulina* and the green mandarin essential oil, measured with the analyses performed as described before.

In order to determine the chromatographic repeatability, the standard mixture (Fig. 1), with the exclusion of the chlorophylls, was injected ten times within two weeks. The chlorophylls were injected five times by preparing the standard solutions just before the analysis. The results are reported in Table 3. The percent standard deviation (% s) calculated for the retention times (t_r) shows very close values with a maximum of 6.6% for lycopene, which is the farthest peak and perfectly resolved from the other peaks. The variation coefficient (% CV), calculated for each peak area, was always lower than 2%. In the case of the peaks corresponding to the two chlorophyll standards, because of the high degradation rate observed for these compounds after dissolution, the % CV was not determined. Instead, a good stability of the peak corresponding to chlorophyll-a from a sample of the blue alga *Spirulina* was observed. For this sample, a content of 128.9 mg/100 g with CV of 1.64% was calculated from the results of five chromatografic analyses performed over a nine-day period. The same good stability of chlorophyll-a and -b was found for a sample of mandarin essential oil, which gave a CV of 1.96% for five analyses over a six-day period. The high instability of the pure standards of chlorophyll-a and -b was also observed after dissolving these compounds in other solvents such as ethyl ether or hexane, besides chloroform and dichloromethane. In fact, in the course of successive analyses, a rapid decrement of peak area was observed with a concomitant appearance of new peaks with lower retention times and with absorption spectra different from those of chlorophyll-a and -b. An increment in the stability of the standard chlorophyll solutions was obtained by adding, besides BHT, a known amount of β-carotene that seemed to exert a protective action.

Generally, when analyzing carotenoids, the sample extraction is followed by a saponification step. This is done in order to hydrolyze carotenoid esters and to remove chlorophylls. However, if the chromatographic procedure separates the chorophylls from the carotenoids of interest, the saponification is unnecessary (HART & SCOTT, 1995).

Table 2

The carotenoid content of various sources (mg/100 g)

	Lutein	Zeaxanthin	β-Cryptox.	α-Carotene	β-Carotene	Lycopene	Chlorophyll-b	Chlorophyll-a
Artichokes	0.15	0.02	0.01	0.00	0.03	0.00	0.09	<0.01
Vegetables cocktail	0.38	0.05	0.01	1.44	2.57	0.00	0.98	0.00
Eggplants and peppers	0.70	0.13	0.21	0.40	0.96	0.02	0.65	3.39
Dried tomatoes	0.55	0.04	0.00	0.01	1.17	35.1	0.00	0.00
Tomato puree	0.06	<0.01	0.00	0.03	0.24	28.9	0.00	0.00
Spirulina blue alga	0.00	5.27	0.37	0.12	9.99	0.00	0.00	128.9
Peaches	<0.01	0.01	0.02	0.06	0.17	0.00	0.00	0.00
Peaches canned	<0.01	0.01	0.01	<0.01	0.12	0.00	0.00	0.00
Olive oil	0.10	0.01	0.01	0.00	0.00	0.00	0.00	0.00
Yellow orange juice	0.03 (0.06)	0.04 (0.06)	0.05 (0.07)	<0.01 (0.01)	0.03 (0.03)	0.00 (0.00)	Not determined	Not determined
Orange based beverage A	0.05 (0.09)	0.08 (0.11)	0.05 (0.08)	0.01 (0.01)	0.04 (0.04)	0.00 (0.00)	Not determined	Not determined
Orange based beverage B	0.04 (0.07)	0.06 (0.10)	0.15 (0.21)	<0.01 (<0.01)	0.19 (0.17)	0.00 (0.00)	Not determined	Not determined

Figure 3, in fact, shows the chromatographic analysis performed on a green mandarin essential oil. The good resolution of chlorophylls-a and -b peaks from the other carotenoid peaks can be easily seen. However, we employed the saponification step according to ROUSEFF and co-workers (1996) to investigate the possible differences in chromatograms of samples treated with and without the saponification procedure, especially regarding the peak area of lutein, α-carotene, β-carotene and lycopene for tomato products and of zeaxanthin and β-cryptoxanthin for orange derivatives.

Table 3

Repeatability of the retention time (% s) and reproducibility of the peak area (% CV) of the standard carotenoid mixtures

	t_R (% s)	Peak area (% CV)
Lutein	2.08	1.62
Zeaxanthin	2.64	1.67
β-Apo-8'-carotenal	2.63	1.24
β-Cryptoxanthin	1.53	1.47
Chlorophyll-b	2.52	–[a]
α-Carotene	1.55	1.04
Chlorophyll-a	2.50	–[a]
β-Carotene	1.53	1.81
Lycopene	6.56	1.53
Mean	2.62	1.48

[a] Not measured.

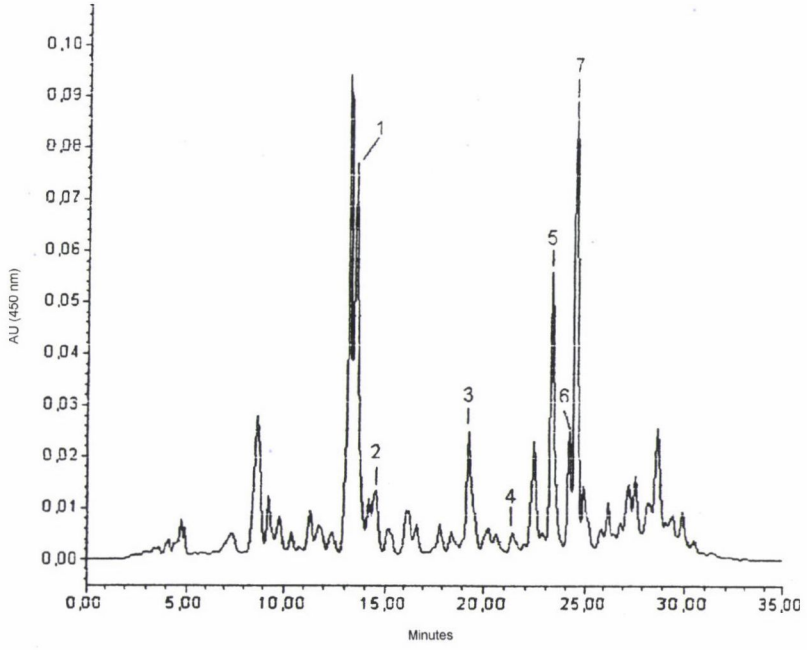

Fig. 3. Reversed phase HPLC analysis of green mandarin essential oil. Peak 1: lutein; peak 2: zeaxanthin; peak 3: β-cryptoxanthin; peak 4: chlorophyll-b; peak 5: α-carotene; peak 6: chlorophyll-a; peak 7: β-carotene. The essential oil was injected without any treatment. The component quantification is reported in Table 2

In the case of tomato puree and tomato paste the chromatograms of saponified and non-saponified samples were found substantially identical. For orange juice and other beverages based on orange juice, after saponification, a mean increment of 100% for lutein and 50% for zeaxanthin and β-cryptoxanthin was found. A similar observation was already reported by WINGERATH and co-workers (1996).

3. Conclusions

The procedure described in this paper allows a rapid and sensitive HPLC separation of the main carotenoids present in vegetables. The analysis time appears lower than other reported methods which utilize reversed phase column with elution gradient and, also highly hydrophobic compounds, such as lycopene, are eluted as sharp a peak with rather low retention time. The good resolution obtained with this procedure the chlorophylls from caroteinoids could also allow, in favorable cases, to skip the saponification step of the extracts from the green vegetables to be analyzed for their carotenoid content.

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The authors would like to express their gratitude to Ing. D. CACACE of Stazione Sperimentale per l'Industria delle Conserve Alimentari, Angri (SA), Italy, for the support in the statistical analysis of data.

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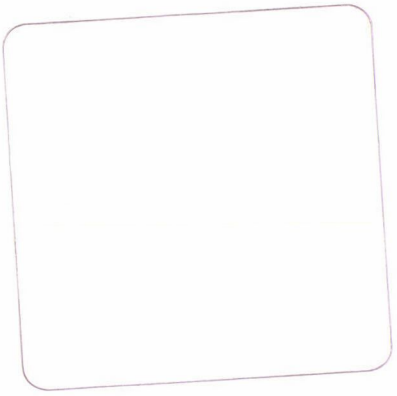

Acta Alimentaria, Vol. 30 (4), pp. 407–414 (2001)

Preliminary communication

DIETARY FIBER CONTENT OF BULGUR AS AFFECTED BY WHEAT VARIETY

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(Received: 1 June 2000; accepted: 17 June 2001)

Dietary fiber content of bulgurs prepared from different wheat varieties was investigated. Grains of 29 Turkish wheat cultivars and advanced breeding lines (23 of durum and 6 of common wheat) were used in this study. The average values for ADF and NDF (+amylase) contents of investigated durum wheats were 3.4% and 9.9%, respectively and the corresponding values of common wheats were 3.4% and 11.5%. In this study, the average values for ADF and NDF (+amylase) contents of bulgurs made of durum wheats were found to be 5.4% and 10.3%, respectively and the corresponding values of bulgurs made of common wheats were 5.8% and 11.7%. The minimum and maximum values for ADF and NDF (+amylase) contents of bulgurs made of durum wheats were found to be 4.1%–6.8% and 7.9%–11.8%, respectively and the corresponding values of bulgurs made of common wheats were 5.1%–6.4 and 10.6%–12.4%. The processing of wheat into bulgur generally increased the levels of ADF and NDF(+amylase) contents. It can be concluded that bulgur is at least as good as a raw wheat in terms of dietary fibre content. Although there is no essential change in the total protein content, ash and β-carotene contents of the bulgurs were lower than the ones in the original wheats as a result of debranning.

Keywords: dietary fiber, bulgur, wheat

Bulgur is one of the oldest cereal-based staple foods in Turkey and Near Eastern countries (WILLIAMS et al., 1984; BAYRAM, 2000). In 1996, 950 thousand tons of bulgur was produced in Turkey (ANON, 1996). Bulgur has been processed by a series of steps of soaking, cooking, drying, milling to remove the outer bran coating, and cracking. Therefore, bulgur is a parboiled, dry and partially debranned whole wheat product. Traditionally, bulgur has been made by boiling or simmering whole wheat in open pans for 1–3 hours, then drying the product in the sun or in simple dryers. To avoid leaching of soluble nutrients, minimum amount of water is used. The loose outer bran is removed by beating in a large stone mortar with a wooden pestle. The dried kernels are cracked

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0139-3006/2001/$ 5.00

and sieved to obtain a coarse, a medium and a fine granulation. In the modern, large-scale and continuos bulgur production method, pressure is used for cooking wheat for bulgur (PENCE et al., 1964; SMITH et al., 1964; HARRIS et al., 1978). Since it is a relatively unrefined product, its nutritional value has been regarded as similar to that of whole wheat (ÖZKAYA et al., 1996). That means the nutritive value of bulgur remains relatively unaltered. According to NEUFELD and co-workers (1957), debranning was generally continued until about 7% – by weight –, of the kernels had been removed. In addition, removal of part of the outer bran from cooked and dried wheats and subsequent cracking and size-grading led to a 15–20% decrease in crude fiber (PENCE et al., 1964). The importance of the intake of dietary fiber in relation to health and disease prevention has been the subject of a great deal of researches (TROWELL, 1976; SOUTHGATE et al., 1978; ANON, 2001) but there is no published information on the dietary fiber (DF) contents of bulgur. Therefore, the objective of this study is to compare the dietary fiber contents and the chemical composition of the bulgur samples and the wheats which are used in the bulgur production. The increasing interest in recent years in the consumption of dietary fiber rich foods attracted us to this study on the dietary fiber contents of bulgur.

1. Materials and methods

1.1. Materials

Grains of 29 Turkish wheat cultivars and advanced breeding lines (23 of durum and 6 of common wheat) were used in this study.

1.2. Bulgur-making process

The wheat (1 kg) was soaked to raise the moisture content to about 45% by adding water and kept in water bath at 60 °C for 3 h. The soaked grain was cooked in an autoclave (Funke Gerber, Webeco, Germany) under a steam pressure of 20 p.s.i., at 121 °C for 15 min, which was sufficient to gelatinize the starch completely. After cooling for 10 min, cooked product was dried at 60±2 °C to a moisture content of 10–12%. Dried product was conditioned with 2% additional water for half an hour. The loose outer bran was removed by beating in a plastic mortar. The debranned product was cracked by using Falling Number Laboratory Mill Type-KT 30 (adjusted to produce the coarsest particle size), aspirated to remove residual bran material and sifted through a 0.5 mm sieve to remove the fine particles (ÖZKAYA et al., 1996).

1.3. Analytical methods

Moisture, protein, ash and β-carotene contents of the wheat and bulgur samples were determined by A.A.C.C. methods (1990). Acid-detergent fiber (ADF) was determined according to the original procedure (VAN SOEST, 1963). Neutral-detergent fiber (NDF) content was determined by using additional amylase enzymes in order to remove starch (NDF+amylase). For NDF+amylase determination (BAŞMAN & KÖKSEL, 1999), samples of known moisture content were weighed accurately (≈0.5 g) into polypropylene tubes and 10 ml of phosphate buffer (0.08 mol l^{-1}, pH 6.0) and 100 µl of Termamyl 120L (Novo, Denmark) were added. Tubes were capped and incubated in a water bath (93 °C) for 0.5 h with stirring every 5 min. They were cooled and 250 µl of HCl (to adjust to pH 4.1–4.8) and 150 µl of amyloglucosidase 300L (Novo) were added. They were incubated at 60 °C for 20 min with stirring in every 5 min and centrifuged at 2000 r.p.m. (Heraeus Sepatech Labofuge Ae) for 5 min. The supernatants were discarded and the residue was treated with neutral detergent solution according to the original NDF procedures (VAN SOEST & WINE, 1967). All analyses were performed on wheat and bulgur samples. The values represent means of duplicate analyses. The data were analyzed using the SPSS for Windows Release 5.0.1 (SPSS, Inc., Chicago, IL).

2. Results and discussion

2.1. Composition of wheat and bulgur samples

The mean, minimum and maximum values of the protein, ash and β-carotene contents of wheats and the bulgurs are given in Table 1.

When wheat was converted to bulgur under the conditions used in this study, which minimize leaching of soluble nutrients, there was essentially no change in the total protein content (Table 1). The outer bran tissues are normally removed and discarded during bulgur manufacture. These tissues have higher fiber and ash contents than the deeper layers of bran that are normally retained in bulgur. Unavoidably, ash and β-carotene contents of the bulgurs were lower than the ones in the original wheats as a result of debranning and leaching (Table 1). The degree of debranning, of course, will also affect retention of these factors (PENCE et al., 1964; SHAMMAS & ADOLPH, 1954).

Table 1

The mean, minimum and maximum values of the protein, ash and β-carotene contents of wheat (durum and common) and bulgur samples

Samples		Mean ± S.D.		Minimum		Maximum	
		Durum n=23	Common n=6	Durum n=23	Common n=6	Durum n=23	Common n=6
Protein[a] (Nx5.7) (%)	W	14.0±1.40	12.6±1.13	11.4	11.5	16.6	14.4
	B	14.0±1.31	12.6±1.23	11.5	11.3	16.6	14.5
Ash[a] (%)	W	1.58±0.32	1.72±0.28	1.12	1.34	2.08	1.98
	B	1.51±0.30	1.58±0.28	1.11	1.18	2.02	1.80
β-carotene (ppm)	W	3.80±0.72	2.30±0.39	2.90	1.50	5.90	2.50
	B	3.40±0.60	2.10±0.34	2.20	1.40	4.60	2.30

[a] Dry basis; W: wheat; B: bulgur

2.2. Dietary fiber contents of wheat samples

The ADF and NDF(+amylase) contents of durum (n=23) and common wheat (n=6) samples are given in Tables 2 and 3, respectively. The average values for ADF and NDF(+amylase) contents (dry basis) of durum wheats were 3.4% and 9.9%, respectively and the corresponding values of common wheats were 3.4% and 11.5%.

The ADF contents reported in the present study (3.4% for both durum and common wheats) were slightly higher than the literature values (DONG & RASCO, 1987). The NDF values published by SOUTHGATE and co-workers (1978) and VAN SOEST & WINE (1967) were 10.3%, and 12.5%, respectively, and respective values of DONG & RASCO (1987) and MOSS & MUGFORD (1986) were in the range of 10.9–14.5% and 11.7–13.7%.

2.3. Dietary fiber contents of the bulgur samples

The ADF and NDF(+amylase) contents of bulgurs produced from durum (n=23) and common wheat (n=6) samples are presented in Tables 2 and 3, respectively. Previous workers have reported that partial removal of the outer bran from bulgur led to a 15–20% decrease in crude fiber (PENCE et al., 1964). However, there is no published information on the DF contents of bulgur. In this study, the average values for ADF and NDF(+amylase) contents of bulgurs made of durum wheats were found to be 5.4% and 10.3%, respectively and the corresponding values of bulgurs made of common wheats were 5.8% and 11.7%. To assess the potential of dietary fibre contribution of selected

commercial foods and home-prepared breads over 50 samples were analyzed for their NDF+amylase content (JWUANG & ZABIK, 1979). Results showed that the NDF+amylase content in cereals ranged from 3.8–34.0%, in European crispbreads from 16.4–36.3% and in whole wheat bread from 5.2–13.5%. In another study, whole wheat bread has been reported to contain 14.9% NDF (SPILLER & AMEN, 1975).

Table 2

Dietary fiber contents of durum wheat and bulgur samples[a]

Sample (n=23)	Acid detergent fiber (%)		Neutral detergent fiber + amylase (%)	
	Wheat	Bulgur	Wheat	Bulgur
1	4.1	6.0	11.0	11.2
2	2.0	5.2	10.4	11.1
3	3.8	5.0	10.3	10.5
4	3.9	4.9	8.7	9.7
5	4.0	6.2	9.7	9.9
6	1.9	4.7	9.5	9.4
7	1.9	4.5	11.5	11.6
8	4.3	5.7	10.9	11.2
9	5.0	6.8	11.0	11.3
10	4.3	6.3	10.7	11.0
11	4.0	6.3	9.7	9.5
12	3.8	6.1	9.3	11.2
13	4.1	5.7	8.3	9.1
14	3.8	6.1	11.9	11.8
15	4.0	5.7	9.5	9.6
16	2.1	4.6	8.0	9.3
17	5.3	4.9	9.6	10.6
18	4.1	5.1	9.6	9.8
19	2.0	4.6	7.8	7.9
20	4.1	4.9	9.5	11.4
21	2.1	4.1	11.1	11.0
22	1.9	6.1	8.7	9.2
23	2.0	5.0	9.9	9.9
min	1.9	4.1	7.8	7.9
max	5.3	6.8	11.9	11.8
mean	3.4±1.12	5.4±0.73	9.9±1.11	10.3±1.01

[a] Dry basis

Table 3

Dietary fiber contents of bread wheat and bulgur samples[a]

Sample (n=6)	Acid detergent fiber (%)		Neutral detergent fiber + amylase (%)	
	Wheat	Bulgur	Wheat	Bulgur
1	4.3	5.4	12.2	12.4
2	2.1	5.1	10.5	10.6
3	3.9	5.5	10.4	11.1
4	2.1	6.4	11.8	11.7
5	3.9	6.2	11.6	11.8
6	4.0	6.1	12.2	12.4
min	2.1	5.1	10.4	10.6
max	4.3	6.4	12.2	12.4
mean	3.4±1.00	5.8±0.52	11.5±0.81	11.7±0.71

[a] Dry basis

High NDF values in the cereal based products are generally due to the interference from unremoved starch (SCHWEIZER & WÜRCH, 1979). Lower results were obtained after a pretreatment with amylase enzymes (NDF+amylase) due to the digestion of starch in endosperm material. MOSS and MUGFORD (1986) reported that bran particles contained small pockets of starch granules surrounded by endosperm cell walls adjacent to the aleurone layers. The retention of these walls during analysis might have been prevented the complete digestion of starch.

There are various studies investigating the changes in the DF content of the cereal products during processing (SOUTHGATE et al., 1978; BJÖRCK et al., 1984). These researchers have reported that 1.5% and 20% increases in DF content during baking and drum drying, respectively. However, we have not encountered any research studying the changes in DF content in bulgur processing. In the present study, the processing of raw wheat into bulgur was found to have no detrimental effect on the dietary fiber content of the bulgur. In order to determine whether or not bulgur production is effective on the dietary fiber content of wheat samples, DF content of samples were determined before and after bulgur production and the data were evaluated by using t-test for paired-samples. From the results, we rejected the hypothesis of no effect at the 5% level for ADF and NDF values of durum wheat samples and ADF value of common wheat samples. It can be concluded that the bulgur production has increased the ADF and NDF contents of durum wheat and ADF content of common wheat samples ($P<0.05$). For the common wheat samples, we can conclude that bulgur production has no significant effect on the NDF value.

The increase in the dietary fiber contents of bulgurs might also be explained by the formation of enzyme resistant starch (RS) (RANHOTRA et al., 1999). The effect of drum-drying on starch availability in white wheat flour was studied and compared with boiling and pressure cooking (BJÖRCK et al., 1984). The process conditions with respect to steam pressure and drum speed were as follows: 4.9 bar, 13 r min^{-1} for mild conditions and 9.8 bar, 4 r min^{-1} for severe conditions. In this study, they have found that the dietary fiber content in raw wheat flour and in wheat flour processed under mild conditions was similar. However, under severe drying conditions, the dietary fiber content in wheat flour increased by about 20%. They have suggested that the increase in dietary fibre resulting from severe drum-drying conditions was due to the formation of a form of starch that was resistant to digestion by the alpha-amylase (Termamyl) used in the dietary fiber assay. The mechanism behind the formation of RS during processing is believed to be related to the retrogradation of amylose. In another study, the amount of RS formed during baking (≤3%) or autoclaving (≤10%) covers the range of dietary fibre contents in most staple foods (BJÖRCK et al., 1987).

3. Conclusions

Previous studies have shown that bulgur is a product which resembles, in its nutritional properties (e.g. mineral, vitamin, protein contents), whole wheat more closely than refined white flour. It has therefore been given a good deal of attention by nutritionists. Effects of bulgur processing on the DF contents of wheats were investigated in detail for the first time in the present study. Bulgur is found to be at least as good as raw wheat in terms of ADF and NDF(+amylase) contents. Despite the removal of bran during bulgur production, the NDF(+amylase) contents of bulgurs were usually higher than the corresponding wheat samples which can be explained by the formation of RS during bulgur production. Hence, further studies are needed on this matter.

Cereal products are important components in the diet of most people around the world. However, new and more appealing products are required in the market, to increase the consumption of cereal based foods. Ethnic cereal foods have a potential to provide healthier diets to the people around the world. Hence, the research on the ethnic foods such as bulgur, couscous and flat breads etc. might give the opportunity to increase the dietary fiber consumption.

*

We wish to thank the Research Fund of Hacettepe University (Proj. No. 94 02 010 001) and National Productivity Center (Turkey) for their collaborative financial supports.

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